

EVALUATION OF BACTERIOCINS IN *Xanthomonas perforans* FOR USE IN BIOLOGICAL  
CONTROL OF *Xanthomonas euvesicatoria*

By

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*Xanthomonas perforans* strain 91-118 produces at least three different bacteriocin-like compounds (BcnA, BcnB, BcnC) antagonistic toward *X. euvesicatoria* strains. Previous research showed that deletion of one bacteriocin (BcnB) produced the highest level of antagonism toward sensitive *X. euvesicatoria* strains. One aspect for this study was to further characterize each bacteriocin by deletion mutagenesis to establish which open reading frames (ORFs) were responsible for bacteriocin activity for each bacteriocin as well as determining their possible functions. BcnA has been shown to contain at least four essential genes for activity and a model has been created to suggest the role of each gene. BcnB and BcnC were both found to be proteinases (endoproteinase Arg-C and extracellular metalloproteinase, respectively).

A second aspect of this study was to develop a viable biocontrol strategy by creating pathogenicity-attenuated mutants such that these attenuated mutants on the plant surface would suppress bacteriocin-sensitive strains. Several candidate genes were chosen based on mutant phenotypes in either *X. perforans* (OpgH<sub>Xcv</sub>) or the closely related *X. euvesicatoria* strain 85-10 (hpaA, hpaB, hpaC, xopA, xopD, avrBs2 and gumD). Each candidate gene was amplified and PCR-assisted deletion mutagenesis was performed for final marker exchange into wild-type (wt)

*X. perforans* to create attenuation mutants. Each mutant was tested for growth rate, disease severity and antagonism toward *X. euvesicatoria*-sensitive strains. Mutations in *opgH* and *gumD* gave the most significant reduction in disease and growth rate while maintaining the ability to reduce *X. euvesicatoria* populations.

One attenuated mutant, 91-118::*ΔopgH*, was chosen for further investigation.

Greenhouse and field experiments were conducted using 91-118::*ΔopgHΔbcnB* to determine its ability to reduce *X. euvesicatoria* populations. Greenhouse and field experiments indicate 91-118::*ΔopgHΔbcnB* significantly reduced *X. euvesicatoria* populations. In the field, weekly application of 91-118::*ΔopgHΔbcnB* consistently reduced *X. euvesicatoria* populations as compared to the standard control (application of copper + manzate and actigard every two weeks). 91-118::*ΔopgHΔbcnB* applied every two weeks also significantly reduced *X. euvesicatoria* populations in one season, but were not significantly different from the grower standard control.

## CHAPTER 1 INTRODUCTION

Bacterial spot of tomatoes and peppers is caused by the phytopathogenic bacterium *Xanthomonas euvesicatoria*, *X. vesicatoria* and *X. perforans* (69). It is a worldwide disease and is a major problem in Florida, particularly during periods of high temperatures and high humidity. Pohronezny *et al.* (115) estimated as high as 50% loss of marketable fruit due to bacterial spot on tomatoes.

The pathogen is a Gram-negative, rod-shaped bacterium which can readily be isolated from diseased tissue. It is motile, possessing a single polar flagellum, strictly aerobic, and measures 0.7 to 1.0  $\mu\text{m}$  by 2.0-2.4  $\mu\text{m}$ . On nutrient agar the bacterium produces a characteristic yellow water-insoluble pigment called xanthomonadin and an extracellular polysaccharide (EPS) termed xanthan gum (149).

Bacterial spot of tomato affects the aerial portions of the plant, with symptoms consisting of numerous small (1 to 5 mm) circular lesions on leaves, stems and fruit. Bacterial spot can be distinguished from fungal leaf spots by a greasy, water-soaked appearance on the abaxial side of leaves. Chlorosis and epinasty of leaves occurs, eventually leading to complete necrosis of tomato leaflets (140).

### **History, Etiology, and Strain Diversity**

Bacterial spot of tomato and pepper is one of the earliest recorded bacterial diseases. *X. euvesicatoria* was first described as bacterial canker in South Africa by Doidge in 1921 (33). That same year Gardner and Kendrick (52) in the United States discovered a similar organism and referred to it as bacterial spot. Doidge performed a comprehensive study on the etiology of bacterial spot, which she termed tomato canker, and identified the causal agent as *Bacterium vesicatorium*, whereas in the United States, Gardner and Kendrick (52) originally named the

organism *Bacterium exitiosa*. Over time, as a result of comprehensive studies (51), the two bacteria were discovered to be very similar and, in the mid 1920s, were designated *Bacterium vesicatoria*. Since that time the bacterium was renamed several times, changing genera from *Bacterium* to *Pseudomonas* to *Phytomonas* and finally to *Xanthomonas* (71).

Once transferred to *Xanthomonas*, the bacterium underwent several species and pathovar changes. In 1980 it was transferred to *X. campestris* pv. *vesicatoria* (*X.c. vesicatoria*) (35). In the 1990s, major changes occurred in the taxonomy of the genus *Xanthomonas* which resulted in the renaming of many species. Extensive comparison of strains using DNA-DNA hybridizations resulted in the identification of two groups, A and B (140, 157). Group A (tomato race 1) strains were transferred to *X. axonopodis* and designated *X. axonopodis* pv. *vesicatoria*, while Group B strains (tomato race 2) were placed in *X. vesicatoria* (157). In the 1990s a new group of strains was identified in Florida (72) that was phylogenetically most closely related to group A, but was phenotypically and genotypically distinct enough from group A that it was designated as group C. Because group C is most closely related to group A based on DNA-DNA hybridization this new group was designated within *X. axonopodis* pv. *vesicatoria*. In 2004, the most recent changes occurred in nomenclature (69). Group A (tomato race 1) and Group C (tomato race 3) strains were removed from *X. axonopodis*, since they shared less than 70% DNA relatedness to other *X. axonopodis* strains, and were placed in *X. euvesicatoria* and *X. perforans*, respectively.

Several avirulence genes have been characterized in xanthomonads associated with tomato. In 1993, Whalen *et al.* (166) found *X. euvesicatoria*, tomato race 1 (T1), strains to carry the avirulence gene *avrRxv*, which induces an incompatible reaction that activates localized cell death also known as a hypersensitive response (HR) on the genotype H7998 carrying the corresponding resistance gene *Rxv*; *X. perforans*, tomato race 3 (T3), strains were determined to

carry *avrXv3*, which induces an HR in H7981 that contains the resistance gene *Xv3*(4, 100). In 2000, a new avirulence gene *avrXv4* was described in *X. perforans* strains based on reactions on the tomato genotype, LA716 (*Lycopersicon pinnellii*) which carries the *Xv4* resistance gene (3, 4). Therefore, *X. perforans* strains carrying this new avirulence gene (*avrXv4*) but lacking a functional *avrXv3*, have been designated as tomato race 4.

### **Epidemiology**

Leben (84) introduced the concept of a resident phase where the pathogen is able to replicate in the phyllosphere (leaf surface) without causing visible symptoms. *Xanthomonas euvesicatoria* has been shown to colonize pepper and tomato leaves epiphytically (93, 137). Long-term survival occurs in crop residue and volunteer plants (70, 140). Seed contamination was proposed as an important mechanism for transmission by Bashan *et al.* (6); however, Jones *et al.* (70) concluded that survival on seed occurs at extremely low levels and may be less important in the epidemiology of the disease than other inoculum sources when the pathogen is endemic. In soils artificially infested with *X.c. vesicatoria*, survival is poor with the bacterium being detected for only 16 days (6).

The bacterium gains entry into the plant when: (I) conditions are favorable for disease development (57), or (II) a threshold of epiphytic populations is reached (94) or (III) if a plant is compromised by wounding (156). *Xanthomonas euvesicatoria* enters the plant in many ways. Routine farming operations damage the plant causing wounds that act as entry points (6, 116). Epidermal abrasions, leaf hair breakage and water congestion of the intercellular spaces increase entry of the bacterium up to 100-fold over healthy plants (156). The bacterium can also enter through natural openings such as stomates and hydathodes (96, 123, 145). High humidity is conducive to bacterial ingress and survival; high relative humidity has been shown to increase

infection of *X. euvesicatoria* by 10- to 100-fold on tomato leaves compared to low humidity (151).

Dissemination of the bacterium is a major factor in the epidemiology of the disease. Overhead irrigation enhances dissemination compared to furrow irrigation (160). Dissemination also occurs in the form of aerosols and wind-blown rain (95, 162). Infected seed serves as a mechanism for dissemination (116). Farming practices, such as thinning, tying, and mechanized spraying also serve as factors in dissemination (116).

### **Disease Control**

Bacterial spot of tomato is difficult to control when high temperatures and high moisture exist. Bactericides, such as fixed coppers and streptomycin, have provided the major means of control (90, 142). Streptomycin-resistant mutants were rapidly selected on streptomycin-treated plants (142).

As a result of rapid selection for streptomycin-resistant mutants, copper compounds have been used almost exclusively. However, Marco and Stall in the 1980s (90) showed that many *X. euvesicatoria* strains were tolerant to copper and determined that copper resistance is mediated by genes located on a self-transmissible plasmid (143). Adding mancozeb, a fungicide, to copper sprays was shown to improve control efficiency (25) and was shown by Marco and Stall (90) to control copper-tolerant strains. However, they also showed that this treatment is insufficient when conditions favorable for disease development exist.

Because of the presence of copper-tolerant strains, other control strategies need to be considered. Identification of resistance genes and introgression into commercial genotypes has been a focus of breeding programs (135). There are currently no commercially available tomato varieties resistant to all races of bacterial spot.

## **Bacteriocins**

Bacteriocins are proteins or peptides with antibacterial properties, in most instances targeting related bacteria belonging to the same species or genus (135). Bacteriocins of Gram-positive bacteria, such as lactobacilli, typically are small peptides (40, 92). Bacteriocins from Gram-negative bacteria are often larger proteins among which the colicins from *Escherichia coli* represent the best-known examples (14, 66, 83, 91). There is considerable structural diversity among them which is reflected in widely different modes of action, including membrane disruption, non-specific degradation of nucleic acids and inhibition of peptidoglycan synthesis, and proteases.

Reports on the production of bacteriocin-like compounds by phytopathogenic bacteria are scarce. The observed report of a phytopathogenic bacterium producing bacteriocin-like compounds was reported by Okabe in the early 1950s (110). He reported that strains of *Pseudomonas (Ralstonia) solanacearum* were inhibitory exclusively to other *P. solanacearum* strains. Since then, these types of compounds have been reported for several other genera: *Agrobacterium* (77), *Clavibacter* (36), *Erwinia* (22, 37, 80) and *Pseudomonas* (30, 48, 82, 139, 152). There have been reports of xanthomonads producing bacteriocins as well (45, 155, 169).

In 1991, *X. perforans* were first identified in Florida. In fields where both *X. euvesicatoria* and *X. perforans* were present, the *X. perforans* strains became predominant (72). *In vitro* assays have shown that *X. perforans* strains inhibit growth of *X. euvesicatoria* strains (39). Jones *et al.* (71) characterized this relationship in greenhouse experiments tested on three genotypes including a T3 resistant genotype. Under field conditions *X. perforans* strains had a competitive advantage over *X. euvesicatoria* strains (71, 117). Tudor (153) identified at least three antagonistic compounds in *X. perforans* strains that closely resembled bacteriocins. These compounds were determined to have narrow inhibition spectra (restricted to *Xanthomonas*

strains) and fit the definition of a bacteriocin described by Reeves (125) based on the following criteria: (I) the presence of a biologically active protein moiety, (II) inducibility with mitomycin C and (III) non-self inhibition. All three bacteriocin-like groups (BcnA, BcnB and BcnC) were unique in activity and specificity against *X. euvesicatoria* strains (155).

### **The Type III Secretion System (T3SS)**

Pathogenicity of bacterial spot of tomato is determined by the type III secretion system (T3SS), which is highly conserved in most Gram-negative bacterial pathogens of plants and animals (149). The T3SS is composed of a secretion apparatus and an array of diverse proteins, known as effectors, that are injected into plant cells via the secretion apparatus (103).

The T3SS is encoded by a gene cluster termed the hrp (hypersensitive response and pathogenicity) cluster (1). One group of genes located within the hrp cluster encodes for a secretion apparatus known as the hrp pilus. The hrp pilus serves as a secretion apparatus for the translocation of T3SS effector proteins (59, 131). Hrp pilus mutants no longer cause disease in susceptible plants and are unable to induce resistance in resistant host and non-host plants (12, 16). Inside the host cell, type III effectors have specific functions and interact with specific targets in the host (17, 58, 63, 106); however, the functions of many effectors are unknown and their deletion produces no detectible phenotype.

Some plants have developed resistance to these invading pathogenic bacteria via resistance genes (R genes) which recognize specific effector proteins called avirulence (avr) genes. When the pathogenic bacterium injects an avirulence gene into a resistant plant carrying the corresponding R gene, an incompatible reaction, or HR, occurs which localizes the invading bacteria and limits secondary infection of surrounding cells (103).

## Biological Control

Biological control is another important approach for control of the disease. Pathogen resistance to fungicides has prompted interest in development of biocontrol agents (19), to provide additional tools for disease management. Unlike biocontrol of insects, biocontrol of plant diseases is a relatively new field. In the last 25 years biocontrol has become an established sub-discipline in plant pathology (113). *Agrobacterium radiobacter* strain K84, registered with the United States Environmental Protection Agency (EPA) for control of crown gall in 1979 (EPA registration number 11,4201), and was the first commercially available biological control agent against a bacterial plant disease (77). Since then, a total of 14 bacteria and 12 fungi have been registered with EPA for control of plant diseases (47, 112).

Several promising biological control approaches that include antagonistic microorganisms, natural fungicides and induced resistance are available for use in disease control today (38). However, achieving success using biocontrol agents for many bacterial diseases has been difficult. Some success has been achieved in this area through empirical selection of biocontrol agents, as indicated by the commercialization of the products Agriphage™ (a mixture of bacteriophages for control of bacterial spot of tomato (46)), Galltrol™, for control of crown gall, and BlightBan™ A506, for control of fire blight and frost injury (86). For bacterial spot of tomato, field experiments have been conducted utilizing a non-pathogenic bacteriocin-producing *X. perforans* strain to control disease incited by *X. euvesicatoria* strains (88). The non-pathogenic strain was able to reduce *X. euvesicatoria*-incited disease incidence and severity when applied prophylactically; however, the disease was still above acceptable levels (forty percent) (88).

For *Ralstonia solanacearum*, efforts to obtain a biological control strategy utilizing bacteriocin-producing non-pathogenic *hrp*<sup>-</sup> mutants gave low to moderate levels of control of

wildtype (wt) *R. solanacearum* (152). However, control using a partially pathogenic hrp mutant (*hrcV*), which is capable of higher levels of colonization of the root and stem tissue, achieved better control levels (49). Research into colonization has been conducted to understand the possible relationship between invasion efficiency of the biocontrol agent and its ability for disease control. Etchebar *et al.* (41) suggested that there was a positive correlation between colonization of the xylem by the hrp mutant and the level of control of the wt *R. solanacearum*.

The goal of this study was to evaluate a new biological control strategy utilizing pathogenicity-attenuated, bacteriocin-producing *X. perforans* strains for control of bacteriocin-sensitive strains of *X. euvesicatoria*. The objectives of this study were: (I) to further characterize the bacteriocins associated with *X. perforans*, (II) to identify and individually delete genes that contribute to pathogenicity to create less virulent mutants of *X. perforans*, and (III) to determine the ability of these pathogenicity-attenuated mutant strains to antagonize *X. euvesicatoria* *in vitro*, *in planta* and under field conditions.

## CHAPTER 2 CHARACTERIZATION OF GENETIC DETERMINANTS AND EVALUATION OF THEIR ROLE IN ANTAGONISM

Bacterial spot of tomato is incited by four *Xanthomonas* species: *X. euvesicatoria*, *X. vesicatoria*, *X. perforans* and *X. gardneri*. The first three bacterial species were previously known as tomato races 1 (T1), 2 (T2) and 3 (T3), respectively, based on their reaction on three tomato genotypes: Hawaii 7998 (H7998), Hawaii 7981 (H7981) and Bonny Best (71, 72, 139). *X. gardneri* has only been found in former Yugoslavia, Costa Rica and Brazil (10, 123, 143).

In 1991, *X. perforans* was first identified in Florida (72). In fields where both *X. euvesicatoria* and *X. perforans* were present, *X. perforans* became predominant (72). This phenomenon was due to bacteriocin-like activity of *X. perforans* strains (152). Bacteriocins are substances, usually proteinaceous, that are inhibitory or harmful toward closely related bacteria (124). Bacteriocins of Gram-negative bacteria represent a diverse group of proteins in terms of size, microbial target, mode of action and immunity mechanism. They are high molecular weight proteins that gain entry into susceptible cells by binding to surface receptors. Their mode of action varies from degradation of cellular DNA, to disruption of cleavage of 16S RNA, inhibition of synthesis of the peptidoglycan and pore formation in the cytoplasmic membrane (26).

The most extensively studied bacteriocins are the colicins produced by *Escherichia coli* (14, 66, 83, 91, 120, 121, 126, 163). A model system has been developed for proteinaceous bacteriocin production consisting of three components: the toxin, the immunity gene and a mechanism for delivery (126). Several known bacteriocins are transcribed in an inactive form (pre-bacteriocin), which, upon secretion, is processed to its active form (eg. Colicin V; (169)) (111).

Reports on the production of bacteriocin-like compounds by phytopathogenic bacteria are limited. In the 1950s Okabe *et al.* (110) published the first article on phytopathogenic bacteria, where strains of *Pseudomonas (Ralstonia) solanacearum* were inhibitory only to other *P. solanacearum* strains. Since the original description, production of such compounds has been reported for several other genera: *Agrobacterium* (77), *Clavibacter* (36), *Erwinia* (22, 38, 80), *Pseudomonas* (30, 82, 138, 151) and *Ralstonia* (41, 48). There have been a few reports of xanthomonads producing bacteriocins (45, 154, 168).

In order to further characterize the bacteriocin-like activity of *X. perforans*, a genomic library was screened to localize bacteriocin activity (154). Three groups of clones were identified that showed unique bacteriocin activity and all three bacteriocin-like groups (BcnA, BcnB and BcnC) were unique in activity and specificity based on *X. euvesicatoria*-sensitive strains (154). None of the clones conferred immunity to the other bacteriocins.

BcnA was localized to an 8.0-kb fragment containing seven open reading frames (ORF) identified in the sequenced region. The largest ORF (ORFA), approximately 3.6-kb, is required for BcnA<sup>+</sup> activity. The ORFA protein contains 1012 amino acids with a theoretical molecular weight of approximately 111-kDa. BcnA<sup>+</sup> activity was detected in unconcentrated, cell-free extracts of strains expressing ORFA. In some bacteria, an immunity function is necessary in order to avoid self-inhibition of the producing strain (111, 169). The putative immunity function of BcnA was mapped to a 4.5-kb *Bam*HI/*Eco*RI fragment downstream of ORFA (154).

Southern hybridization analysis using an ORFA-specific probe indicated that among bacterial spot strains tested, only *X. perforans* strains hybridized. Hybridization of the probe to a chromosomal location suggests that BcnA<sup>+</sup> is in the chromosomal DNA. Homology searches using the deduced amino acid sequence of the ORF revealed significant homology to only two

known proteins, WapA and Rhs. Both of these proteins contain multiple copies of an almost identical ligand-binding motif, thought to be involved in carbohydrate binding. Seven copies of a similar motif were found in ORFA.

*Xanthomonas campestris* pv. *glycines* (*X.c. glycines*) is one of the few xanthomonads that produces multiple bacteriocin-like compounds with activity against selected xanthomonads (45). According to Tudor (153) at least one of the *X.c. glycines* bacteriocin-like compounds is very similar in activity to BcnA. In *X.c. glycines*, bacteriocin-like compounds were heat sensitive and trypsin resistant (45), suggestive of the involvement of a high molecular weight protein.

Relatively little is known about BcnB and BcnC. BcnB and BcnC were previously subcloned to 8.9-kb and 5.1-kb fragments, respectively (154). Both were sequenced (60, 154). No immunity factor was associated with BcnB or BcnC activity. It is unknown how a heterologous strain that expresses either bacteriocin is not inhibited. Although the exact ORF involved in BcnC expression was not identified, one ORF within this fragment showed significant homology to extracellular metalloproteases secreted by *Aeromonas hydrophila*, *Armillaria mellea*, *Pleurotus ostreatus*, *Grifola frondosa*, *Aspergillus fumigatus* and *Penicillium citrinum* (153). Enzymes produced by bacteria may mimic the action of bacteriocins. The *zooA* gene of *Streptococcus zooepidemicus*, which encodes a bacteriocin-like inhibitory substance, contains a region with significant homology to several known endopeptidases (137).

It was reported that some proteinases and bacteriocins are secreted via the type two secretion pathway (T2SS) (2, 149, 160). In order to determine the involvement of the T2SS in *X. perforans* bacteriocin production, disruption mutants were created in the closely related bacteria, *X. euvesicatoria*, then transformed with a cosmid expressing either BcnA, BcnB, or BcnC. The T2SS is composed of 12 proteins (Xps) for translocating extracellular proteins across the outer

membrane in *Xanthomonas campestris* pv. *campestris* (*X.c. campestris*) (23). *X.c. campestris* secretes several hydrolytic enzymes, including  $\alpha$ -amylase, protease, pectate lyase and cellulase by the type 2 secretion pathway (23). The XpsD T2SS protein, an outer membrane lipoprotein, is required for protein secretion via the T2SS (23). An *XpsD* mutant from *Xanthomonas oryzae* pv. *oryzae* also lost T2SS function (50). Thus, *xpsD* was chosen for disruption to create a T2SS mutant. In this study we further characterized three bacteriocins of *X. perforans* to determine their activity, delivery mechanisms and immunity.

The goals of this study were (I) to further characterize the role of each bacteriocin-like compound secreted by *X. perforans*; (II) to determine the delivery mechanism of each bacteriocin and; (III) to understand the possible functions of each bacteriocin.

## **Materials and Methods**

### **Bacterial Strains, Plasmids and Culture Conditions**

Strains of *X. euvesicatoria* and *X. perforans* (Table 2-1) were grown on nutrient agar (NA) medium (Difco Laboratories, Detroit, MI) at 28°C. Strains of *Escherichia coli* (Table 2-1) were grown on Luria-Bertani (LB) medium at 37°C (97). All strains were stored in 20% glycerol in sterile tap water at -80°C. Antibiotics were used to maintain selection for resistance markers at the following concentrations: tetracycline (Tc) 12.5  $\mu$ g/mL; rifampicin (Rif) 100  $\mu$ g/mL; spectinomycin (Sp) 50  $\mu$ g/mL; kanamycin (Km) 25  $\mu$ g/mL; chloramphenicol (Cm) 34  $\mu$ g/mL; streptomycin (Sm) 200  $\mu$ g/mL and nalidixic acid (Nal) 50  $\mu$ g/mL.

### **DNA Manipulations**

Standard techniques for molecular cloning were conducted as described by Sambrook *et al.* (133). Restriction endonuclease digestions were performed according to manufacturer's specifications. All enzymes were obtained from Promega (Madison, WI) or Biolab (Ipswich,

MA). All DNA extractions were done as described by Sambrook *et al.* (135). T4 DNA ligase (M180A) was used according to manufacturer's specifications (Promega). Constructs were transformed into competent *Escherichia coli* DH5 $\alpha$  cells prepared as described in Sambrook *et al.* (135) and stored at  $-80^{\circ}\text{C}$  until transformations.

### **Construction of Bacteriocin Mutants**

According to an ORF search, there are 5 genes in the BcnA fragment, designated ORFA, ORF2, ORF3, ORF4, and ORF5. To characterize the function of BcnA, genes ORFA, ORF2, and ORF3 were disrupted either by deletion or transposon mutagenesis to create 91-118:: $\Delta$ ORFA, 91-118:: $\Delta$ ORF2, and 91-118:: $\Delta$ ORF3.  $\Delta$ ORF4 was previously disrupted (153). The 91-118:: $\Delta$ ORFA mutant was constructed by deleting an *EcoRV* and *BglIII* fragment.  $\Delta$ ORF2 and  $\Delta$ ORF3 mutants were created by using surrounding sequences up and downstream of the target ORFA. For ORFA, PCR was performed with primers A5 and A3 (Table 2-2), then the resulting PCR product was inserted upstream of ORF3 subcloned in the phagemid vector pBluescript II KS (pBS) (Stratagene, La Jolla, CA) (Figure 2-1a). Final  $\Delta$ ORF2 was moved into suicide vector pOK1 using restriction enzymes *ApaI* and *SpeI* (Figure 2-1b). To make 91-118:: $\Delta$ ORF3, PCR was performed with primers ORF2F and ORF3R, then the resulting PCR product was inserted upstream of ORF4 in pBS:ORF4 to create pBS: $\Delta$ ORF3. The fragment containing  $\Delta$ ORF3 was subcloned into suicide vector pOK1 with *ApaI* and *SalI* creating pOK1: $\Delta$ ORF3. The final plasmid constructs were mated into 91-118 to make each mutant via suicide vector-assisted mutagenesis as described previously (74). Candidates were screened using PCR primers designed to amplify flanking regions of the cross-over region (Table 2-2). Each mutant was tested for bacteriocin activity against *X. euvesicatoria* strain 91-106.

BcnB was disrupted by adding an insertion stop codon (TAA) using Quick-change XL Site Directed Mutagenesis kit (Stratagene).

### **Construction of T2SS Mutant**

Mutations were created to determine if BcnA, B and C are secreted by T2SS. In order to clone *xpsD* gene, primers *xpsDF* and *xpsDR* were designed using 85-10 genome sequence (149). A 2,229 bp *xpsD* gene was amplified and subcloned into pGEM vector (Promega). A chloramphenicol-resistance cassette from pRKP10 (123) was inserted in a *kpnI* site to disrupt *xpsD*. This disrupted *xpsD* gene was subcloned into suicide vector pOK1 with *ApaI* and *XbaI*. The final plasmid constructs were mated into 91-106 to make 91-106:: $\Delta xpsD$  via suicide vector-assisted mutagenesis as described previously (74). Candidates were screened using PCR primers designed to amplify flanking regions of the cross-over region (Table 2-2).

### **Bioinformatics Characterization of Bcn<sup>+</sup> Cosmid Clones**

Each ORF was analyzed for sequence homology (BlastP, <http://www.ncbi.nlm.nih.gov/BLAST/>), signal peptides and hydrophobicity (SOSUI, <http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html>), and localization (PSORT, <http://www.psort.org/>).

### **Subcloning of BcnB and BcnC**

The BcnB<sup>+</sup> clone pXV6.0 (6.0-kb BcnB<sup>+</sup> fragment) was subcloned using a *KpnI/EcoRI* fragment from pXV442 (BcnB<sup>+</sup>). The 6.0 kb fragment was sequenced (Appendix A-1). For sequence analysis (Figure 2-2), the *KpnI/EcoRI* region was cloned into pBS using *KpnI/EcoRI* enzymes and was sequenced using T7 and SP6 primers from pGEM and custom designed oligonucleotides generated by the DNA Sequencing Core Laboratory of the Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville, FL (UF-ICBR). DNA sequencing was performed by the DNA Sequencing Core Laboratory of UF-ICBR using the

Applied Biosystems model 373 system (Foster City, CA). Further subcloning of this fragment was performed using PCR primers (Table 2-2).

The BcnC pXV1.7 clone (1.7-kb BcnC<sup>+</sup> *Sall/EcoRI* fragment) was directionally cloned for expression in the plus and minus direction for expression of an extracellular metalloprotease (plus direction) or a glycine-rich protein (minus direction) (Figure 2-3).

### **Protease Activity Assay**

Proteolytic activity was measured by a diffusion assay in agar plates containing skim milk (casein) as substrate as described previously (34). Five microliters of each bacterial suspension were applied onto the surface of plates containing 20 mL of 0.5% (wt/vol) skim milk, 2% (wt/vol) agar and 50mM Tris hydrochloride (pH 8.0) and allowed to incubate for 24 h at 28°C. Zones of clearing around the bacteria due to the degradation of the substrate were measured.

### **BcnA Timing of Activation and Size Analysis**

Bacteriocin activity was assessed to determine which fraction(s) contain active BcnA protein. Supernatants and cells were collected from 18 h nutrient broth (NB) cultures. Cells were collected via centrifugation, suspended with phosphate buffered saline (PBS) (135) and sonicated using a digital Sonifier® unit model S-150D (Branson Ultrasonics Corporation, Danbury, CT). Fractions were sonicated for 30 s two times on ice. Supernatant and sonicated cell fractions were assessed for bacteriocin activity by plate assay described below.

Once fractions were prepared, size analysis was performed by separating cell fractions by Microcon protein filtration system (Millipore, Billerica, MA) with filter cut-offs of 50 kDa (YM-50) and 100 kDa (YM-100).

### **In vitro Antagonistic Assay**

Each mutant was evaluated for its relative bacteriocin activity produced toward a sensitive *X. euvesicatoria* strain based on an *in vitro* zone of inhibition assay (153). Strains to be tested

were shaken at 28°C overnight in NB. The cells were pelleted and resuspended in sterile tap water. Resuspended cells were then standardized to  $A_{600} = 0.3$  which is approximately  $5 \times 10^8$  CFU/mL. A 25  $\mu$ l sample was spotted on a Petri plate (100  $\times$  15mm) containing 20 mL NA (five samples per plate) and grown for 18 h at 28°C. After 18 h a suspension ( $5 \times 10^7$  CFU/mL) of *X. euvesicatoria* strain 91-106 (sensitive indicator strain) was sprayed over the plate using a Sigma aerosol spray unit (Sigma Chemical, St. Louis, MO). After 24-48 h incubation, zones of inhibition around the test strain were measured.

A second technique involved incubation of the plates for 24 h, killing the test strains by inverting glass plates over 2-3 mL of chloroform until all of the chloroform was evaporated, aerating the plates for 1 h and overlaying the agar surface with 3.5 mL of 0.3% water agar (50 °C) which contained 200  $\mu$ l of a  $5 \times 10^7$  CFU/mL cell suspension of the indicator strain (*X. euvesicatoria* strain 91-106). A clear zone of inhibition around test colonies after 24 - 48 h was considered indicative of antagonism and scored as bacteriocin-like (BcnA<sup>+</sup>) activity.

Cell-free extracts were screened for BcnA activity by growing the test cultures for 18 h in NB followed by centrifugation to pellet cells. The supernatant was then sterilized using a low protein binding Microcon filter (Amicon, Beverly, MA) with a 0.22  $\mu$ m pore size and analyzed for antagonism by the well diffusion assay method (146). Five millimeter diameter wells were cut into 20 mL NA plates. Wells were filled with 100  $\mu$ L of test filtrates and left for 18 h to allow diffusion of the liquid into the medium. Plates were then overlaid with 3.5 mL of soft agar containing 200  $\mu$ l of  $5 \times 10^8$  CFU/mL cell suspension of the indicator strain 91-106. Plates were examined after 24 h at 28°C. Each test was replicated three times.

## Evaluation of Immunity

*In vitro assays.* An immunity in vitro assay was conducted to determine the ORF responsible for BcnA immunity. All ORFs downstream of ORFA, individually (ORF2, ORF3, ORF4, ORF5) and in combination (ORF2 to 3 and ORF2 to 5). were subcloned into pLAFR119 and then mated into 91-106 to create 91-106 + pL:ORFA, 91-106 + pL:ORF2, 91-106 + pL:ORF3, 91-106 + pL:ORF4, and 91-118 + pL:ORF5. Next they were evaluated for immunity to 91-118:: $\Delta$ bcnBC, which expresses BcnA. The test strains were shaken at 28°C overnight in NB. The cells were pelleted and resuspended in sterile tap water. Antagonism assays were conducted using 91-118:: $\Delta$ bcnBC as the producing strain and the mutants with deletion in all ORFs downstream of ORFA as the test strain.

ORF5 was further tested to confirm its immunity. 91-106 containing either pLAFR119 (pL) or pLAFR119:ORF5 (pL:ORF5) were evaluated for sensitivity to 91-118:: $\Delta$ bcnBC (Table 2-1). Strains were shaken at 28°C overnight in NB tubes for 18 h. Cells were then washed, resuspended in sterile tap water and standardized to produce  $5 \times 10^6$  CFU/mL of 91-118:: $\Delta$ bcnBC, then incubated at 28°C. After 6 h incubation,  $5 \times 10^5$  CFU/mL of 91-106 strains (with pL or pL:ORF5) were added to the flasks. Samples were assayed at 24 h intervals for 96 h. Each experiment was conducted three times. Population data were transformed to logarithmic values and standard errors were determined.

*In planta assays.* The *X. perforans* and sensitive test *X. euvesicatoria* and transconjugant strains (91-106 + pL, 91-106 + pL:ORF2, 91-106 + pL:ORF3, 91-106 + pL:ORF4 and 91-106 + pL:ORF5) were inoculated at  $5 \times 10^7$  CFU/mL and  $5 \times 10^6$  CFU/mL, respectively. The 91-118 strains were inoculated into leaflets by infiltration 18 h prior to inoculation with the sensitive strain. Six-week-old seedlings of the tomato cultigen Florida 47 were inoculated (15 leaflets

each plant) using a hypodermic syringe as described previously (68). Following inoculation, plants were incubated at 24°C to 28°C. In order to determine populations of the sensitive test strain and transconjugants in leaflets, 1-cm<sup>2</sup> leaf disks were removed from inoculated areas, macerated in 1 mL sterile tap water and dilution plated onto NA amended with the appropriate antibiotic. Samples were assayed at 24 h intervals from 48 to 96 h. Each experiment was conducted three times. Population data were transformed to logarithmic values and standard errors were determined.

## **Results**

### **Sequence Analysis of Genes Involved in BcnA Activity**

Previously a 12.1 kb fragment (pXV12.1) was shown to contain five ORFs (ORFA, ORF2, ORF3, ORF4 and ORF5) potentially important for expression of and for immunity to BcnA (56). Each putative ORF product was evaluated for presence of a signal peptide and localization (Table 2-3). Based upon sequence analysis ORFA is predicted to be a water soluble protein, with a hydrophobicity value of -0.56. It has a predicted location in the bacterial cytoplasm (0.56) with no predicted signal peptides. The putative ORF2 product has an N-terminal signal peptide, a hydrophobicity value of 0.28 and is predicted to be localized to the bacterial outer membrane (0.926) or the bacterial periplasmic space (0.175). The ORF3 product has an N-terminal signal peptide, a hydrophobicity value of 0.20, and an estimated localization to either the bacterial periplasmic space (0.939) or the bacterial outer membrane (0.326). The ORF4 has an N-terminal signal peptide, a hydrophobicity value of -0.36, and two transmembrane helices, from AA148 to 170 (VTAVAPPPTPTFQPAILTLGAVL) and from AA 176 to 198 (PAAVSWVSPIMGSIVLAPVLYFA). The ORF4 product is predicted to be located in the bacterial inner membrane (0.187). The ORF5 product has no signal peptide, a hydrophobicity

value of 0.166, and is predicted to be a water soluble protein located in the bacterial inner membrane (0.109).

### **BcnA Activity Requires ORFA, ORF2, ORF3 and ORF4**

To further analyze the function of BcnA, ORFA, ORF2, ORF3 and ORF4 were individually disrupted (91-118:: $\Delta$ ORFA, 91-118:: $\Delta$ ORF2, 91-118:: $\Delta$ ORF3, 91-118:: $\Delta$ ORF4). Each mutant was tested for bacteriocin activity against *X. euvesicatoria* strain 91-106. Three mutants, 91-118:: $\Delta$ ORFA, 91-118:: $\Delta$ ORF2 and 91-118:: $\Delta$ ORF4, lost inhibition activity against the T1 strain (Figure 2-4). 91-118:: $\Delta$ ORF3 had inhibition activity, but it was reduced compared to 91-118.

### **Localization of BcnA Activation**

Bacteriocins are produced either in an active or inactive (pre-bacteriocin) form, which is activated during its secretion. In order to determine the location of BcnA activation, different cell fractions of a 24 h broth culture of 91-118 were tested for bacteriocin activity. Bacteriocin activity was only found in the supernatant (Figure 2-5). No activity was observed in supernatant from the less than 50 kDa fraction; however, supernatant from 50 to 100 kDa and above 100 kDa had inhibitory activity (Figure 2-6), confirming previous results (154). In order to determine if bacteriocin activity was associated with the cell fraction of 91-118, cells were disrupted by sonication, intact cells removed by filtration through a 0.22  $\mu$ m filter and then the bacteriocin activity was checked by plate assay. The cell fraction did not have activity (Figure 2-5).

### **Identification of the Immunity Gene**

A 4.5-kb fragment downstream of ORFA was previously found to contain the immunity gene (154). This fragment contains ORF2, ORF3, ORF4 and ORF5 (Figure 2-1). In order to identify which gene was responsible for immunity *X. euvesicatoria* strains were created that expressed each gene under a lac promoter in pLAFR119 (Table 2-1). The positive control

91-106 + pXV12.1 and 91-106 + pL:ORF5 were not sensitive to 91-118:: $\Delta$ *bcnBC* while 91-106 + pL:ORF2, 91-106 + pL:ORF3 and 91-106 + pL:ORF4 were sensitive (Figure 2-7).

In an *in vivo* experiment, bacterial populations of 91-106 + pL:ORF5 and 91-106 + pLAFR119 (empty vector) strains co-inoculated with 91-118:: $\Delta$ *bcnBC* were determined. 91-106 + pL:ORF5 reached concentrations of  $10^5$  to  $10^6$  CFU/cm<sup>2</sup> tomato leaf tissue in the presence of BcnA, whereas 91-106 + pLAFR119 was reduced to thousand-fold less at 100 CFU per mL after 9 h (Figure 2-8). Similarly, greenhouse experiments showed that 91-106 + pL:ORF5 was able to establish population an average of 1.5 log higher than 91-106 + pLAFR119, when co-inoculated with 91-118:: $\Delta$ *bcnBC* in leaf tissue (Figure 2-9). In addition, 91-118:: $\Delta$ ORFA, 91-118:: $\Delta$ ORF2, 91-118:: $\Delta$ ORF3 and 91-118:: $\Delta$ ORF4 mutants all maintained insensitivity to 91-118:: $\Delta$ *bcnBC* in plate antagonism assays. These results clearly demonstrate that ORF5 confers immunity.

### **Sequence Analysis of BcnB and BcnC**

It has been shown previously (154) that plasmid pLAFR3 carrying 5.8-kb (pLB5.8) and 5.1-kb (pLC5.1) DNA fragments, BcnB and BcnC, respectively, conferred bacteriocin activity to sensitive *X. euvesicatoria* strains. In order to identify genes involved in BcnB and BcnC activity, subclones of different regions of those DNA fragments were created in pLAFR119 (Figures 2-2 and 2-3). Each subclone was expressed in *X. euvesicatoria* strains ME90 or 91-106 and the ability to produce inhibition was tested on NA media using strain 91-106 as an indicator. BcnB and BcnC subcloned to 3.0-kb and 1.7-kb DNA fragments, respectively, carried on plasmid pLAFR119, were the smallest fragments that conferred bacteriocin activity (Figure 2-2 and 2-3). The nucleotide sequence revealed two complete ORFs named *bcnB* and *bcnC*. BcnB shows homology to endoprotease Arg-C with a predicted amino acid size and molecular mass of 466 aa and 48, 487 MW. BcnB has no N-terminal signal peptide. BcnB is predicted to be located in the bacterial outer membrane (0.933) or periplasm (0.258). BcnC shows homology to extracellular

metalloprotease with a predicted amino acid size and molecular mass of 401aa and 42, 471 MW, respectively. BcnC has no N terminal signal peptide. BcnC is predicted to be located in the bacterial inner membrane (0.351). The introduction of a stop codon (TAA, Table 2-2 in **bold**) at the 5' end just downstream of the ATG start codon disrupted BcnB activity when expressed in ME90 (ME90 + pL3.0mut) compared to the control (ME90 + pL3.0) (Figures 2-2 and 2-10).

Plasmid pLAFR119 has only lac promoter. Directional cloning of BcnB and C genes was performed in pLAFR119. Plasmids pLB5.8 and pLC5.1 actively expressed BcnB and BcnC, respectively, without aid of the pL lac promoter (Figure 2-2 and 2-3), suggesting that their native promoters are functional. BcnB was subcloned to a 3.0 kb fragment. An ORF with endoprotease Arg-C homology was determined to be responsible for BcnB<sup>+</sup> activity based on analysis with of a stop codon (TAA) insertion in the forward direction (Figure 2-2). For BcnC, a 1.7 kb fragment of BcnC was directionally subcloned in pLAFR119 in both directions. The reverse direction BcnC (pL1.7CR) gave very slight bacteriocin activity compared to under direction of the lac promoter (Figure 2-3).

### **Purification and Characterization of BcnB and BcnC**

Purification was conducted to evaluate BcnB and BcnC activity. Bacterial supernatant was concentrated with Microcon YM-100 (Millipore, Billerica, MA) or TCA precipitation (135). These concentrated samples were run on an SDS-PAGE gel and detected using Coomassie Brilliant Blue 250 (Pierce Biotechnology, Rockford, IL) or Silver staining (BioRad Laboratories; Hercules, CA). No bands were detected.

ME90 expressing ORFA was protease negative; however, ME90 expressing BcnB and BcnC produced clearing zones (2.0 cm and 3.1 cm, respectively) typical of protease activity (Figure 2-11). Size exclusion analysis was conducted using T3 strain 91-118:: $\Delta$ ORFA

supernatants. Protease activity was observed from total cell or filtrates of less than 50-kDa with only minor activity above 50-kDa.

### **Type II Secretion Mutant Lost Secretion of Amylase and Bacteriocins**

The role of the T2SS on delivery was determined for each bacteriocin by plate inhibition assay. Confirmation of deletion of *xpsD* was performed via PCR and analysis of starch hydrolysis media in XpsD mutants (Figure 2-12). The *X. euvesicatoria* T2SS *xpsD* mutant expressing clones, 91-106 + pXV12.1, 91-106 + pL5.8 and 91-106 + pL5.1, were unable to produce a zone of inhibition in plate assays while wt 91-106 expression of each clone produced typical zones for each bacteriocin.

### **Discussion**

In this study, bacteriocins of *X. perforans* were further characterized to determine their activity and possible functions. Disruption of ORFA, ORF2 and ORF4 abolished BcnA activity, suggesting that BcnA is part of a multiple component family of bacteriocins. ORF5 was shown to encode the immunity function for BcnA, making normally sensitive *X. euvesicatoria* strains insensitive. This information and the predicted localization in the inner and outer membrane of the cell suggests that these ORFs make up the necessary parts of a three component system (the toxin, immunity and a mechanism for delivery) of a typical Gram-negative bacteriocin outlined by Riley and Wertz (128). Because of size selection (>100 kDa), ORFA is suggested to be the toxin, ORF2, ORF3 and ORF4 proteins are responsible for delivery and/or possible processing of BcnA (ORFA product) and ORF5 is the immunity function. All bioinformatics results (SOSUI and PSORT) suggest ORFA is a soluble cytoplasmic protein. BcnA was only detected in supernatants and not in detectable levels in the cell fraction of BcnA producing *X. perforans* cells. These results suggest BcnA may be activated upon secretion. ORF3 is included in the model because a mild reduction in antagonism was associated with ORF3 disruption. Based on

predicted localization to the periplasm and outer membrane, perhaps ORF3 aids in transfer of pre-bcnA (once pre-bcnA is in the periplasm) to ORF2 on the outer membrane. ORF2, ORF3 and ORF4 appear to play secondary roles such as in transport, modification or secretion of BcnA. The zone inhibition assay and growth rate *in vitro* and *in planta* experiments strongly suggest that ORF5 is responsible for the immunity function. SOSUI predicted ORF5 would localize to the bacterial inner membrane supported by a positive hydrophobicity value (0.2). This may suggest ORF5 disrupts BcnA or prevents delivery of active BcnA from entry into the cell. This is similar to what has been found for the immunity function of Colicin V (ColV) of *E. coli* (169).

Col V is one of many known multiple component bacteriocins previously described (8, 99, 169). ColV was used as a reference for basic components of a Gram-negative bacteriocin (42, 43, 55, 56, 64, 169). ColV immunity was previously shown to prevent insertion of ColV into the inner membrane of sensitive strains of *E. coli* (169).

Based on localization analysis (PSORT) we predicted the localization of each ORF involved in BcnA production. Based on this information and what is known for ColV, we have developed a basic model for BcnA (Figure 2-13). For the BcnA model, all predicted locations for ORFs involved in BcnA activity were based on predicted localization and deletion analysis. The model suggests four steps: (I): pre-BcnA delivery into the periplasm with help of or chaperoned by ORF4; (II) Processing of pre-BcnA and delivery of the active BcnA outside of the cell by ORF2 and ORF3; (III) Entry of active BcnA into cells (unknown); (IVa) BcnA suppressed by ORF5; and (IVb) BcnA inhibition (either in the periplasm or in the cytoplasm).

Previously BcnB activity was localized to a 5.9 kb fragment (153). Only two ORFs were found within this fragment that contained homology to genes of known function. One was an

amino acid transporter and the other an endoprotease Arg-C. Both genes were isolated and tested for bacteriocin activity. Only fragments containing the intact endoprotease like gene were active. This ORF was confirmed using an inserted STOP codon (TAA) at the 5' end of the fragment which in turn lost activity confirming that the endoprotease was responsible for the bacteriocin-like activity. Endoprotease Arg-C is a family of serine endoproteases which cleaves carboxyl peptide bonds of arginine residues. The enzyme has also been shown to cleave Lys-Lys and Lys-Arg bonds (119).

BcnC was previously localized to a 1.7 kb fragment (61). Two possible ORFs were located within this fragment one in the plus and one in the minus direction. Directional cloning analysis shows that the plus directional ORF was responsible for BcnC activity. This gene had high homology to an extracellular metalloprotease gene family. Metalloproteases are proteolytic enzymes which use a metal for their catalytic mechanism. Most metalloproteases are zinc-dependent, while some use cobalt (3).

BcnB and BcnC were tested for protease activity based on homology data. Our findings show that both BcnB and BcnC exhibited protease activity as determined by casein degradation analysis; however, BcnB produced smaller protease zones than BcnC. The results of the protein size filtration data were consistent with the predicted size of BcnB (48 MW) and BcnC (42 MW).

The ORFs responsible for BcnA activity were identified and their possible roles have been hypothesized. Further research is needed to determine their specific roles. Only one gene was determined to be necessary for expression of BcnB or BcnC. The protease assays have determined their roles as proteases; however, further research is necessary to determine the target of these proteases within sensitive strains.

Table 2-1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference*
<b><i>Xanthomonas euvesicatoria</i></b>		
E3-1	Nal <sup>R</sup> Sm <sup>R</sup>	(61)
ME-90	Rif <sup>R</sup> Km <sup>R</sup>	(154)
91-106		(154)
91-106::Δ <i>xpsD</i>	XpsD <sup>-</sup> Cm <sup>R</sup>	This study
<b><i>X. perforans</i></b>		
91-118	Rif <sup>R</sup>	(154)
91-118::ΔORFA	ORFA <sup>-</sup> Rif <sup>R</sup>	This study
91-118::ΔORF2	ORF2 <sup>-</sup> Rif <sup>R</sup>	This study
91-118::ΔORF3	ORF3 <sup>-</sup> Rif <sup>R</sup>	This study
91-118::ΔORF4	ORF4 <sup>-</sup> Rif <sup>R</sup>	This study
91-118::ΔORF5	ORF5 <sup>-</sup> Rif <sup>R</sup>	This study
<b><i>Escherichia coli</i></b>		
DH5α	F <sup>-</sup> <i>recA</i>	BRL
C2110	Nal <sup>R</sup>	BRL
λPIR	Host for pOK1; Sp <sup>R</sup> oriR6K RK2 replicon	UB
<b>Plasmids</b>		
pBluescript-KS+	Phagemid, pUC derivative; Amp <sup>R</sup>	Stratagene
pLAFR119	Tc <sup>R</sup> rlx <sup>+</sup> RK2 replicon	BJS
pRK2073	Sp <sup>R</sup> tra <sup>+</sup> mob <sup>+</sup>	(28)
pRKP10	Cm <sup>R</sup> cassette	(123)
pOK1	Suicide vector; <i>SacB</i>	(63)
pL5.8	pLAFR119 <i>KpnI/EcoRI</i> 5.8-kb BcnB <sup>+</sup> fragment	This study
pL3.0	pLAFR119 <i>KpnI/XbaI</i> 3.0-kb BcnB <sup>+</sup> fragment	This study
pL3.0mut	pLAFR119 <i>KpnI/XbaI</i> 3.0-kb BcnB <sup>-</sup> fragment	This study
with a TAA stop codon insertion		
pLKH	pLAFR119 <i>KpnI/HinDIII</i> 2.3-kb BcnB <sup>-</sup> fragment	This study
pLHX	pLAFR119 <i>XbaI/HinDIII</i> 2.4-kb BcnB <sup>-</sup> fragment	This study
pL5.1	pLAFR119 <i>HinDIII/EcoRI</i> 5.1-kb BcnC <sup>+</sup> fragment	This study

\* BRL, Bethesda Research Laboratories, Gaithersburg, MD; Stratagene, Stratagene Inc., La Jolla, CA; BJS, B. J. Staskawicz, University of California, Berkeley, CA; UB, U. Bonas, Martin-Luther-Universität, Halle, Germany.

Table 2-2. PCR primer used in *bcnA*, *bcnB* and *bcnC* analyses for genetic manipulation

plasmid	Primer name	restriction site	Primer sequence	length	GC (%)	Tm (C)	
pLAFR	NP5		CCCTTCACCAAGTTCGACGACA	22	54.5	61	
	NP3		GCGGGTGCCGTGCTCGTGTT	20	70	65	
BcnA	A5	<i>XhoI</i>	CCTCGAGATGCGCCACCCGTCGG	16	75	60	
	A3	<i>XhoI</i>	CCTCGAGCAGCAAAAGCTGATAGAGC	19	47.3	54	
	ORF5F	<i>HinDIII</i>	GGGGAAGCTTCAGGGTGGCGGCAAGGGA	27	70.3	70.2	
	ORF5R	<i>HinDIII</i>	GGGGAAGCTTGGGCTTCTCTGGAAGCGGAC	29	65.5	69.5	
	ORF3F	<i>HinDIII</i>	CCCGAAGCTTCCGGTTGACCTCTATGTAGATGGATGC	36	55.6	68.8	
	ORF3R	<i>HinDIII</i>	CCCGAAGCTTCCCAGTGCAAATGTAAGCCGCGAC	33	60.6	69.6	
	ORF2F	<i>HinDIII</i>	GGGGAAGCTTACACAGGACGGGACATGCACAG	31	61.3	68.9	
	ORF2R	<i>HinDIII</i>	GGGGAAGCTTACAACCTCCACATCTCGCACCG	31	61.3	68.9	
	ORF4F	<i>HinDIII</i>	CCCAGCTTGCCGGATGCGACATTGTTGCGC	31	61.3	70.2	
	ORF4R	<i>HinDIII</i>	CCCAGCTTGCTTGTTCAAGTCATCACC	30	53.3	66.5	
BcnB	B5' new	<i>EcoRI</i>	CGGAATCCAATCGCAAGAACGCGATG	21	50	63.4	
	B32	<i>KpnI</i>	CGGGTACCCTGGCCGAAGTAGGTGGAAT	21	52.3	69.3	
	BORF1F		ATGGGCTTGTCGGCCACATAATCGTCACAA	30	50	68.1	
	BORF1R		TTGTGACGATTATGTGGCCGACAAGCCCAT	30	50	68.1	
	BORF2F		AACGAACGAAGTTACTGCTCCACCAT	30	50	68.1	
	BORF2R		ATGGTGGAGCCAGTGTAACCTTCGTTTCGTT	30	50	68.1	
	BORF3F		ATGAATCGCAAGTAAGCGATGTATCTGGCG	30	46.7	66.8	
	BORF3R		CGCCAGATACATCGCTTACTTGCGATTTCAT	30	46.7	66.8	
	BORF4F		ATGGCTGCAAATTGATAATGCGCTCACGGT	30	46.7	66.8	
	BORF4R		ACCGTGAGCGCATTATCAATTTGCAGCCAT	30	46.7	66.8	
	BORF5F		CAGCCGCGGATTAGATGACCATTGCCAT	30	56.7	70.9	
	BORF5R		ATGGCAATGGTCATCTAATCGCGGGGCTG	30	56.7	70.9	
	BORF7F		ATGGACGATCGTAACCGTCGATCCGCTTC	30	56.7	70.9	
	BORF7R		GAAGCGGATCGACGGTTAGCGATCGTCCAT	30	56.7	70.9	
	B5XhoI	<i>XhoI</i>	CCTCGAGATGAATCGCAAGAACGCG	18	52.3	58	
	BcnC	C5'	<i>EcoRI</i>	CGGAATCCGTGAAGAACGTCTTCCTC	27	51.9	54
		C3'	<i>KpnI</i>	GGGTACCCTTGTCGTCATCGTTCTGCGCCGAGTGTT	37	50	54
C5XhoI		<i>XhoI</i>	CCTCGAGGTGAAGAACGTCTTCCTC	18	61.1	58	
T2SS	xpsDF		ATGACGCCGCGCCTGTTTCC	20	65	58	
	xpsDR		CCCTTCTCAAGTGGCTGCAT	20	60	58	

Table 2-3. Characterization of bacteriocin ORFs associated with the expression of BcnA, BcnB and BcnC

		AA <sup>a</sup>	Size (kDa)	Signal Peptide <sup>b</sup>	Localization <sup>c</sup>	Location value <sup>c</sup>	hydrophobicity <sup>b</sup>
BcnA	ORFA	1012	111.0	none	bacterial cytoplasm	0.56	-0.56
	ORF2	124	13.6	MTLCIFLPLLLCAKASAAPYVVMGNIVTR	Outer membrane or periplasmic space	0.926 & 0.175	0.28
	ORF3	100	11.0	MRFYRISLLALIIFASPRASA	Outer membrane or periplasmic space	0.326 & 0.939	0.20
	ORF4	290	31.9	MNKCSDAYGIYLRTLFVFFMYTLFCTSASAVIRY	inner membrane	0.187	-0.36
	ORF5	145	15.9	none	inner membrane	0.109	0.17
BcnB	<i>bcnB</i>	465	48.5	none	Outer membrane or periplasmic space	0.933 & 0.258	-0.07
BcnC	<i>bcnC</i>	400	42.5	none	inner membrane	0.351	-1.44

<sup>a</sup> BcnA ORF predicted amino acid size (AA) were previously described (154).

<sup>b</sup> Prediction of signal peptide was performed with SOSUI (<http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html>).

<sup>c</sup> Prediction of localization was performed using PSORTb v.2.0 (<http://www.psort.org/>).

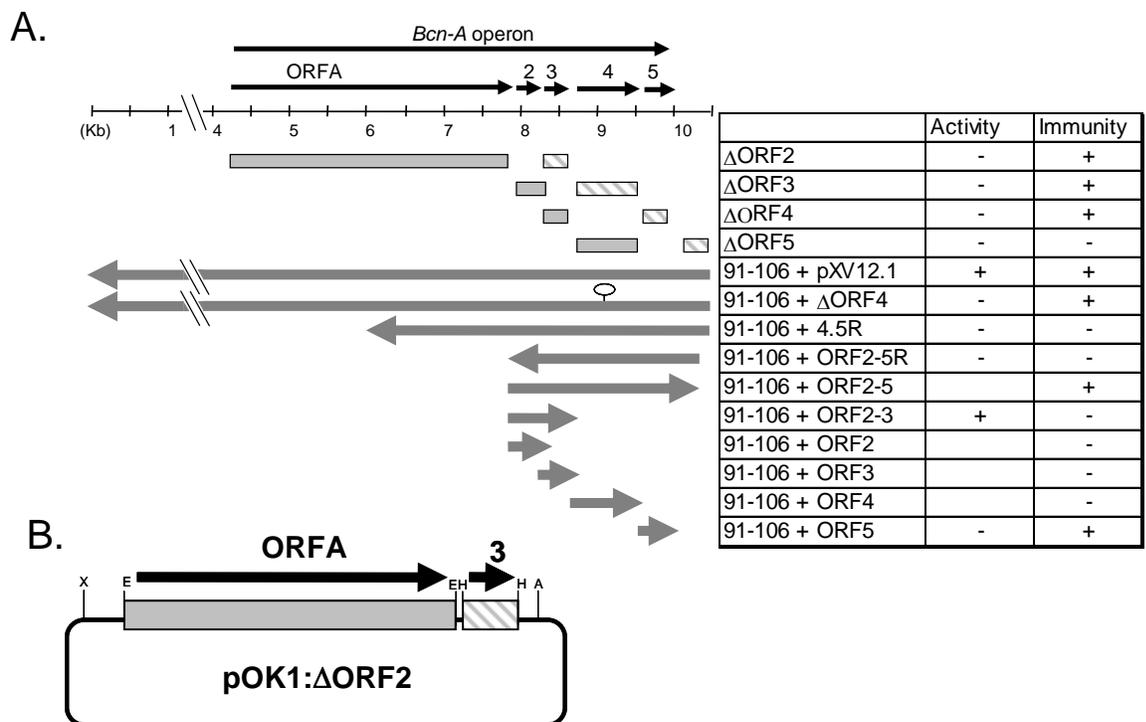


Figure 2-1. BcnA diagram showing position of individual ORFs and positions and fragment constructs. The grey arrows indicate the directional expression of fragments under the lac promoter of pLAFR119. The circle on ME90 +  $\Delta$ ORF4 indicates the location of a transposon insertion. B. Diagram of deletion construct for ORF2.

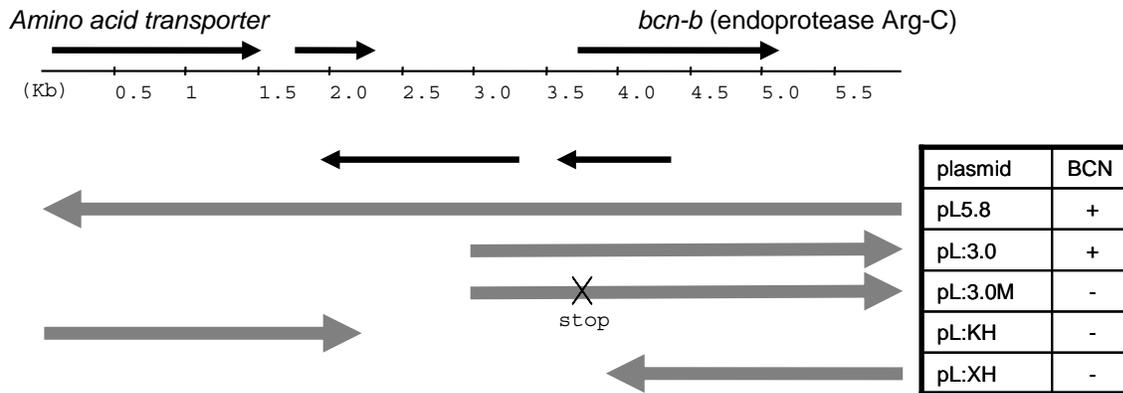


Figure 2-2. BcnB diagram showing position of individual ORFs and directional expression (grey arrows) and activity (table) of fragment constructs under the lac promoter of pLAFR119. The x indicates an artificial stop codon (TAA) insertion in frame of the *bcnB* sequence.

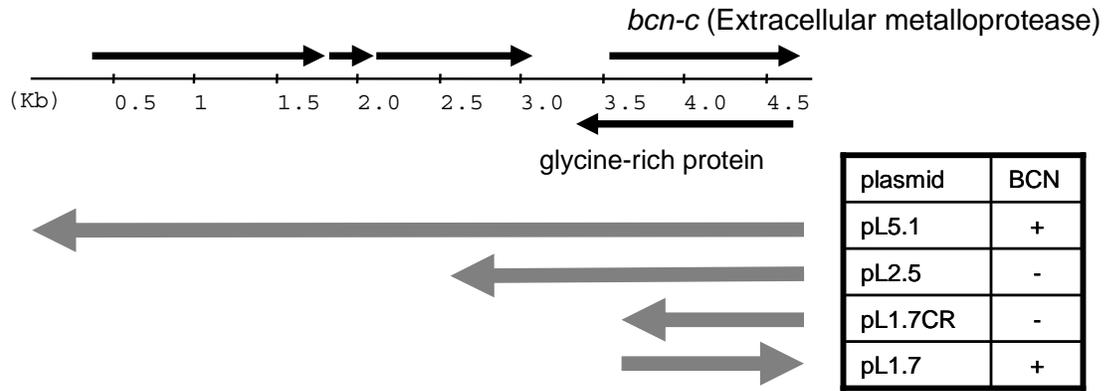
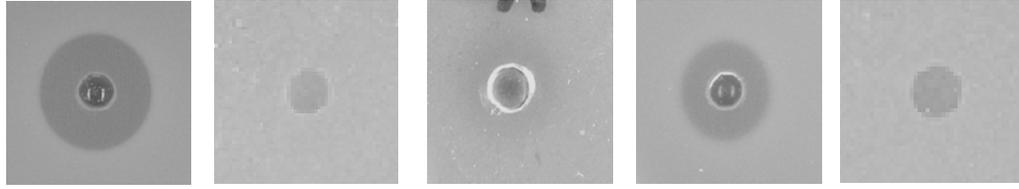


Figure 2-3. BcnC diagram showing position of individual ORFs and directional expression (grey arrows) and activity (table) of fragment constructs under the lac promoter of pLAFR119.



Test Strain	WT T3	$\Delta$ ORFA	$\Delta$ ORF2	$\Delta$ ORF3	$\Delta$ ORF4
inhibition zone	+++	-	-	++	-

Figure 2-4. Antagonism assays of bacteriocin-like activity against *X. euvesicatoria* strain 91-106 were assessed using cell-free supernatants of individual ORF knockout mutants of 91-118.

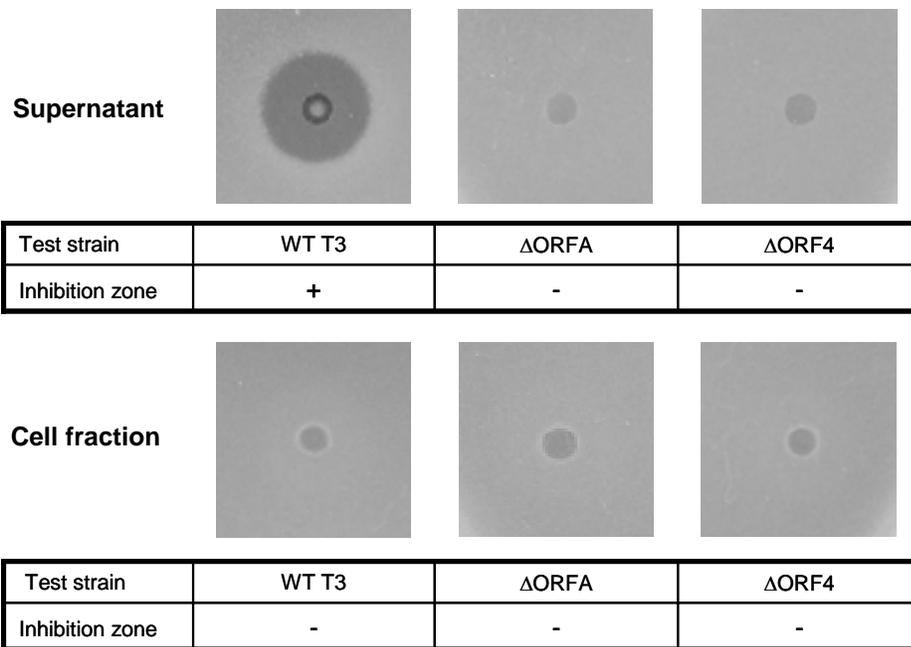
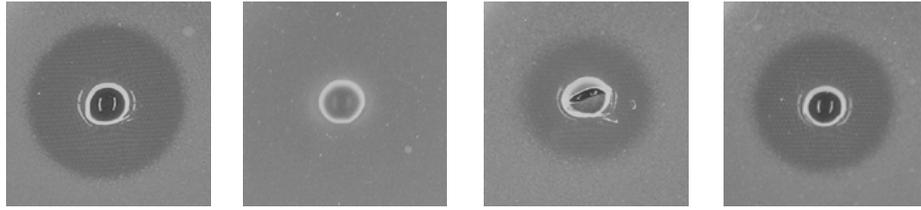


Figure 2-5. Antagonism activation assays of bacteriocin-like activity against *X. euvesicatoria* strain 91-106. Supernatant from *X. perforans* was separated into supernatant and cell fractions.



Size range (kDa)	All	X < 50	50 < X < 100	X > 100
Inhibition Zone	+++	-	++	++

Figure 2-6. Secretion assays of bacteriocin-like activity against *X. euvesicatoria* strain 91-106. Supernatant from *X. perforans* was separated based on size exclusion technique into three fragments, less than 50, 50 to 100 and over 100 kDa.

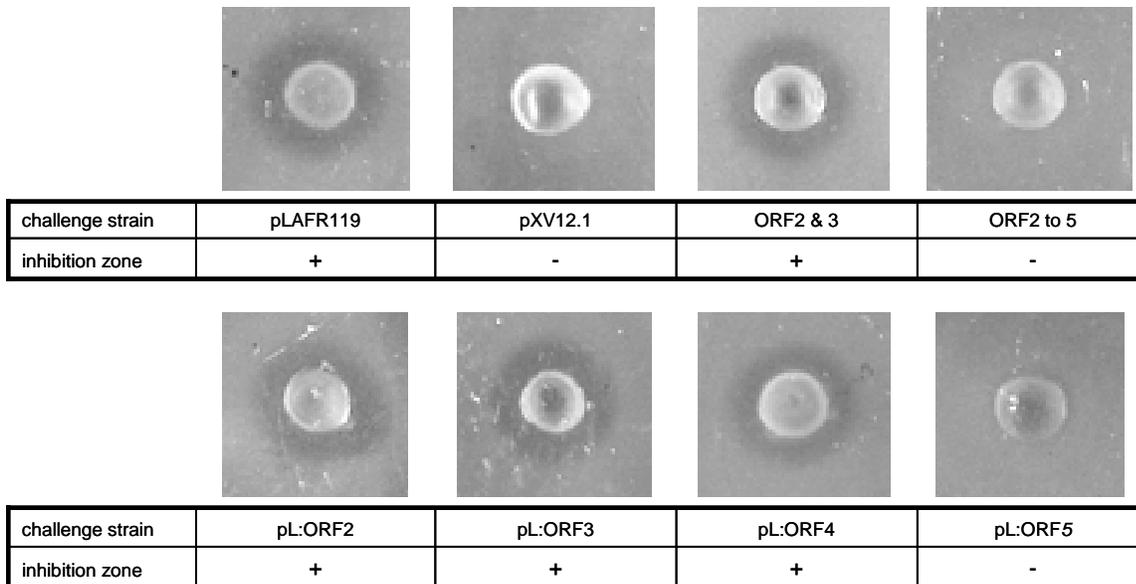


Figure 2-7. Immunity assays of bacteriocin-like activity against *X. euvesicatoria* strain 91-106 using 91-118:: $\Delta bcnBC$  as the producing strain. Bacteriocin sensitivity candidates were screened to identify ORFs expressing immunity.

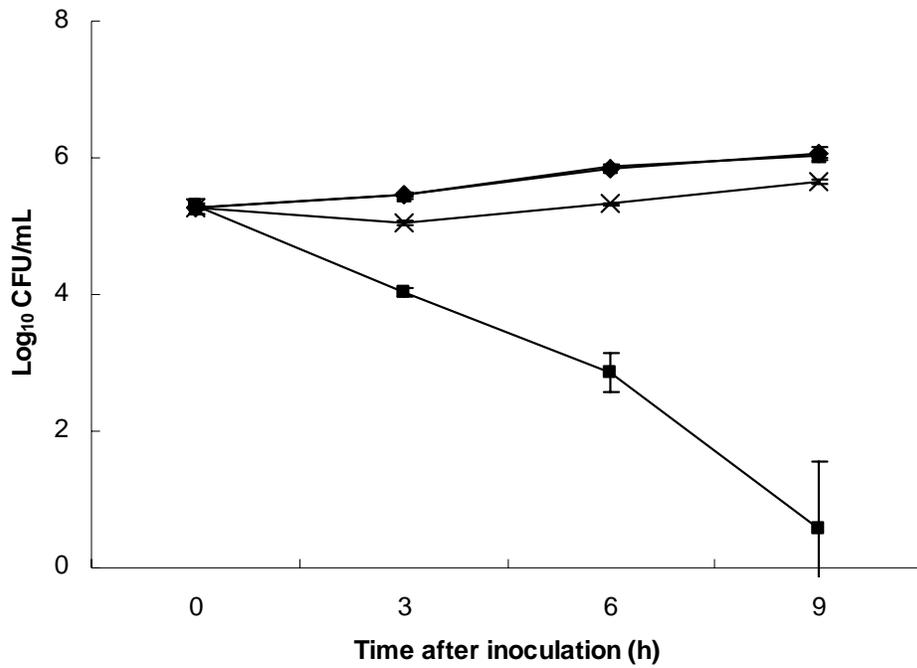


Figure 2-8. *In vitro* populations of *X. euvesicatoria* strain 91-106 transconjugants with [pLAFR119 (■) and pLAFR:ORF5 (x)] and without [pLAFR119 (◆), pLAFR119:ORF5 (▲)] co-inoculation of 91-118:: $\Delta bcnBC$ . Error bars indicate the standard error.

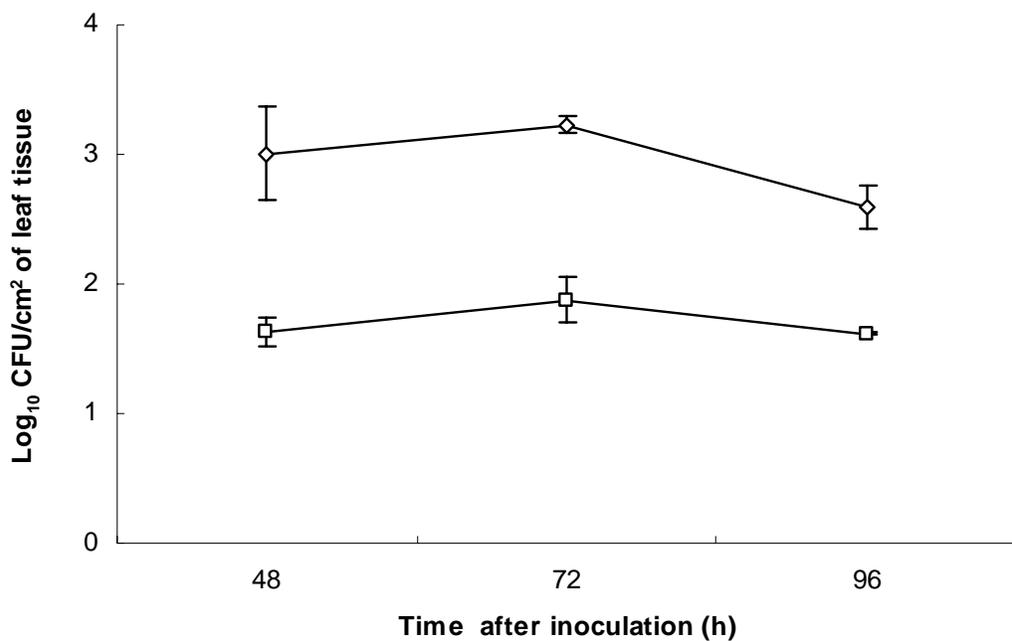
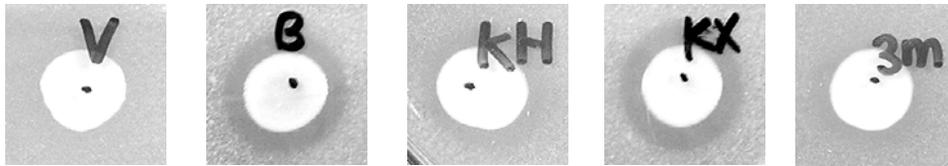


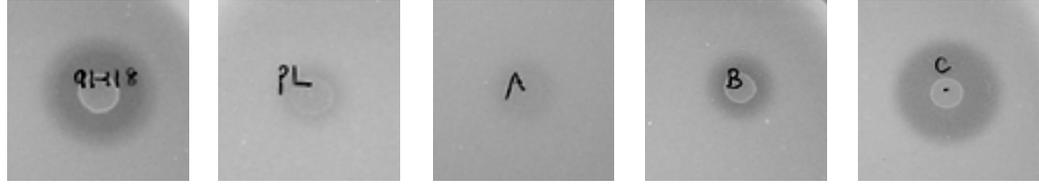
Figure 2-9. *In planta* populations of *X. euvesicatoria* strain 91-106 containing plasmid pLAFR119 (□) or pLAFR119:ORF5 (◇), respectively, when co-inoculated with 91-118::  $\Delta bcnBC$  (BcnA expression only) in leaflets of tomato cultivar Bonny Best. Error bars indicate the standard error.



Test Strain	ME90 + pLAFR119	ME90 + pL5.8	ME90 + pLKH	ME90 + pL3.0	ME90 + pL3.0mut
inhibition zone	-	+	-	+	-

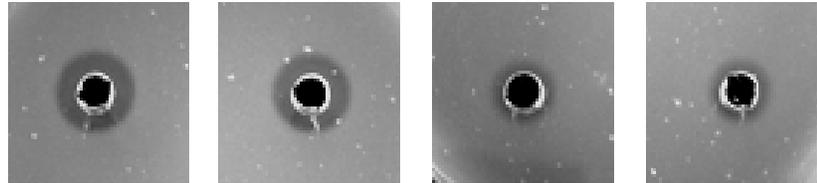
Figure 2-10. Antagonism assays of bacteriocin-like activity against *X. euvesicatoria* strain 91-106 were assessed using cell-free supernatants of individual ORF knockout mutants of 91-118.

**A.**



Strain	WT T3	ME90 + pLAFR	ME90 + A	ME90 + B	ME90 + C
Protease activity	+++	-	-	+	+++

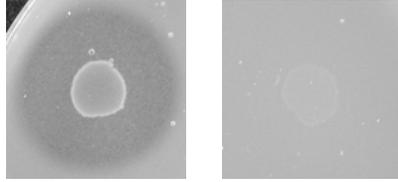
**B.**



Size Range (kDa)	All	X < 50	50 < X < 100	X > 100
Protease activity	+++	+++	+	+

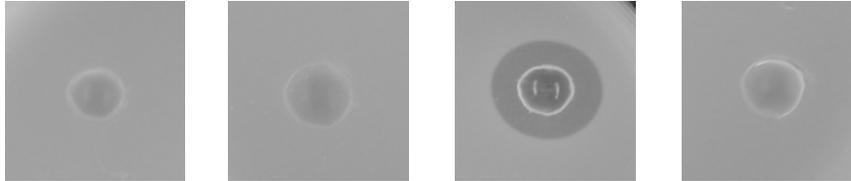
Figure 2-11. Protease activity of bacteriocin candidates. A. Protease assay where 5  $\mu$ L of  $5 \times 10^8$  CFU/mL of each strain was plated onto 0.5% skim milk agar and incubated for 24 h at 28°C. B. Fractions from *X. perforans* were separated based on size exclusion technique into three fragments, less than 50, 50 to 100 and over 100 kDa and analyzed for protease activity.

**A.**



Test strain	91-106	91-106:: <i>ΔxpsD</i>
Protease activity	+	-

**B.**



Test strain	91-106	91-106 + pL	91-106 + pXV12.1	91-106:: <i>ΔxpsD</i> + pXV12.1
BcnA activity	+++	-	++	++

Figure 2-12. Evaluation of type two secretion system mutant *xpsD*. A. Proteinase assay of 91-106 and 91-106::*ΔxpsD*. B. Antagonism assays of BcnA using cell-free supernatants using *X. euvesicatoria* strain 91-106 as an indicator strain.

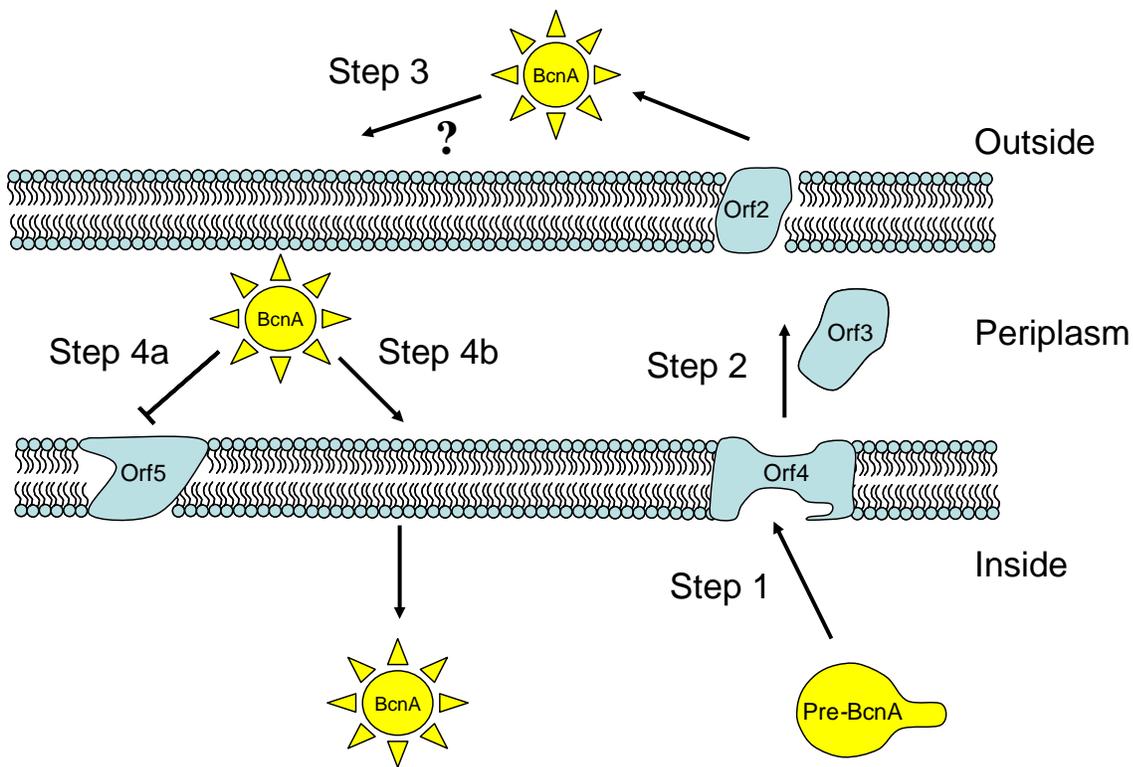


Figure 2-13. BcnA Model for ORFs predicted involved in BcnA activity based on predicted localization and deletion analysis. Step 1: pre-BcnA delivery into the periplasm by ORF4. Step 2: Processing of pre-BcnA and delivery of the active BcnA outside of the cell by ORF2 and ORF3. Step 3: Entry of active BcnA into cells (unknown). Step 4 a: BcnA suppressed by ORF5. Step 4b: BcnA inhibition (either in the periplasm or in the cytoplasm).

### CHAPTER 3

#### ANALYSIS OF PATHOGENICITY MUTANTS OF *XANTHOMONAS PERFORANS* AND THEIR EFFECT ON BACTERIOCIN EXPRESSION

Fresh-market tomato production accounts for more than 50% of the harvested acres and 63% of the national yield in the southeastern U.S., with approximately 40,000 acres in Florida (29). Bacterial spot of tomato, incited by *Xanthomonas euvesicatoria*, is a devastating disease of tomato in Florida, the Caribbean and worldwide (11, 108, 114, 118, 132). There are at least three important management strategies to reduce severity and incidence of bacterial tomato diseases: reducing initial inoculum, minimizing plant susceptibility (natural resistance) and chemical control (copper-based chemicals and antibiotics). Although chemical control using copper bactericides is routinely used to control bacterial spot, these efforts are often futile because of the presence of copper-tolerant strains of the bacterium (90). Adding mancozeb, a fungicide, to copper sprays was shown to improve control efficiency and was shown by Marco and Stall (90) to control copper-tolerant strains. However, they also showed that this treatment is insufficient when conditions favorable for disease development exist.

Recently, new strategies have emerged that could be utilized as alternative management practices. These include using bacteriophages specific to the target bacterium (5), application of plant activators that induce systemic acquired resistance (SAR) in the plant to bacterial pathogens (89) and application of bacterial biological control agents (49, 67). These new strategies focus on reducing initial epiphytic or internal populations of the potential pathogen. Several studies on xanthomonads have shown a relationship between epiphytic populations of the pathogen and disease severity (73, 81). Lindemann *et al.* (86) demonstrated a strong correlation between a threshold level of epiphytic populations of *Pseudomonas syringae* and the occurrence of disease in the field. Reducing epiphytic populations may play a key role in a successful biological control strategy.

Recently Hert *et al.* (61) demonstrated that populations of *X. euvesicatoria* strains were reduced significantly by bacteriocin-producing *X. perforans* strains in the field. These results are supportive of the potential of bacteriocins for controlling bacteriocin-sensitive strains and may provide a new approach to biological disease control. Non-pathogenic Hrp mutants of *X. perforans* provided significant levels of control (88, 102) against bacteriocin sensitive strains; however, there were still unacceptable levels of disease. In other studies, Hrp mutants were created in a bacteriocin expressing *Ralstonia solanacearum* strain for control of wild-type (wt) *R. solanacearum* strains (41, 49, 152). These studies revealed that slightly pathogenic hrp mutants that were able to achieve higher levels of root and stem tissue colonization provided higher levels of control than other hrp mutants with minimal colonization capabilities (49, 152). Etchebar *et al.* (41) suggested that there was a relationship between the degree of colonization of the xylem by the mutant and the level of control of the wt *R. solanacearum*. *X. perforans* also causes disease, therefore if *X. perforans* is to be used as a biological control agent, it is necessary to reduce the virulence of *X. perforans* to levels that are not deleterious to the plant.

There has been progress in identifying genes involved in bacterial virulence of *Xanthomonas* species (3, 4, 44, 58, 64, 165). Identification of these genes as potential targets to create strains attenuated in virulence has become much easier as a result of genome sequencing of xanthomonads. Thieme *et al.* (150) sequenced the *X. euvesicatoria* genome and estimated that 480 putative pathogenicity factors and associated genes are found in the genome. These factors were placed into six categories: (I) secretion systems, (II) flagellum, (III) secreted proteins (via type III secretion system (T3SS)), (IV) detoxification, (V) surface structure and adhesion and (VI) quorum sensing. Based on previous studies, several candidate genes (*opgH*, *avrBs2*, *hpaA*,

*hpaB*, *hpaC*, *xopA*, *xopD* and *gumD*) appeared interesting for use in this study (18, 17, 21, 63, 76, 101, 106).

One known *X. perforans* pathogenicity-attenuating mutant served as our model candidate, *opgH<sub>Xcv</sub>*. The *opgH* mutant was previously shown to have an attenuated virulence phenotype (101), exhibiting reduced disease severity and growth curve in susceptible tissue and triggering delayed hypersensitive reaction (HR) in a resistant plant genotype. A second candidate gene, the effector *avrBs2*, was chosen as another candidate as a result of its role in virulence of *X. euvesicatoria* in pepper (76). It has been shown that *avrBs2* mutants are less virulent on susceptible hosts (53, 76, 146).

Genes *hpaA*, *hpaB* and *hpaC* were shown to be involved in virulence. These genes play a role in pathogenicity as an effector (*hpaA*) and as chaperones (*hpaB* and *hpaC*) of effectors of the T3SS (18, 17, 63). HpaA appears to function as an effector molecule in *X. euvesicatoria* since disruption of *hpaA* eliminates disease symptoms in tomato and pepper plants without affecting the ability to elicit a hypersensitive response. HpaB and HpaC were shown to form an oligomeric protein complex and interact with two classes of effectors (class A containing XopJ and XopF1 and class B containing AvrBs3 and XopC) and HrcV of the T3SS (18).

Two genes that code for proteins that are designated *Xanthomonas* outer proteins (Xop) were also chosen as potential candidates that may affect virulence. XopA and XopD are secreted by the T3SS and thus represent putative effector proteins. XopA is necessary for both *in planta* growth and full virulence (106).

GumD of the *gum* operon is involved in xanthan gum biosynthesis (7, 75). Xanthan gum is a high molecular weight extracellular heteropolymer produced by xanthomonads and has been

implicated as a virulence factor based on deletion analysis in *X. c. pv. campestris*. Xanthan gum minus *gumD* mutants exhibited dramatic delay in disease symptoms (21).

In this study, mutations were created in the candidate genes described above to determine their role in pathogenicity and possible use as pathogenically-attenuated bacteriocin-expressing biological control agents.

## **Materials and Methods**

### **Bacterial Strains, Plasmids and Culture Conditions**

Strains of *X. perforans* and *X. euvesicatoria* were grown on nutrient agar (NA) medium (Difco Laboratories, Detroit, MI) at 28°C (Table 3-1). Strains of *Escherichia coli* were grown on Luria-Bertani (LB) medium at 37°C (Table 3-1) (97). All strains were stored in 20% glycerol in sterile tap water at –80°C. Bacterial cultures for plant inoculations were grown in nutrient broth (NB) (Difco Laboratories, Detroit, MI) for 18 h at 28°C with shaking (100 rpm). Cells were pelleted by centrifugation (4,000 × *g*, 15 min) and resuspended in sterile tap water. Bacterial suspensions were standardized to an optical density at 600 nm (OD<sub>600</sub>) = 0.3 (5 × 10<sup>8</sup> CFU/mL) with a Spectronic 20 spectrophotometer (Spectronic UNICAM, Rochester, NY) and subsequently diluted in sterile tap water to appropriate cell densities for individual experiments. Antibiotics were used to maintain selection for resistance markers at the following concentrations: tetracycline (Tc) 12.5 µg/mL; rifampicin (Rif) 100 µg/mL; spectinomycin (Sp) 50 µg/mL; kanamycin (Km) 50 µg/mL; chloramphenicol (Cm) 34 µg/mL; streptomycin (Sm) 200 µg/mL; and nalidixic acid (Nal) 50 µg/mL.

### **Plant Material**

Seeds of tomato (*Lycopersicon esculentum*) cv. Bonny Best were planted in Plugmix (W. R. Grace & Co., Cambridge, MA). After 2 weeks, the emerged seedlings were transplanted to

Metromix 300 (W. R. Grace & Co.) in 10-cm plastic pots. Seedlings were grown in the greenhouse at temperatures ranging from 25 to 35°C.

### **Primer Design**

Candidate genes (Table 3-2) were amplified using primers designed as follows. BLAST search analysis was conducted to locate conserved regions flanking the candidate gene sequence by scanning genomic sequences of closely related *Xanthomonas* species (i.e. *X. euvesicatoria* strain 85-10 (NC\_007508.1), *X. oryzae* pv. *oryzicola* (AY875714.3), *X. oryzae* pv. *oryzae* strain KACC10331 (NC\_006834.1), *X. axonopodis* pv. *citri* strain 306 (NC\_003919.1) and *X. campestris* pv. *campestris* strain ATCC 33913 (NC\_003902.1). Primers were designed to conserved regions for amplification of the corresponding regions in *X. perforans* strain 91-118 (Table 3-2). Candidates were confirmed by sequence analysis. Sequencing of the clones was conducted at the ICBR sequencing facility (University of Florida, Gainesville, FL) with the Applied Biosystems model 373 system (Foster City, CA).

### **Generation of Mutants**

Candidate genes for creating attenuated mutants of *X. perforans* (*avrBs2*, *hpaA*, *hpaB*, *hpaC*, *xopA* and *xopD*) were disrupted using either restriction digestion or PCR-assisted deletion mutagenesis. All candidate genes were amplified and deleted using PCR primers described in Table 3-2. Each attenuated candidate was individually cloned into pGEM (Promega, Madison, WI) or pTOPO (Invitrogen, Carlsbad, CA) then an internal fragment was deleted using restriction digestion or PCR assisted deletion mutagenesis and replaced with a chloramphenicol resistance cassette.

An *avrBs2* mutant was created as follows. A fragment containing *avrBs2* was amplified by PCR using primers *avrBs2F* and *avrBs2R* and cloned into pTOPO to create pTOPO:*avrBs2*. For disruption of *avrBs2*, two primer pairs, *avrBs2F*, *avrBs2DD2F*, *avrBs2DD2R* and *avrBs2R*

were utilized to amplify portions of the 5' and 3' ends separately to delete a 174-bp fragment not amplified in either set of primers (Figure 3-1A). Both fragments (5' and 3' products) were cloned into pTOPO creating pTOPO:avrBs2F and pTOPO:avrBs2DR. Primers avrBs2DD2F and avrBs2DD2R contain artificial *SalI* restriction sites. The pTOPO:avrBs2-3 was then digested with *SalI* (artificially created by avrBs2DD2R) and pTOPO restriction site *SpeI*, then ligated into pTOPO:avrBs2DF, as illustrated in Figure 3-1A, to create pTOPO: $\Delta$ avrBs2. Next, the artificial *SalI* restriction enzyme sites from avrBs2DD2F and avrBs2DD2R were utilized to insert a Cm resistance gene cassette (124) to create pTOPO: $\Delta$ avrBs2. Finally, using pTOPO's MCS restriction sites *BamHI* and *ApaI*, the deleted *avrBs2* was ligated into pOK1 to create pOK1: $\Delta$ avrBs2. The final construct was then mated into 91-118 as described later in this section.

The *hpaA* gene was amplified by PCR using primers hpaAF and hpaAR, containing artificial *HinDIII* restriction sites and cloned into pGEM to create pGEM:*hpaA*. For disruption of *hpaA* divergent PCR primers hpaADF and hpaADR were utilized to delete a 446-bp internal fragment of *hpaA* (Figure 3-1B). Artificial *SmaI* restriction enzyme sites were added by primers hpaADF and hpaADR, then utilized to insert a Cm resistance gene cassette (124) to create pGEM: $\Delta$ *hpaA*. Finally, using pGEM's MCS restriction sites *SpeI* and *ApaI*, the deleted *hpaA* was ligated into the pOK1 to create pOK1: $\Delta$ *hpaA*. The final construct was then mated into 91-118 as described later in this section.

For deletion of *hpaB*, a fragment containing *hpaB* was amplified by PCR using primers hpaBF and hpaBR, containing artificial *HinDIII* restriction sites (not used in this study) and cloned into pGEM to create pGEM:*hpaB*. For disruption of *hpaB*, a Cm resistance gene cassette (124) was inserted into a native *KpnI* restriction site within *hpaB* to create pGEM: $\Delta$ *hpaB*.

Finally, using pGEM's MCS restriction sites *SpeI* and *ApaI*, the deleted *hpaB* was ligated into the pOK1 to create pOK1: $\Delta$ *hpaB*. The final construct was then mated into 91-118 as described later in this section.

A fragment containing *hpaC* was amplified by PCR using primers *hpaCF* and *hpaCR* and cloned into pGEM to create pGEM:*hpaC*. For disruption of *hpaC*, divergent PCR primers *hpaCDF* and *hpaCDR* were utilized to delete a 58-bp internal fragment of *hpaC*. Artificial *HinDIII* restriction enzyme sites were added by *hpaCDF* and *hpaCDR* and utilized to insert a Cm resistance gene cassette (124) to create pGEM: $\Delta$ *hpaC*. Finally, using pGEM's MCS restriction sites *SpeI* and *ApaI*, the deleted *hpaC* was ligated into the pOK1 to create pOK1:  $\Delta$ *hpaC*. The final construct was then mated into 91-118 as described later in this section.

A fragment containing *xopA* was amplified by PCR using primers *XopAF* and *XopAR*, containing artificial *HinDIII* restriction sites, and cloned into pGEM to create pGEM:*xopA*. For disruption of *xopA* two *AvaI* restriction sites, located within pGEM:*xopA* were utilized. This *AvaI* deletion completely deleted *xopA* leaving flanking DNA for marker exchange. Finally, using pGEM's MCS restriction sites *SpeI* and *ApaI*, the deleted *xopA* was ligated into the pOK1 to create pOK1: $\Delta$ *xopA*. The final construct was then mated into 91-118 as described later in this section.

A fragment containing *xopD* was amplified by PCR using primers *xopDF* and *xopDR* and cloned into pGEM to create pGEM:*xopD*. For disruption of *xopD* divergent PCR primers *xopDDF* and *xopDDR* were utilized to delete a 134-bp internal fragment of *xopD*. Artificial *HinDIII* restriction enzyme sites were added by *xopDDF* and *xopDDR* were utilized to insert a Cm resistance gene cassette (124) to create pGEM: $\Delta$ *xopD*. Finally, using pGEM's MCS

restriction sites *SpeI* and *ApaI*, the deleted *xopD* was ligated into the pOK1 to create pOK1: $\Delta$ *xopD*. The final construct was then mated into 91-118 as described later in this section.

A fragment containing *gumD* was amplified by PCR using primers GumDF and GumDR and cloned into pGEM to create pGEM:*gumD*. Two *NcoI* restriction sites within pGEM:*gumD* were used for disruption of *gumD*. This *NcoI* deletion removed a 400bp segment of the *gumD* gene leaving 5' and 3' portions of flanking DNA for marker exchange. Finally, using pGEM's MCS restriction sites *SpeI* and *ApaI*, the deleted *gumD* was ligated into the pOK1 to create pOK1: $\Delta$ *gumD*. The final construct was then mated into 91-118 as described later in this section.

All candidates were confirmed by PCR using primers pOK1F and pOK1R (Table 3-2) for sequencing of each candidate deletion at the DNA Sequencing Core Laboratory, as mentioned previously. Once sequence analysis confirmed deletion of each candidate, suicide vector assisted mutagenesis was performed as described previously (74). Candidates were screened using PCR primers designed to amplify flanking regions of the cross-over region (Table 3-2).

Deletion mutant 91-118:: $\Delta$ *avrBs2* was also confirmed in resistant cultivars of tomato (transgenic VS36 containing 35S::*Bs2* (148)) and pepper (ECW-20R) for loss of HR. Leaves were infiltrated with a bacterial suspension of  $5 \times 10^8$  CFU/mL using a hypodermic syringe as described previously (68) and scored for presence or absence of a hypersensitive response (HR) after 24 h and observed for 72 h.

### **Growth Room Growth Curve Assays**

Growth room assays were conducted to compare the growth curve of the deletion mutants with that of parent strain 91-118. The strains were inoculated at  $3 \times 10^5$  CFU/mL into leaflets of 6-week-old seedlings of the tomato cultivar Bonny Best. Leaflets were infiltrated (15 leaflets per strain) using a hypodermic syringe and needle, as described previously (68). Following

inoculation, plants were incubated at 24°C to 28°C. Three samples were taken for each treatment every 24 h for 96 h. Populations were quantified by macerating 1-cm<sup>2</sup> leaf disks in 1 mL sterile tap water and dilution plating onto NA medium amended with the appropriate antibiotic. Plates were incubated at 28°C and colonies were counted after 48 to 72 h. Population data were log transformed and standard errors were determined. The overall growth curve was determined by calculating the area under the population progress curve (AUPPC). The AUPPC is a modification of the area under the disease progress curve (AUDPC) which has been used to analyze population progress (136): standardized AUPPC =  $\sum [(x_i + x_{i-1})/2](t_i - t_{i-1})$ , where  $x$  is population density in log<sub>10</sub> CFU per cm<sup>2</sup> and  $t$  is time in hours. The AUPPC values for the strains were compared by analysis of variance and subsequent separation of sample means by Waller-Duncan multiple range test using SAS version 9.0 (SAS Inc., Cary, NC). Each experiment was conducted three times.

### **Greenhouse Disease Severity Assay**

Greenhouse pathogenicity assays were conducted to compare symptom development incited by the mutants and wt 91-118. In each test, four young Bonny Best plants (four-true-leaf stage) were inoculated with each strain by dipping into sterile tap water suspensions containing  $3 \times 10^6$  CFU/mL of bacteria and 0.025% Silwet L-77 (Loveland Industries, Inc., Greeley, CO) for 15 s. Plants were maintained in the greenhouse during the evaluation period. The plants were assessed for disease severity 14 to 21 days after inoculation. Disease assessments were made based on leaf and stem ratings compiled from three separate greenhouse inoculation tests.

### **Growth Room Antagonism Assay**

Antagonism assays were performed to determine the effect of wt and mutant *X. perforans* strains on a sensitive *X. euvesicatoria* strain E3-1. Internal and external/leaf surface (phyllosphere) populations were evaluated using two different antagonism assay techniques.

*Internal antagonism assay.* Six-week-old seedlings of the tomato cultivar Florida 47 were inoculated with a  $5 \times 10^7$  CFU/mL suspension of *X. perforans* (15 leaflets per strain) using a hypodermic syringe as described previously (68) followed 12 h later by injecting a  $5 \times 10^6$  CFU/mL suspension of bacteriocin-sensitive *X. euvesicatoria* strain E3-1. Each treatment consisted of three replications. Following inoculation, plants were incubated at 24°C to 28°C. In order to determine populations of the sensitive strain (E3-1<sup>SmNal</sup>) in leaflets, 1-cm<sup>2</sup> leaf disks were removed from inoculated areas, macerated in 1 mL sterile tap water and dilution plated onto NA amended with Nal and Sm to quantify E3-1 populations. Samples were assayed at 24 h intervals for 96 h. Each experiment was conducted three times. Population data were log transformed and standard errors were determined. AUPPC values (calculated as described above) were compared by analysis of variance and subsequent separation of sample means by Waller-Duncan multiple range test using SAS version 9.0 (SAS Inc., Cary, NC).

*Phyllosphere antagonism assay.* Growth room phyllosphere antagonism assays were conducted to determine if the gene deletions affected the levels of antagonism toward external leaf populations of *X. euvesicatoria* strain E3-1 by comparing the antagonistic ability of the mutants with the parent strain 91-118. Six-week-old Bonny Best tomato seedlings were dipped into a suspension of the wt or mutant strain of *X. perforans* adjusted to  $5 \times 10^7$  CFU/mL amended with Silwet L77 (0.025%). Seven days later the plants were sprayed with a  $5 \times 10^7$  CFU/mL suspension of *X. euvesicatoria* strain E3-1. Following spray inoculation, plants were incubated at 24°C to 28°C. Leaf tissue was sampled every 24 h for 96 h to quantify E3-1 populations. Three leaflets were taken at each time point. Each leaflet was weighed, placed in a polyethylene bag containing 10 mL of sterile tap water and shaken on a wrist action shaker (Burrel Co., Oakland, CA) for 20 min. The leaf-wash was then dilution plated on NANalSm to

selectively determine the concentration of E3-1 colonies. Population data were analyzed following log<sub>10</sub> transformation and standard errors were determined. AUPPC values (calculated as described above) were compared by analysis of variance and subsequent separation of sample means by Waller-Duncan multiple range test using SAS version 9.0 (SAS Inc., Cary, NC). Each experiment was conducted three times.

## Results

### Sequence Analysis of Attenuated Mutant Candidate Genes

The eight candidate genes selected for disruption (Table 3-3), were amplified and cloned from *X. perforans* strain 91-118 and sequenced (Appendix A-2, A-3, A-4, A-5, A-6 and A-7). Sequence analysis of each gene was conducted to determine relatedness between *X. perforans* AA sequence and other proteins using BLAST search protocol (Blastp) at the NCBI website (<http://www.ncbi.nlm.nih.gov/BLAST/>). For *hpaA*, *hpaB*, *hpaC* and *avrBs2* the nucleotide and deduced amino acid sequences of these genes had very high homology (75% to 100%) (Table 3-4) to the corresponding genes in other xanthomonads such as *X. euvesicatoria* strain 85-10 (NC\_007508.1), *X. o. oryzicola* (AY875714.3), *X. o. oryzae* KACC10331 (NC\_006834.1), *X. a. citri* strain 306 (NC\_003919.1), *X. c. campestris* strain ATCC 33913 (NC\_003902.1) and *X. c. glycines* (AF499777.1) (Appendix A-8, A-9, A-10 and A-11 and Table 3-4). XopA and XopD had less AA homology than all other attenuated mutant candidate genes (Appendix A-12 and A-13 and Table 3-4). XopA only had high homology to *X. euvesicatoria* strain 85-10 (100%) and much lower homology with *X. c. glycines* (47%). XopD only had high homology to *X. euvesicatoria* strain 85-10 (85%) and *X. c. campestris* strain ATCC 33913 (74%). XopD was also found to have 86% nucleotide homology with *P. syringae* pv. *eriobotryae* gene *psvA* (AB018553).

## Population Dynamics and Pathogenicity Assays

In growth room experiments, there were three separate groups according to overall AUPPC values. The first group includes wt 91-118, 91-118:: $\Delta hpaB$  and 91-118:: $\Delta avrBs2$ . Although 91-118:: $\Delta avrBs2$  was not considered significantly different overall, it was significantly different at the 96 and 120 h time points according to standard error. Populations of 91-118 and 91-118 $\Delta hpaB$  exhibited a normal growth curve over the 120 h sample period and, based on the AUPPC (Table 3-5). The second group included 91-118:: $\Delta xopA$  and 91-118:: $\Delta gumD$ . These mutants were not significantly different from each other and grew 0.5 to 0.75 log<sub>10</sub> CFU/mL lower than wt 91-118 throughout the experiment (Figure 3-2). 91-118:: $\Delta hpaC$  was between groups according to overall significant difference and was not considered significantly different according to standard error at 48 and 72 h from 91-118:: $\Delta xopA$  and 91-118:: $\Delta gumD$ . The third group consisted of only 91-118:: $\Delta opgH$ . 91-118:: $\Delta hpaC$  was considered significantly different from 91-118:: $\Delta opgH$  at 24, 48 and 72 h according to standard error. 91-118:: $\Delta opgH$  consistently grew 1 to 1.5 log<sub>10</sub> CFU/mL lower than wt 91-118. 91-118:: $\Delta xopA$  and 91-118:: $\Delta avrBs2$ . The largest reduction was observed for 91-118:: $\Delta opgH$  which was consistently significantly lower populations over the experiment.

Greenhouse disease severity experiments were conducted to determine the effects of the mutations on the ability of 91-118 to cause disease *in planta*. 91-118:: $\Delta xopA$  and 91-118:: $\Delta opgH$  mutants induced 1.5 to 3.2 times less disease than 91-118 (Figure 3-15 and Table 3-5). The largest reduction in disease severity was observed in 91-118:: $\Delta opgH$  (Table 3-5). The *avrBs2* disruption mutant was confirmed on pepper and tomato genotypes expressing the *Bs2* corresponding *R* gene. A resistance response (HR) was only observed in tomato leaves infiltrated with wt 91-118, infiltration with 91-118:: $\Delta avrBs2$  did not give an HR (Figure 3-3).

## Antagonism Assays

Growth room (internal and external) antagonism assays were conducted to determine the antagonistic ability of deletion-mutant candidates toward E3-1. Treatment with water prior to *X. euvesicatoria* resulted in a normal growth curve over the 96-h sampling period in both experiments based on the AUPPC (Figures 3-4 and 3-5).

All attenuated mutant candidates significantly reduced *X. euvesicatoria* populations in the internal antagonism experiment (Figures 3-5). Wt 91-118 gave the most significant reduction in E3-1 populations. There were 2 groups of mutants from this experiment, however all were not significantly different from one another. 91-118:: $\Delta hpaB$ , 91-118:: $\Delta xopA$  and 91-118:: $\Delta gumD$  gave the most reduction of the mutants tested, however they were not significantly different from any the other strains overall. Looking at standard error, however, they appeared to be significant from *hpaC* at 96 h. The second group of mutants (91-118:: $\Delta hpaC$  and 91-118:: $\Delta avrBs2$ ) was non-significantly different from the *X. euvesicatoria* 91-106 strain treatment.

In the external antagonism assay, all attenuation mutant candidates tested were similar to wt 91-118 in antagonism, however they were significantly different overall (Table 3-6). At 48 and 72 h, however, 91-118:: $\Delta opgH$  was not significantly different according to standard error. Overall, wt 91-118 and all 91-118 mutants tested were significantly effective at reducing populations of E3-1.

## Discussion

The goal of this project was to identify genes mutants that would provide a dramatic reduction in disease symptoms while still maintaining the significant expression levels of BcnA and BcnC. Several pathogenicity related genes (*hpaA*, *hpaB*, *hpaC*, *xopA*, *xopD*, *avrBs2*, *gumD*)

were evaluated for their possible attenuating effects when in *X. perforans*. 91-118:: $\Delta$ *opgH* served as the model system. This *opgH* mutant had an attenuated phenotype in disease severity and growth curve experiments, as observed previously (101), and maintained its ability to reduce *X. euvesicatoria* significantly better than water and *X. euvesicatoria* controls. The *opgH* mutant was selected as a model system for these experiments because it was also chosen for further investigation in the form of field experiments in Chapter 4.

Growth curve analysis suggests mutants (91-118:: $\Delta$ *xopA*, 91-118:: $\Delta$ *gumD*, 91-118:: $\Delta$ *hpaC* and 91-118:: $\Delta$ *opgH*) were effected in overall fitness within the plant by exhibiting a reduced growth curve peak compared to wt 91-118. There are a few hypotheses that may explain why we observed this reduction. One explanation could be associated with the effect of the mutants on the overall fitness of the bacterium. A second hypothesis may be that there is recognition of the pathogen by the plant due to the mutations created. It does not appear that these mutants would eventually reach the levels based on the stationary phase of the curve at 96 to 120 h supporting the second hypothesis. Further research is needed to solidify which or if both hypotheses is correct.

Of the mutants created in this study, 91-118:: $\Delta$ *gumD* and 91-118:: $\Delta$ *opgH* exhibited the overall characteristics we were looking. Both mutants caused significant reductions in growth curve and disease severity while maintaining relatively high levels of antagonism in internal and external antagonism experiments. Xanthan gum biosynthesis appears to be important in pathogenicity for *X. perforans* as described previously in *X. c. campestris* (21). This gene may be of interest for further investigation in designing a pathogenicity-attenuated biological control agent.

Mutant 91-118::*ΔxopA* incited intermediate levels of disease severity and reduced growth curve compared to wt 91-118. Deletion of *xopA* affected both internal and external antagonism compared to wt *X. perforans*, however, the bacteria still maintained relatively high levels of antagonism. Overall XopA is necessary for full virulence and *in planta* growth as previously described in *X. euvesicatoria* (106), however, disease levels caused by the *xopA* mutant were still too high (>25%) to be a viable pathogenicity-attenuated mutant for use as a biocontrol agent.

91-118::*ΔhpaC* and 91-118::*ΔavrBs2* were significantly different in overall growth rate, however, they were the most affected in overall antagonism according to internal antagonism experiments. Both only reduced E3-1 levels similar to a, non-bacteriocin producing, *X. euvesicatoria* 91-106 strain and were not significantly different from the other mutants tested or the 91-106 treatments (Table 3-6).

Two candidates, 91-118::*ΔhpaB* and 91-118::*ΔavrBs2*, exhibited growth curve and disease severity with overall similarity to wt 91-118. These candidates could, however, be distinguished from wt, based on antagonism in internal antagonism assays. Although these genes were previously shown to be involved in virulence in *X. euvesicatoria* (18, 17, 53, 63, 76, 146), they do not appear to affect disease severity sufficiently to be feasible for creating a pathogenicity-attenuated biological control agent. Furthermore, these mutants dramatically reduced bacteriocin antagonism; however, further research is needed to determine if this reduction is due to overall fitness of the bacteria or partial recognition and suppression of the bacterium by the plant as hypothesized earlier.

Two genes, *opgH* and *gumD*, exhibited the desired attenuation and bacteriocin activity when mutated. Although the other mutants did not reduce pathogenicity sufficiently alone, they may provide a more dramatic effect when in combination with one another. HpaB and HpaC are

chaperones for effectors to the T3SS channel (HpaB and HpaC) (18, 17), and have been shown to directly interact forming an oligomeric protein complex and interact with two classes of effectors (class A containing XopJ and XopF1 and class B containing AvrBs3 and XopC) and HrcV of the T3SS (18). Based on their known interaction in *X. euvesicatoria*, HpaB and HpaC may provide a greater reduction in disease when both are knocked out within the same bacterium (18).

Although eight pathogenicity related genes were selected to study, there is an abundant source of genes involved in pathogenicity that could be exploited to create further pathogenicity-attenuated mutants. Many xanthomonads and other pathogenic bacteria have been recently sequenced such as *X. euvesicatoria strain 85-10* (150), *X. axonopodis pv. citri strain 306* (27), *Xanthomonas campestris pv. campestris* (27), *X. oryzae pv. oryzae* (85), *P. syringae pv. tomato* (15) and *R. solanacearum* (133). These sequences will provide essential information for understanding how bacteria develop a pathogenic relationship with the host. For instance, Thieme *et al.* has estimated that there are ~480 putative pathogenicity factors in *X. euvesicatoria strain 85-10* (150). This represents a large pool of putative pathogenicity genes, and therefore, opportunity to utilize these genes to create pathogenicity-attenuated mutants. Although there are a number of reports about mutants with reduced pathogenicity in *X. euvesicatoria* (17, 18, 54, 76, 105, 106, 107, 130, 146, 171), there are only a few reports of attenuated phenotypes in *X. perforans* (101). This information was utilized to create a biological control strategy to allow a bacteriocin-producing *X. perforans* strain to effectively colonize the plant and deliver bacteriocins while causing little to no disease.

Other secretion systems may prove to be important in pathogenicity as well. *X. euvesicatoria* has many substrates delivered via the type II secretion system such as cellulases,

$\beta$ -glucosidases, pectate lyases, polygalacturonases and xylanases are proposed to exhibit plant cell wall-degrading activity (150). Deletion of the type II secretion system may be too detrimental to the bacterium; however, deletion of a number of the delivered cell wall-degrading enzymes (eg. polygalacturonate lyase,  $\alpha$ -amylase, or endoglucanase) may provide a desirable pathogenicity-attenuated phenotype. A putative type IV secretion system in *X. euvesicatoria* has been shown to have homology to the Icm/Dot system of human pathogens (20). The essential role of Icm/Dot type IV secretion system of *Legionella* species may suggest a possible role in virulence in *Xanthomonas* as well (20, 150). The type IV pilus is also thought to be involved in movement by retraction to mediate adhesion to plant tissue (107).

Inactivation or over expression of the quorum sensing auto-inducers may also provide an alteration in pathogenicity. Several genes encoding this system that are found in *X. euvesicatoria* (*rpfA* to *H*) (150). Diffusible signal factors (DSFs) have been shown to be involved in regulation of the synthesis of extracellular enzymes, exopolysaccharides and cyclic glucans (98, 163).

The genes mentioned here along with many others may provide optimal attenuation for our system. Another possibility may be to create deletions in multiple pathogenicity related genes (as discussed with *hpaB* and *hpaC*) to determine their combined mutant phenotypes. This may be useful as a tweaking tool to create the optimal level of colonization and infection of the host plant. One hurdle to overcome concerning characterization of these genes is functional redundancy. Mutations in most effector genes do not show significant effects on bacterial virulence when deleted, presumably because of functional redundancy of some effectors (9, 24, 31, 78). Overall, there is a great deal of potential for utilization of these pathogenicity related genes for creation of a pathogenicity-attenuated biological control agent.

Table 3-1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference*
<b><i>Xanthomonas euvesicatoria</i></b>		
E3-1	Nal <sup>R</sup> Sm <sup>R</sup>	(61)
91-106		(155)
ME-90	Rif <sup>R</sup> Kan <sup>R</sup>	(155)
<b><i>X. perforans</i></b>		
91-118	Rif <sup>R</sup>	(155)
91-118:: $\Delta$ opgH	OpgH <sup>-</sup> Rif <sup>R</sup>	(101)
91-118:: $\Delta$ avrBs2	AvrBs2 <sup>-</sup> Rif <sup>R</sup> Cm <sup>R</sup>	This study
91-118:: $\Delta$ xopA	XopA <sup>-</sup> Rif <sup>R</sup>	This study
91-118:: $\Delta$ xopD	XopD <sup>-</sup> Rif <sup>R</sup> Cm <sup>R</sup>	This study
91-118:: $\Delta$ hpaA	HpaA <sup>-</sup> Rif <sup>R</sup> Cm <sup>R</sup>	This study
91-118:: $\Delta$ hpaB	HpaB <sup>-</sup> Rif <sup>R</sup> Cm <sup>R</sup>	This study
91-118:: $\Delta$ hpaC	HpaC <sup>-</sup> Rif <sup>R</sup> Cm <sup>R</sup>	This study
91-118:: $\Delta$ gumD	GumD <sup>-</sup> Rif <sup>R</sup>	This study
<b><i>Escherichia coli</i></b>		
DH5 $\alpha$	F <sup>-</sup> rec A	BRL
C2110	Nal <sup>R</sup>	BRL
$\lambda$ PIR	Host for pOK1; Sp <sup>R</sup> oriR6K K2 replicon	UB
<b>Plasmids</b>		
pBluescript-KS+	Phagemid, pUC derivative; Amp <sup>R</sup>	Stratagene
pLAFR3	Tc <sup>R</sup> rlx <sup>+</sup> RK2 replicon	BJS
pRK2013	Km <sup>R</sup> tra <sup>+</sup> mob <sup>+</sup>	(28)
pOK1	Suicide vector; SacB	(63)
pOK1: $\Delta$ opgH	OpgH <sup>-</sup> Sm <sup>R</sup>	This study
pOK1: $\Delta$ avrBs2	AvrBs2 <sup>-</sup> Sm <sup>R</sup> Cm <sup>R</sup>	This study
pOK1: $\Delta$ xopA	XopA <sup>-</sup> Sm <sup>R</sup>	This study
pOK1: $\Delta$ xopD	XopD <sup>-</sup> Sm <sup>R</sup> Cm <sup>R</sup>	This study
pOK1: $\Delta$ hpaA	HpaA <sup>-</sup> Sm <sup>R</sup> Cm <sup>R</sup>	This study
pOK1: $\Delta$ hpaB	HpaB <sup>-</sup> Sm <sup>R</sup> Cm <sup>R</sup>	This study
pOK1: $\Delta$ hpaC	HpaC <sup>-</sup> Sm <sup>R</sup> Cm <sup>R</sup>	This study
pOK1: $\Delta$ gumD	GumD <sup>-</sup> Sm <sup>R</sup>	This study

\* BRL, Bethesda Research Laboratories, Gaithersburg, MD; Stratagene, Stratagene Inc., La Jolla, CA; BJS, B. J. Staskawicz, University of California, Berkeley, CA; UB, U. Bonas, Martin-Luther-Universität, Halle, Germany.

Table 3-2. PCR primers used in *hpaA*, *hpaB*, *hpaC*, *xopA*, *xopD*, *avrBs2* and *gumD* analyses for genetic manipulations

Gene	primer name	restriction site	Primer sequence	length	GC (%)	Tm (C)
<i>hpaA</i>	hpaAF	<i>HinDIII</i>	<u>AAGCTT</u> GCTCAAGCTGGTGGTG	22	54.5	56.0
	hpaAR	<i>HinDIII</i>	<u>AAGCTT</u> ATCTAATCGTGCCTGC	24	50.0	
	hpaA2F		ACGCAAACGAGCAGGAAG	18	55.6	55.0
	hpaA2R		AGCAGGATCAGTGGAAAGCAG	20	55.0	
	hpaADF	<i>SmaI</i>	<u>CCCGGGG</u> TTTGGCTTCGATCTCTTCTGCTC	31	61.3	54.0
	hpaADR	<i>SmaI</i>	<u>CCCGGG</u> ATCTCTGCTTCCACTGATCCTGCT	31	61.3	
<i>hpaB</i>	hpaBF	<i>HinDIII</i>	GGG <u>AAGCTT</u> GCGACGCTGCGCGACA	26	65.4	60.0
	hpaBR	<i>HinDIII</i>	GGGGA <u>ATTCA</u> AGGCTGCCATGGAGGAGGG	29	62.1	
	hpaB2F		CAGAAGTCCATGAACAACAAGATCACG	27	44.4	57
	hpaB2R		ATCTCCCGCCAAACCTGTATCG	22	54.5	
<i>hpaC</i>	hpaCF		GGCATCGAGATCGCCCAG	18	66.7	58.5
	hpaCR		CGCATACCGCAACCCGAG	18	66.7	
	hpaC2F		GATAGCCAGCCACGCTTCCC	20	61.5	58.5
	hpaC2R		CGCACAGCTCGCGCTTCC	18	61.7	
	hpaCDF	<i>HinDIII</i>	GGG <u>AAGCTT</u> GACAGATTGCGACCGAGTGGATAC	33	54.5	61& 67
	hpaCDR	<i>HinDIII</i>	GGG <u>AAGCTT</u> GATACGTAAGGGTGGGTCCGTTTG	33	54.5	
<i>xopA</i>	XopAF	<i>HinDIII</i>	GGG <u>AAGCTT</u> TGCTGGAAGAGGAAAAGCG	28	53.6	52& 60
	XopAR	<i>HinDIII</i>	GGGGA <u>ATTCA</u> ATCCGCGCGTGCGA	24	62.5	
	XopA2F		GAGAGGCTGAGGCTAGT	17	56.0	57.0
	XopA2R		TCATTGAATACGTCCGACC	22	57.0	
	XopA3F		AAGTGGATAACGGCAGTGAG	23	56.0	54.5
	XopA3R		CGGAAAGCGACACAGCAG	18	57.2	
<i>xopD</i>	xopDF		TGCTGCCTTTTGTATGGAC	19	47.4	52.0
	xopDR		TCCTGCCAACCTACTTTAC	20	50.0	
	xopD2F		TCCAAAAAGCAAGCCCAC	18	52.6	52.0
	xopD2R		GACGAGCAATGACCAATGAG	20	55.4	
	xopD3F		GAGCCAACTTCAGAATGCG	19	55.2	55.0
	xopD2R		GACGAGCAATGACCAATGAG	20	55.4	
	xopDDF	<i>HinDIII</i>	CCC <u>AAGCTT</u> CTGAAATCACTGCTCACCCAGAC	33	51.5	59& 66
	xopDDR	<i>HinDIII</i>	CCC <u>AAGCTT</u> GGTTCTTCTTCTATTCGTCCCTGTTC	33	51.5	
<i>avrBs2D</i>	avrBs2F		ATCGCCCGCATCGCCTTC	18	59.5	60.0
	avrBs2R		CACGCAGTCGCCTCCACC	18	61.7	
	avrBs2DD2F	<i>SalI</i>	<u>GTCGAC</u> CCTCGTAGGCATGATCGATGGAC	28	57.1	53& 59
	avrBs2DD2R	<i>SalI</i>	<u>GTCGAC</u> GGAACCTACGTCAAGACCGACC	28	57.1	
<i>gumD</i>	gumDF		TCGTTCTCTTCGTGCAGC	20	60	60
	gumDR		TCCCGTATGTTTCGGGCTCCT	20	60	

Table 3-3. List of attenuation candidate genes and their published, corresponding mutant phenotype

Candidate Genes	Organism	Phenotype	Reference
<i>opgH<sub>xcv</sub></i>	<i>X. perforans</i>	moderate reduction in <i>in planta</i> growth.	Minsavage <i>et al.</i> 2003
<i>hpaA</i>	<i>X. euvesicatoria</i>	moderate reduction in <i>in planta</i> growth.	Huguet <i>et al.</i> 1998
<i>hpaB</i>	<i>X. euvesicatoria</i>	moderate reduction in <i>in planta</i> growth.	Büttner <i>et al.</i> 2004
<i>hpaC</i>	<i>X. euvesicatoria</i>	significant reduction in <i>in planta</i> growth.	Büttner <i>et al.</i> 2005
<i>xopA</i>	<i>X. euvesicatoria</i>	moderate reduction in <i>in planta</i> growth.	Noël <i>et al.</i> 2001 & Noël <i>et al.</i> 2002
<i>xopD</i>	<i>X. euvesicatoria</i>	no reduction in <i>in planta</i> growth.	Noël <i>et al.</i> 2002
<i>avrBs2</i>	<i>X. euvesicatoria</i>	Significant reduction in <i>in planta</i> growth.	Kearney and Staskawicz 1990
<i>gumD</i>	<i>X. c. campestris</i>	delayed disease symptom development.	Chou <i>et al.</i> 1997

Table 3-4. Homology of *X. perforans* genes chosen to be deleted to create pathogenicity-attenuated mutants to genes of other closely related xanthomonads

<b>Xanthomonads</b>	<b>% Homology (AA)</b>					
	<b>HpaA</b>	<b>HpaB</b>	<b>HpaC</b>	<b>AvrBs2</b>	<b>XopA</b>	<b>XopD</b>
<i>X. euvesicatoria</i> (85-10) <sup>a</sup>	98	100	99	98	100	85
<i>X. oryzae</i> pv. <i>oryzicola</i>	88	94	88	88		
<i>X. oryzae</i> pv. <i>oryzae</i> (KACC10331)	87	95	89	89		
<i>X. axonopodis</i> pv. <i>citri</i> (306)	75	93	86	95		
<i>X. campestris</i> pv. <i>campestris</i> (ATCC 33913)	75	85	55	76		74
<i>X. campestris</i> pv. <i>glycines</i>	75	93	87		47	

<sup>a</sup> Strains are designated in parenthesis

Table 3-5. *In planta* growth and aggressiveness of *X. perforans* strain 91-118 mutants as measured by area under the population progress curve (AUPPC) and percent disease severity, respectively, following inoculation of Bonny Best tomato plants

Strain	Growth curve [AUPPC] <sup>a</sup>	Disease severity [% disease severity] <sup>b</sup>
<i>X. perforans</i> (91-118)	118.1 a <sup>c</sup>	38.7 a
91-118:: $\Delta$ <i>opgH</i>	98.5 c	12.3 c
91-118:: $\Delta$ <i>xopA</i>	105.5 b	25.7 b
91-118:: $\Delta$ <i>hpaB</i>	116.4 a	36.3 a
91-118:: $\Delta$ <i>hpaC</i>	104.4 bc	33.7 a
91-118:: $\Delta$ <i>avrBs2</i>	113.7 a	38.0 a
91-118:: $\Delta$ <i>gumD</i>	106.4 b	13.7 c

<sup>a</sup> AUPPC from the growth curve of internal wt and mutant strains of *X. perforans* inoculated at  $5 \times 10^6$  CFU/mL over a 120 h period.

<sup>b</sup> Percent disease severity 14 days after dip inoculation of each bacterium at  $5 \times 10^6$  CFU/mL amended with 0.025% Silwet L-77.

<sup>c</sup> Values followed by the same letter are not significantly different based on Waller-Duncan multiple range test ( $P = 0.05$ ).

Table 3-6. Growth room *in planta* internal and phyllosphere antagonism experiments measuring *X. euvesicatoria* strain E3-1 populations when co-inoculated with water, *X. euvesicatoria* strain E3-1 or wt and mutants of *X. perforans* strain 91-118 measured as area under the population progress curve (AUPPC)

Strain	Internal antagonism [AUPPC] <sup>a</sup>	Phyllosphere antagonism [AUPPC]
water control	115.3 a <sup>b</sup>	-
<i>X. euvesicatoria</i> (91-106)	101.7 b	120.8 a
<i>X. perforans</i> (91-118)	67.0 d	69.0 c
91-118:: $\Delta$ <i>opgH</i>	86.7 c	79.1 b
91-118:: $\Delta$ <i>xopA</i>	87.2 c	84.7 b
91-118:: $\Delta$ <i>hpaB</i>	89.2 c	-
91-118:: $\Delta$ <i>hpaC</i>	94.6 bc	-
91-118:: $\Delta$ <i>avrBs2</i>	95.7 bc	-
91-118:: $\Delta$ <i>gumD</i>	86.7 c	-

<sup>a</sup> AUPPC from antagonism assay over a 96 h period based on recovered populations of *X. euvesicatoria* strain E3-1.

<sup>b</sup> Values followed by the same letter are not significantly different based on Waller-Duncan multiple range test (P = 0.05).

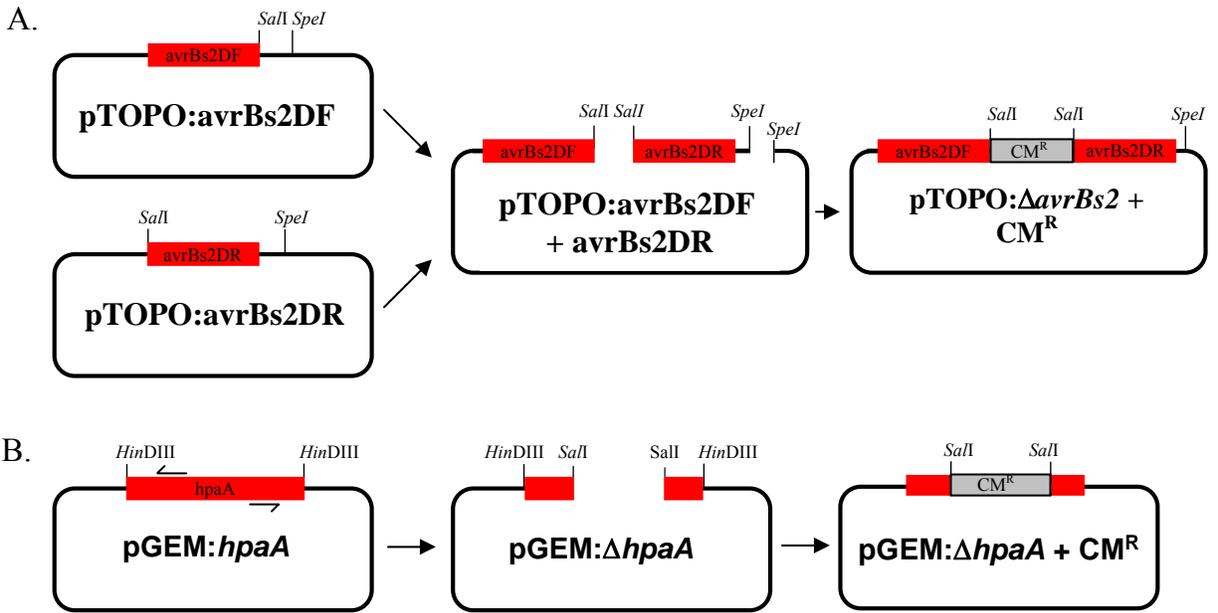


Figure 3-1. Illustration of deletion constructions. A. Deletion strategy for *avrBs2*. B. Deletion strategy for *hpaA*, *hpaC*, *xopA* and *xopD*.

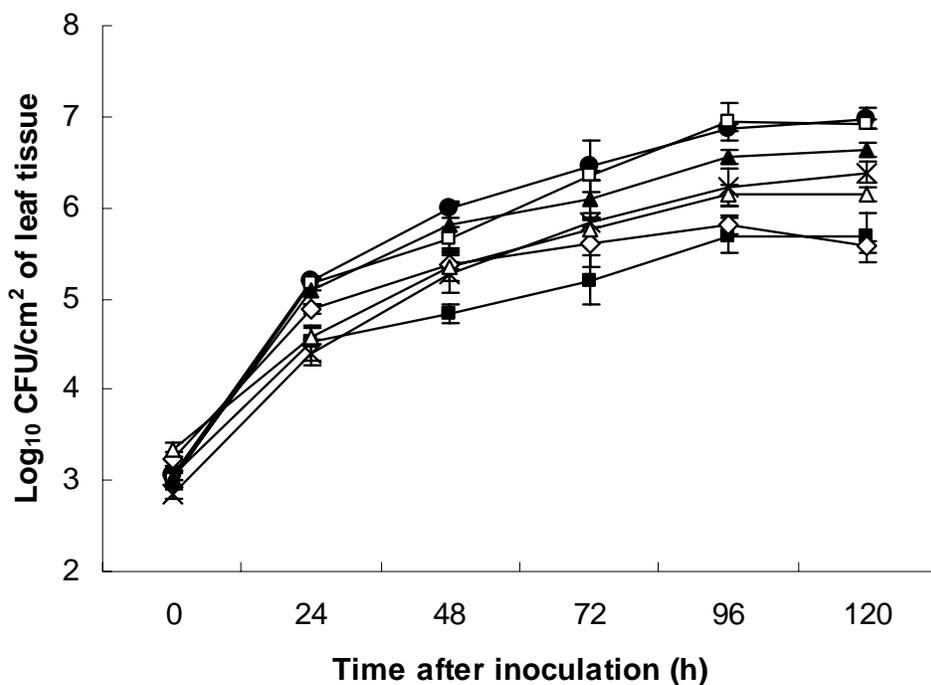
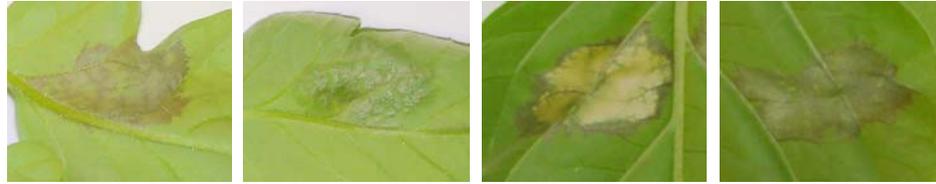


Figure 3-2. *In planta* growth of wild-type (wt) and mutant *X. perforans* strains. Plants were inoculated at  $5 \times 10^5$  CFU/mL of 91-118:: $\Delta$ opgH (■), 91-118:: $\Delta$ hpaB (□), 91-118:: $\Delta$ hpaC (◇), 91-118:: $\Delta$ xopA (x), 91-118:: $\Delta$ gumD (Δ), 91-118:: $\Delta$ avrBs2 (▲), wild-type 91-118 (●) in tomato genotype Bonny Best. Error bars indicate the standard error.



Figure 3-3. Disease severity on Bonny Best leaflets 2 weeks after dip inoculation ( $5 \times 10^6$  CFU/mL + 0.025% Silwet L-77) with *X. perforans* strains. Top: wt 91-118 (left), 91-118:: $\Delta xopA$  (left center) and 91-118:: $\Delta opgH$ , (right center) and 91-118:: $\Delta hpaB$  (right). Bottom: 91-118:: $\Delta hpaC$  (left), 91-118:: $\Delta avrBs2$  (center) and 91-118:: $\Delta gumD$  (right).



	Tomato [VF36 + Bs2]		Pepper [ECW-20R]	
strain	91-118	91-118:: $\Delta$ avrBs2	91-118	91-118:: $\Delta$ avrBs2
HR	HR	-	HR	-

Figure 3-4. Phenotype in leaves of Bs2 transgenic tomato VS36 and pepper (ECW-20R) inoculated with  $5 \times 10^9$  CFU/mL of *X. perforans* strains 91-118 (left) and 91-118: $\Delta$ avrBs2 (right). Phenotypes were recorded 24 h after inoculation. Browning of the tissue is associated with a hypersensitive response (resistance).

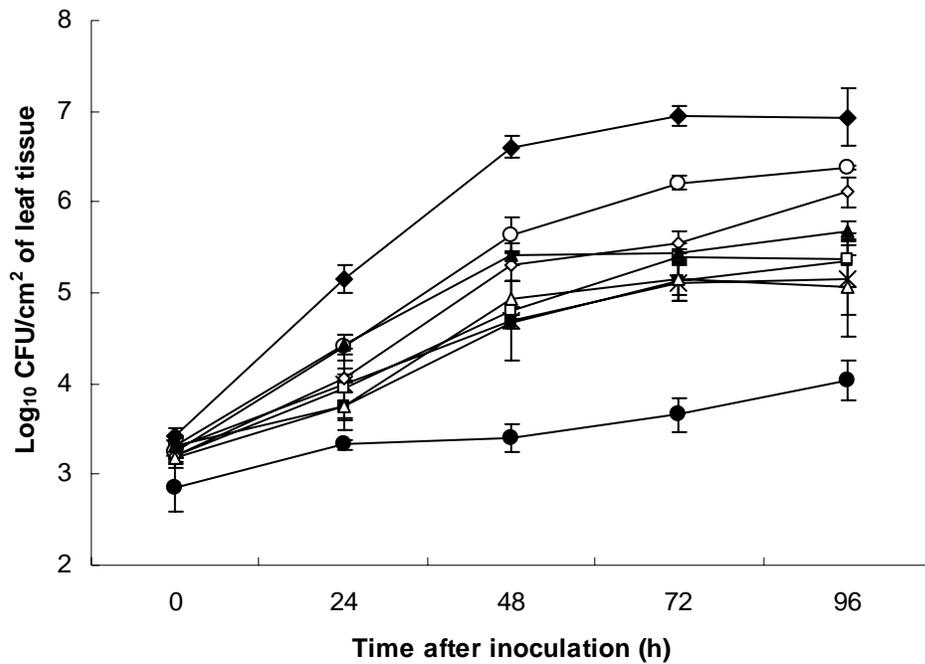


Figure 3-5. Growth room internal antagonism assay measuring *X. euvesicatoria* strain E3-1 in leaflets. Plants were infiltrated with  $5 \times 10^7$  CFU/mL of 91-118:: $\Delta opgH$  (■), 91-118:: $\Delta hpaB$  (□), 91-118:: $\Delta hpaC$  (◇), 91-118:: $\Delta xopA$  (x), 91-118:: $\Delta gumD$  (Δ), 91-118:: $\Delta avrBs2$  (▲), wild-type 91-118 (●), *X. euvesicatoria* strain 91-106 (○) and water (◆), followed 18 h later by  $5 \times 10^6$  CFU/mL of E3-1 in tomato genotype Bonny Best. Error bars indicate the standard error.

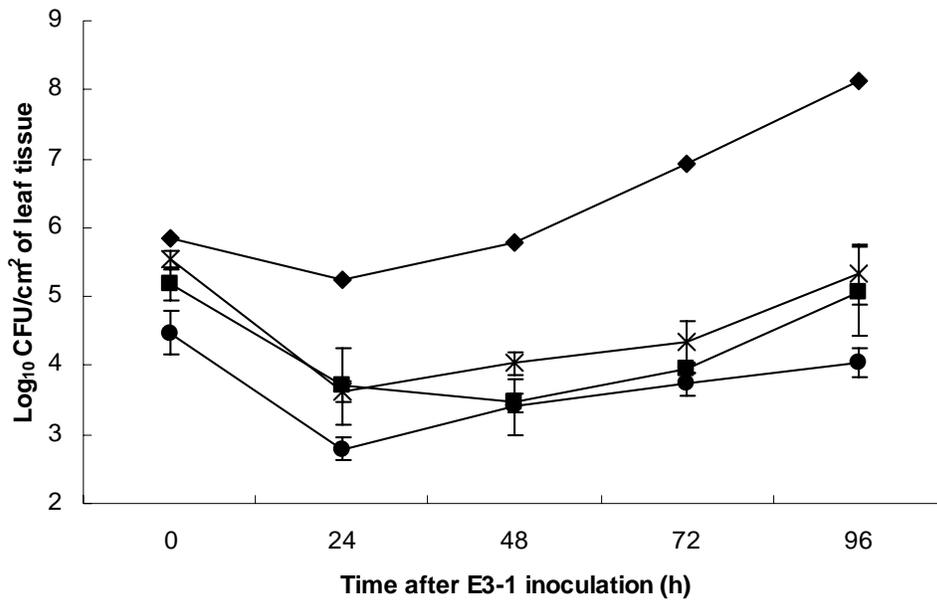


Figure 3-6. Growth room phyllosphere antagonism assay measuring *X. euvesicatoria* strain E3-1 in leaflets. Plants were dip inoculated with suspensions of  $5 \times 10^7$  CFU/mL (amended with 0.025% Silwet L-77) of: 91-118:: $\Delta opgH$  (■), 91-118:: $\Delta xopA$  (x), wild-type 91-118 (●) and water (◆), followed 7 d later by spray inoculation of  $5 \times 10^7$  CFU/mL of E3-1 on tomato genotype Bonny Best. Error bars indicate the standard error.

CHAPTER 4  
EVALUATION OF *XANTHOMONAS PERFORANS* MUTANTS IN CONTROLLING *X. EUVESICATORIA* IN GREENHOUSE AND IN THE FIELD

Bacterial spot of tomato is incited by four *Xanthomonas* species; *X. euvesicatoria*, *X. vesicatoria*, *X. perforans* and *X. gardneri*. The first three bacterial species were previously known as tomato races T1, T2 and T3, respectively, based on their reaction on three tomato genotypes: Hawaii 7998 (H7998), Hawaii 7981 (H7981) and Bonny Best (71, 72, 141). *X. gardneri* has only been found in Yugoslavia, Costa Rica and Brazil (10, 122, 144).

Control of bacterial spot of tomato is difficult when high temperatures and high moisture conditions exist. The disease has been demonstrated to cause significant damage to the crop resulting in major losses. Pohronezny and Volin (118) estimated as high as 50% loss of marketable fruit due to bacterial spot on tomatoes. There are currently no commercially available tomato varieties resistant to bacterial spot. Scott and Jones (135) identified significant resistance in H7998 in which *X. euvesicatoria* strains induce an HR. In 1993 Whalen et al. (166) determined that *X. euvesicatoria* strains carry the avirulence gene *avrRxv*, which induces an hypersensitive response (HR) on the genotype H7998 carrying the corresponding resistance gene *Rxv*; however, *X. perforans* T3 strains carry *avrXv3* which induces an HR in H7981 containing the resistance gene *Xv3* (100). Astua-Monge (4) characterized *avrXv3* and found it to elicit an HR in some tomato and pepper varieties. In 2000 a new avirulence gene *avrXv4* was described in *X. perforans* strains based on an HR in tomato genotype LA716 (*Lycopersicon pinnellii*) carrying the *Xv4* resistance gene (3, 4). *X. perforans* strains carrying this new avirulence gene (*avrXv4*) and a non-functional *AvrXv3* are designated as tomato race 4.

Bactericides, such as fixed coppers and streptomycin, have provided the primary means of chemical control (90, 142, 143); however, streptomycin-resistant mutants and copper-tolerant strains became prevalent (143). Marco and Stall (90) reported widespread emergence of copper-

tolerant *X. euvesicatoria* strains and that addition of the fungicide mancozeb to copper sprays improved disease control caused by copper-tolerant strains (25, 90). Chemical control alone is insufficient to control the disease under optimal conditions for the pathogen. Additionally, the use of copper compounds led to soil contamination in some instances (79).

Recently, there has been increased interest in integrated biological control strategies for bacterial diseases, which are difficult to control with conventional management practices. Some success has been achieved in this area through empirical selection of biocontrol agents, as indicated by the commercialization of the products Agriphage<sup>TM</sup>, a mixture of bacteriophages for control of bacterial spot of tomato (46), Galltrol<sup>TM</sup> for control of crown gall, and BlightBan<sup>TM</sup> A506 for control of fire blight and frost injury (89). However, achieving success using biocontrol agents for many bacterial diseases has been difficult. This failure may in part be due to the very narrow focus on the almost exclusive use of nonpathogenic, saprophytic bacteria as biocontrol agents. While our understanding of the ecology of nonpathogenic saprophytes is increasing, our knowledge is limited to labor-intensive protocols for identifying potential biocontrol agents. New integrated biological control strategies are currently being sought including the use of bacteriocins, attenuated plant pathogens and/or bacteriophages (28, 32, 46, 61, 67, 102, 108, 168) as part of an integrated biological control strategy.

One recent approach for biological control has been the use of bacteriocins (77, 152). Bacteriocins are substances produced by bacteria that are inhibitory or harmful toward only closely related bacteria (125). Bacteriocins and bacteriocin-like compounds encompass an array of structurally different substances including enzyme inhibition, nuclease activity and pore formation in cell membranes (125, 129, 128). Bacteriocins produced by *Escherichia coli* and several Gram-positive bacterial species have been extensively characterized (65, 125, 147). For

bacterial spot of tomato at least three distinct bacteriocin-like activities (BcnA, BcnB and BcnC) were identified in *X. perforans* that are antagonistic toward *X. euvesicatoria* strains (154). Hert et al. previously (61) demonstrated that two of these bacteriocins previously discovered in *X. perforans* (BcnA and BcnC) (154) could effectively control *X. euvesicatoria* populations in greenhouse and field experiments.

Although early attempts for biocontrol of plant diseases using bacteriocin-producing strains were made, few have been implemented (158, 159). For bacterial spot of tomato, Liu (88) conducted biological control studies utilizing a non-pathogenic bacteriocin-producing *X. perforans* strain to control disease incited by *X. euvesicatoria* strains. The non-pathogenic strain reduced bacterial spot disease incidence and severity by 10 to 15 percent in the field when applied prophylactically when compared to *X. euvesicatoria* alone; however, these levels were still unacceptable levels of control (40% disease) (88).

For *Ralstonia solanacearum*, efforts to obtain a biological control strategy utilizing bacteriocin-producing non-pathogenic *hrp*<sup>-</sup> mutants gave low to moderate levels of control of wild-type (wt) *R. solanacearum* (152). However, control using a partially pathogenic *hrp* mutant (*hrcV*<sup>-</sup>), which is capable of higher levels of colonization of the root and stem tissue, achieved better control (49). Research into colonization has been conducted to understand the possible relationship between invasion efficiency of the biocontrol agent and its ability for disease control. Etchebar et al. (41) suggested that there was a positive correlation between colonization of the xylem by the *hrp* mutant and the level of control of the wt *R. solanacearum*.

Based on previous studies in which non-pathogenic strains provided low levels of biological control (41, 49, 88), it was hypothesized that moderate invasion by the biological control agent using a partially pathogenic bacteriocin-producing strain of *X. perforans* rather

than a non-pathogenic strain may increase the efficiency of control under field conditions. These mutants may colonize the leaf tissue internally more effectively than non-pathogenic strains and this could potentially result in more effective biological control. A known *X. perforans* mutant gene *opgH<sub>Xcv</sub>* was selected to create the pathogenicity-attenuated mutant (101). OpgH mutants have a reduced disease severity and growth curve in susceptible tissue and delayed avirulent HR phenotype in resistant plant tissue.

The objective of this study was to evaluate the ability of a pathogenically-attenuated bacteriocin-producing strain (91-118:: $\Delta$ *opgH* $\Delta$ *bcnB*) to reduce the populations and of the disease caused by bacteriocin-sensitive strains of *X. euvesicatoria*. Previous research showed (61) that deletion of BcnB produces lower recovery of sensitive *X. euvesicatoria* strains than wt *X. perforans* when co-inoculated with *X. euvesicatoria*.

## **Materials and Methods**

### **Bacterial Strains, Plasmids and Culture Conditions**

Strains of *X. perforans* and *X. euvesicatoria* were grown on nutrient agar (NA) medium (Difco Laboratories, Detroit, MI) at 28°C (Table 4-1). Strains of *E. coli* were grown on Luria-Bertani (LB) medium at 37°C (97). All strains were stored in 20% glycerol in sterile tap water at –80°C. Bacterial cultures for plant inoculations were grown in nutrient broth (NB) (Difco Laboratories, Detroit, MI) for 18 h at 28°C with shaking (100 rpm). Cells were pelleted by centrifugation ( $4,000 \times g$ , 15 min) and resuspended in sterile tap water. Bacterial suspensions were standardized to an optical density at 600 nm ( $OD_{600}$ ) = 0.3 ( $5 \times 10^8$  CFU/mL) with a Spectronic 20 spectrophotometer (Spectronic UNICAM, Rochester, NY) and subsequently diluted in sterile tap water to appropriate cell densities for individual experiments. Antibiotics were used to maintain selection for resistance markers at the following concentrations:

tetracycline (Tc) 12.5 µg/mL; rifampicin (Rif) 100 µg/mL; spectinomycin (Sp) 50 µg/mL; kanamycin (Km) 50 µg/mL; chloramphenicol (Cm) 34 µg/mL; streptomycin (Sm) 200 µg/mL; and nalidixic acid (Nal) 50 µg/mL.

### **Generation of the 91-118:: $\Delta$ opgH $\Delta$ bcnB Attenuation Mutant**

Triparental matings were performed using *E. coli* DH5 $\alpha$  containing pRK2013<sup>Km</sup> as the helper plasmid (Table 4-1), *E. coli* DH5 $\alpha$  containing pXV442-255 (pXV442<sup>Tc</sup> with insertion of a Km<sup>R</sup> cassette for inactivation of BcnB) as the donor and 91-118:: $\Delta$ opgH as the recipient (Recipient received from Gerald Minsavage). Marker exchange was achieved using standard methods (134). The candidate colonies were screened for loss of BCN activity and confirmed for insertion by Southern hybridization (using subclone BcnB as the probe) and PCR (with primers BCN-1 and BCN-2) (61).

### **Plant Materials**

Seeds of tomato (*Lycopersicon esculentum*) cv. Bonny Best were planted in Plugmix (W. R. Grace & Co., Cambridge, MA). After 2 weeks, the emerged seedlings were transferred to Metromix 300 (W. R. Grace & Co., Cambridge, MA) in 10-cm plastic pots. Seedlings were grown in the greenhouse at temperatures ranging from 25 to 35°C.

### **Growth Room Growth Curve Assays**

Growth room assays were conducted to compare the growth curves of 91-118:: $\Delta$ opgH and 91-118:: $\Delta$ opgH $\Delta$ bcnB mutants with the wt parent strain 91-118. Strains were grown in NB for 18 h, harvested by centrifugation and resuspended in sterile tap water. Strains were inoculated at  $3 \times 10^5$  CFU/mL into leaflets of 6-week-old tomato seedlings. Leaflets were infiltrated (15 leaflets per strain) using a hypodermic syringe and needle, as described previously (68). Following inoculation plants were kept at 24°C to 28°C. Three samples were taken for each

treatment every 24 h for 5 d. Bacterial populations were quantified by macerating 1-cm<sup>2</sup> leaf disks in 1 mL sterile tap water and dilution plating onto NA medium amended with the appropriate antibiotic. Plates were incubated at 28°C and colonies were counted after 48 to 72 h. Population data were log<sub>10</sub> transformed and standard errors were determined. The overall growth curve was determined by calculating the area under the population progress curve (AUPPC). The AUPPC is a modification of the area under the disease progress curve (AUDPC) which has been used to analyze disease progress (136):  $\text{standardized AUPPC} = \sum [(x_i + x_{i-1})/2](t_i - t_{i-1})$ , where  $x$  is population density in log<sub>10</sub> CFU per cm<sup>2</sup> and  $t$  is time in hours. The AUPPC values for the strains were compared by analysis of variance and subsequent separation of sample means by Waller-Duncan multiple range test using SAS version 9.0 (SAS Inc., Cary, NC). Each experiment was conducted three times.

### **Greenhouse Disease Severity Assay**

Greenhouse disease severity assays were conducted to determine the effect of *X. perforans* mutant and wt 91-118 strains on symptom development. In each test, four young (four-true-leaf stage) plants were inoculated with each strain by dipping into sterile tap water containing bacterial suspensions of 91-118, 91-118:: $\Delta bcnB$ , 91-118:: $\Delta opgH$ , 91-118:: $\Delta opgH\Delta bcnB$  and *X. euvesicatoria* strain 91-106 ( $3 \times 10^6$  CFU/mL of bacteria amended with 0.025% Silwet L-77 (Loveland Industries, Inc., Greeley, CO)) for 15 s. Plants were maintained in the greenhouse during the evaluation period. The plants were assessed for disease severity 14 to 21 days after inoculation. Disease assessments for wt and attenuated mutant candidates of *X. perforans* strains were made based on leaf and stem ratings compiled from three separate greenhouse inoculation tests.

## Growth Room Antagonism Assay

Antagonism assays were performed to determine the effect of wt and mutant *X. perforans* strains on the bacteriocin sensitive *X. euvesicatoria* strain, E3-1. Internal and external (phyllosphere) populations were separately evaluated using two antagonism assay techniques.

*Internal antagonism assays.* Strains were grown in NB for 18 h, harvested by centrifugation and resuspended in sterile tap water. *X. perforans* and *X. euvesicatoria* strains were inoculated at  $5 \times 10^7$  CFU/mL and  $5 \times 10^6$  CFU/mL, respectively. Six-week-old seedlings of the tomato cultigen Florida 47 were inoculated (15 leaflets per strain) using a hypodermic syringe as described previously (68). The mutant and wt *X. perforans* strains were inoculated into leaflets by infiltration 12 h prior to inoculation with the sensitive strain (E3-1). Each treatment consisted of three replications. Following inoculation, plants were incubated at 24°C to 28°C. In order to determine populations of the sensitive strain (E3-1<sup>Sm<sup>Nal</sup></sup>) in leaflets, 1-cm<sup>2</sup> leaf disks were removed from inoculated areas, macerated in 1 mL sterile tap water and dilution plated onto nutrient agar amended with the appropriate antibiotic. Samples were assayed at 24 h intervals for 96 h. Each experiment was conducted three times. Population data were log transformed and standard errors were determined. AUPPC values (calculated as described above) were compared by analysis of variance and subsequent separation of sample means by Waller-Duncan multiple range test using SAS version 9.0 (SAS Inc., Cary, NC). Each experiment was conducted three times.

*Phyllosphere antagonism assays.* Growth room phyllosphere antagonism assays were conducted to determine if the gene deletions affected the levels of antagonism toward external leaf populations of *X. euvesicatoria* strain E3-1 by comparing the antagonistic ability of the mutants with the parent strain 91-118. Strains were grown in NB for 18 h, harvested by

centrifugation and resuspended in sterile tap water. Six-week-old Bonny Best tomato seedlings were dipped into  $5 \times 10^7$  CFU/mL suspension of the wt and mutant strains of *X. perforans* amended with Silwet L-77 (0.025%) 7 days prior to spray inoculation with a  $5 \times 10^7$  CFU/mL suspension of *X. euvesicatoria* strain E3-1. Following spray inoculation, plants were incubated at 24°C to 28°C. Leaf tissue was sampled every 24 h for 96 h for quantification of E3-1 populations. Three leaflets were taken at each time point. Each leaflet was weighed, placed in a polyethylene bag containing 10 mL of sterile tap water shaken on a Wrist Action shaker (Burrell Co., Oakland, CA) and shaken vigorously for 20 min. The leaf-wash was then dilution plated on NA<sup>NalSm</sup> to selectively determine the bacterial population of E3-1. Population data were analyzed following log<sub>10</sub> transformation and standard errors were determined. AUPPC values (calculated as described above) were compared by analysis of variance and subsequent separation of sample means by Waller-Duncan multiple range test using SAS version 9.0 (SAS Inc., Cary, NC). Each experiment was conducted three times.

### **Field Experiments**

*Field plot design.* The field experiments were set up in a completely randomized block design consisting of four replications. Raised beds were 0.91 m wide and were covered with black plastic mulch. Plots were arranged in paired beds that were 1.83 m from center to center and each set of paired beds was 7.32 meters apart. Plots within the paired beds were spaced 6.1 m apart. Each plot containing 20 plants were spaced 457 cm apart.

*Bacterial strains, inoculum production, inoculation and plant material.* Field experiments were performed using *X. perforans* mutant 91-118:: $\Delta$ opgH $\Delta$ bcnB and *X. euvesicatoria* strain E3-1 to evaluate antagonism of those strains to the *X. euvesicatoria* strain E3-1 (Table 4-1). Strains were grown in NB for 24 h, harvested by centrifugation and resuspended in sterile tap water.

The bacterial suspensions were adjusted to  $5 \times 10^7$  CFU/mL and the surfactant Silwet L-77 was added to a final concentration of 0.025%. Plants were dipped into suspensions of the 91-118:: $\Delta$ opgH $\Delta$ bcnB strain 24 h prior to spray inoculation with a  $5 \times 10^7$  CFU/mL suspension of E3-1. Six-week-old seedlings of the tomato genotype Florida 47 (Asgrow, Oxnard, CA) were used in all experiments.

*Incidence of strains in lesions.* In 2004, field experiments were conducted at the North Florida Research and Education Center (NFREC) in Quincy, FL to evaluate recovery of wt *X. euvesicatoria* strain E3-1 and 91-118:: $\Delta$ opgH $\Delta$ bcnB from symptomatic leaf tissue. The experiment consisted of six treatments: (1) uninoculated control; (2) E3-1 + growers standard; (3) E3-1 alone; (4) 91-118:: $\Delta$ opgH $\Delta$ bcnB alone; (5) E3-1 and 91-118:: $\Delta$ opgH $\Delta$ bcnB (applied bi-weekly); and (6) E3-1 and 91-118:: $\Delta$ opgH $\Delta$ bcnB (applied weekly). Plants in the grower standard was treated on weekly rotations of acibenzolar-S-methyl (0.055 g/L) (Actigard 50WG; Syngenta Crop Protection Inc., Greensborough, NC) or copper hydroxide (3.6 g/L) (Kocide 2000; Griffin Corp., Valdosta, GA) plus mancozeb (2.5 g/L) (Manzate 75DF; Griffin Corp., Valdosta, GA) every two weeks. Symptomatic leaf tissue was collected every 2 weeks beginning 35 days after transplanting. Ten to twenty leaflets were randomly collected in each plot and bacteria were isolated from thirty lesions. Individual lesions were macerated in 75  $\mu$ L of sterile deionized water and the suspensions were streaked on NA amended with 134  $\mu$ g/mL of pentachloronitrobenzene (PCNB) (126) and 50  $\mu$ g/mL of cycloheximide to eliminate fungal contaminants from the samples. Individual colonies were plated onto two media to differentiate *X. perforans* and *X. euvesicatoria* (NA amended with the appropriate antibiotics for 91-118:: $\Delta$ opgH $\Delta$ bcnB<sup>Rif Km</sup> and E3-1<sup>Nal Sm</sup>). The overall strain incidence was expressed by calculating the area under the incidence progress curve (AUIPC). The AUIPC is a modification

of AUDPC: standardized AUIPC =  $\Sigma [(x_i + x_{i-1})/2](t_i - t_{i-1})$ , where  $x$  is the arcsin of the percent recovery (to normalize the data) and  $t$  is days after inoculation. The AUIPC values were compared by analysis of variance and subsequent separation of sample means by Waller-Duncan multiple range test using SAS version 9.0 (SAS Inc., Cary, NC).

*Incidence of phyllosphere populations.* In 2005, field experiments were conducted at two locations (NFREC and the Citra Research Farm) to evaluate recovery of E3-1 and 91-118:: $\Delta$ opgH $\Delta$ bcnB from the surface of asymptomatic leaf tissue. Asymptomatic leaf tissue was sampled every 2 weeks beginning ~20 days after transplanting (DAT). Seven leaflets were collected from each plot. Each sample was weighed, placed into a polyethylene bag (Becton Dickinson, Rutherford, New Jersey) containing 5 to 10 mL of sterile tap water and shaken at 200 rpm for 30 to 45 minutes. Serial ten-fold dilutions were made in sterile tap water. A 50  $\mu$ l aliquot of each dilution was plated two NA plates, one amended with 134  $\mu$ g/mL of PCNB and/or 50  $\mu$ g/mL of cycloheximide with addition of antibiotics for selection of E3-1 (Sm and Nal) and the second for the 91-118:: $\Delta$ opgH $\Delta$ bcnB mutant (Rif and Km). After incubation at 28°C for 4-5 days, colonies typical of *Xanthomonas* were counted and populations were calculated. Data was analyzed for statistical significance by using the AUIPC. AUIPC values (calculated as described above) were compared by analysis of variance and subsequent separation of sample means by Waller-Duncan multiple range test using SAS version 9.0 (SAS Inc., Cary, NC). Each experiment was conducted three times.

## Results

### 91-118 and Mutants Reduce E3-1 in Growth Room and in the Greenhouse

Growth room experiments were conducted to determine the effects of the mutations to grow *in planta*. The 91-118:: $\Delta$ opgH $\Delta$ bcnB mutant reached populations 1 to 1.5 log units lower

than wt 91-118 (Figure 4-1). The 91-118 population exhibited a normal growth curve during the 120-h sampling period and, based on the AUPPC results (Table 4-2), and had significantly higher populations over time than both 91-118::*ΔopgH* mutants. The 91-118::*ΔopgHΔbcnB* mutant was not significantly different from 91-118::*ΔopgH* suggesting that there was no pronounced effect on growth curve based on the *ΔbcnB* deletion.

Greenhouse disease severity experiments were conducted to determine the effects of the mutations on the ability of 91-118 to cause disease *in planta* (Figure 4-2). Disease severity incited by 91-118::*ΔopgHΔbcnB* and 91-118::*ΔopgH* strains (12% and 14 %, respectively) was significantly lower than that of 91-118 (39 %) (Table 4-2).

Both internal (Figure 4-3) and phyllosphere (Figure 4-4) antagonism assays under growth room conditions were performed to determine the antagonistic activity of 91-118 and 91-118::*ΔopgHΔbcnB* and 91-118::*ΔopgH* strains toward the E3-1 strain. For both assays mutants 91-118::*ΔopgH* and 91-118::*ΔopgHΔbcnB* were moderately antagonistic, whereas wt 91-118 and 91-118::*ΔbcnB* provided the greatest reduction in E3-1 populations. The water control treatment consistently had significantly higher population levels than all other treatments (Figures 4-3 and 4-4 and Table 4-3). Treatment of a wt *X. euvesicatoria* strain (91-106) prior to *X. euvesicatoria* strain (E3-1<sup>NalSm</sup>) reduced populations by ~0.5 log<sub>10</sub> CFU/mL in both assays compared to the water control. Differences in the levels of antagonism was observed between internal and phyllosphere antagonism assays.

### **Field Study**

The ability of the attenuated mutant, 91-118::*ΔopgHΔbcnB*, to reduce E3-1 populations was assessed in the field. Controls included non-inoculated control, a E3-1 alone control and 91-118 alone control plot. In 2004, symptomatic leaf tissue was sampled at the NFREC in

Quincy, FL and *X. euvesicatoria* populations were significantly reduced by both 91-118:: $\Delta$ *opgH* $\Delta$ *bcnB* control treatments (weekly and two week application) (Figure 4-5). E3-1 was recovered from less than 5 percent of the samples in 91-118:: $\Delta$ *opgH* $\Delta$ *bcnB* plots compared to 26 percent from E3-1 alone (Table 4-4).

In 2005 at Quincy (Figure 4-6) and Citra (Figure 4-7) phyllosphere populations were sampled. In both locations E3-1 populations were significantly reduced by the 91-118:: $\Delta$ *opgH* $\Delta$ *bcnB* mutant when applied weekly throughout the growing season (Figures 4-6 & 4-7). In Quincy, E3-1 was recovered from 30% of the samples in plots where E3-1 was applied alone (Table 4-4). In the treatment where 91-118:: $\Delta$ *opgH* $\Delta$ *bcnB* was applied every two weeks, the frequency of recovery of E3-1 populations was not significantly different from plots where E3-1 was applied alone. Weekly application of 91-118:: $\Delta$ *opgH* $\Delta$ *bcnB*, however, significantly reduced recovery of E3-1 populations compared to plots where the E3-1 was applied alone (approximately 65% reduction) (Table 4-4).

In Citra (2005), both 91-118:: $\Delta$ *opgH* $\Delta$ *bcnB* weekly and biweekly treatments significantly reduced recovery of E3-1 populations (Figure 4-7). The AUEPC of 91-118:: $\Delta$ *opgH* $\Delta$ *bcnB* weekly and biweekly treatments had significantly reduced E3-1 incidence compared to the grower standard (37%) and E3-1 alone (54%) plots (Table 4-4).

## Discussion

In this study, we sought to create a pathogenically attenuated *X. perforans* mutant to: (I) express two of the three previously described bacteriocins (based on previous field analysis (61) and (II) maintain itself at a level to maintain antagonism toward *X. euvesicatoria* strains while causing minimal disease. We decided to examine the previously described osmoregulated periplasmic glucan gene, *opgH<sub>xcv</sub>* (101). In greenhouse experiments the growth curve of both

attenuated mutants 91-118::*ΔopgH* and 91-118::*ΔopgHΔbcnB* exhibited reduced growth curves compared to 91-118. The 91-118::*ΔopgHΔbcnB* mutant also caused significantly less disease than wt which was similar to a previously study by Minsavage et al. (101). The 91-118::*ΔopgH* still maintained significant levels of antagonism toward E3-1 populations as observed in the antagonism assay. Overall, mutants 91-118::*ΔopgH* and 91-118::*ΔopgHΔbcnB* were significantly effective in reducing *X. euvesicatoria* populations in the greenhouse. Interestingly, 91-118::*ΔopgH* and 91-118::*ΔopgHΔbcnB* were more effective in reducing external E3-1 populations than internal populations. Lindemann et al. (86) previously concluded that there is a direct correlation between phyllosphere populations and occurrence of disease. The existence of this correlation together with the reduction in *X. euvesicatoria* phyllosphere populations during antagonism experiments suggest that a biological control strategy for *X. euvesicatoria* by 91-118::*ΔopgHΔbcnB* may be effective at reducing disease by reducing phyllosphere populations below the threshold level necessary to cause lesion development.

In 2004, two hurricanes during the season introduced high external populations of wt *X. perforans*, which reduced both E3-1 and 91-118::*ΔopgHΔbcnB* populations. Although naturally occurring populations of *X. perforans* were introduced into the plots, early sampling data along with AUDPC data suggest that weekly and biweekly treatments with 91-118::*ΔopgHΔbcnB* significantly reduced *X. euvesicatoria* populations. Similar trends were also observed in our 2005 field data evaluating phyllosphere levels. In 2005 experiments 91-118::*ΔopgHΔbcnB* effectively reduced *X. euvesicatoria* populations by up to 85 percent. In both years, weekly application of 91-118::*ΔopgHΔbcnB* at  $5 \times 10^6$  CFU/mL significantly reduced *X. euvesicatoria* populations compared to plots receiving *X. euvesicatoria* alone. In two of three experiments, bi-weekly application was found to significantly reduce E3-1 populations. This reduction in *X.*

*euvesicatoria* populations is similar to previous field data using 91-118::*ΔbcnB* (61). Hert et al. (61) found that co-inoculation of 91-118::*ΔbcnB* and an *X. euvesicatoria* in the field yielded less than 5 percent recovery of the *X. euvesicatoria* strain over all seasons tested. These results suggest that the 91-118::*ΔopgHΔbcnB* attenuated mutant has potential as a biological control agent of reducing *X. euvesicatoria* populations.

The reduction in *X. euvesicatoria* populations by 91-118::*ΔopgHΔbcnB* in the antagonism assays does appear to be less inhibitory compared to 91-118::*ΔbcnB*. This suggests that bacteriocin expression was also affected by the 91-118::*ΔopgH* mutation. The repeated treatment of 91-118::*ΔopgHΔbcnB* in the field experiments was sufficient for maintaining 91-118::*ΔopgHΔbcnB* levels and consequently sufficient bacteriocin levels to suppress *X. euvesicatoria* populations. Weekly application of 91-118::*ΔopgHΔbcnB* was more effective in reducing *X. euvesicatoria* populations than previously observed in similar experiments using hrp-*X. perforans* mutants (88). The 91-118::*ΔopgHΔbcnB* mutant appears to colonize within the leaf tissue more effectively than non-pathogenic strains and is similarly to what was observed in a previous study with *R. solanacearum hrcV* mutants (41).

Although the *opgH<sub>Xcv</sub>* mutant was effective in suppressing *X. euvesicatoria* populations, there is potential for identifying other gene targets that can help improve biological control efficacy. Several other pathogenicity factors and associated genes have previously been described in *X. euvesicatoria* with other genes associated with the hrp system (*hpaA*, *hpaB*, *hpaC*), avirulence genes (*avrBs2*, *xopA*, *xopD*) and pathogenicity factors (*gumD*) (17, 18, 63, 106, 105, 167). In the previous chapter (Chapter 3) we tested these pathogenicity-associated genes for their phenotype in *X. perforans* and the potential use for creating further pathogenicity-attenuated biocontrol agents. When several of the genes were mutated in *X. perforans* strain

91-118 there was an associated reduction in pathogenicity and growth curve *in planta*. These mutants or other pathogenicity associated genes may improve our pathogenicity attenuated biological control model system by allowing better internalization and subsequent competition between *X. euvesicatoria* and *X. perforans* populations without detrimental effects to the plant.

Sequencing of the *X. euvesicatoria* genome has provided significant new possibilities for developing pathogenicity attenuated candidates. In 2005, Thieme et al (150) published the *X. euvesicatoria* genome sequence and estimated over 480 putative pathogenicity factors and associated genes. These genes were grouped into 6 categories: (I) secretion systems, (II) flagellum, (III) secreted proteins (via type III secretion system), (IV) detoxification, (V) surface structure and adhesion and (VI) quorum sensing. Genomic sequencing of bacteria provides an opportunity to exploit these genes for our utilization.

Further research is needed to optimize this system to create a weakly aggressive biological control agent that is as antagonistic as wild-type. Recent information from related bacteria and genomic sequences can be used to provide opportunities to improve our understanding of how pathogenic bacteria colonize and subsequently infect the host. Continued exploration of new innovative ideas will help us to utilize this knowledge in effective ways.

Table 4-1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference*
<b><i>Xanthomonas euvesicatoria</i></b>		
E3-1	Nal <sup>R</sup> Sm <sup>R</sup>	(61)
91-106		(155)
ME-90	Rif <sup>R</sup> Kan <sup>R</sup>	(155)
<b><i>X. perforans</i></b>		
91-118	Rif <sup>R</sup>	(155)
91-118:: $\Delta$ <i>bcnB</i>	BcnB <sup>-</sup> Rif <sup>R</sup> Km <sup>R</sup>	(61)
91-118:: $\Delta$ <i>opgH</i>	OpgH <sup>-</sup> Rif <sup>R</sup>	(101)
91-118:: $\Delta$ <i>opgH</i> $\Delta$ <i>bcnB</i>	OpgH <sup>-</sup> BcnB <sup>-</sup> Rif <sup>R</sup> Km <sup>R</sup>	This study
<b><i>Escherichia coli</i></b>		
DH5 $\alpha$	F <sup>-</sup> rec A	BRL
C2110	Nal <sup>r</sup>	BRL
$\lambda$ PIR	Host for pOK1; Sp <sup>R</sup> <i>oriR6K</i> RK2 replicon	UB
Plasmids		
pOK1	Suicide vector; SacB	(63)
pBluescript-KS <sup>+</sup>	Phagemid, pUC derivative; Amp <sup>R</sup>	Stratagene
pLAFR3	Tc <sup>r</sup> <i>rlx</i> <sup>+</sup> RK2 replicon	BJS
pRK2013	helper plasmid; Km <sup>r</sup> tra <sup>+</sup>	(28)

\*BRL, Bethesda Research Laboratories, Gaithersburg, MD; Stratagene, Stratagene Inc., La Jolla, CA; BJS, B. J. Staskawicz, University of California, Berkeley, CA; UB, U. Bonas, Martin-Luther-Universität, Halle, Germany.

Table 4-2. *In planta* growth and aggressiveness of *X. perforans* strain 91-118 mutants as measured by area under the population progress curve (AUPPC) and percent disease severity, respectively, following inoculation of Bonny Best tomato plants

Strain	Growth curve [AUPPC] <sup>a</sup>	Disease severity [% disease severity] <sup>b</sup>
<i>X. perforans</i> (91-118)	118.3 a	38.7 a
91-118:: $\Delta$ <i>opgH</i>	104.2 b	12.3 b
91-118:: $\Delta$ <i>opgH</i> $\Delta$ <i>bcnB</i>	111.6 b	14.1 b

<sup>a</sup> AUPPC from growth curve of internal *X. perforans* strains inoculated at  $5 \times 10^6$  CFU/mL over a 120 h period.

<sup>b</sup> Percent disease severity 14 days after dip inoculation of each bacterium at  $5 \times 10^6$  CFU/mL amended with 0.025% Silwet L-77.

<sup>c</sup> Values followed by the same letter are not significantly different based on Waller-Duncan multiple range test ( $P = 0.05$ ).

Table 4-3. Growth room *in planta* internal and phyllosphere antagonism experiments measuring *X. euvesicatoria* strain E3-1 populations when co-inoculated with water, *X. euvesicatoria* strain E3-1 or wt and mutants of *X. perforans* strain 91-118 measured as area under the population progress curve (AUPPC)

Strain	Internal antagonism [AUPPC] <sup>a</sup>	Phyllosphere antagonism [AUPPC]
water control	115.3 a <sup>b</sup>	-
<i>X. euvesicatoria</i> (91-106)	101.7 b	120.8 a
<i>X. perforans</i> (91-118)	67.0 d	69.0 c
91-118:: $\Delta$ <i>opgH</i>	86.7 c	79.1 b
91-118:: $\Delta$ <i>opgH</i> $\Delta$ <i>bcnB</i>	90.8 c	86.3 b

<sup>a</sup> AUPPC from antagonism assay over a 96 h period based on recovered populations of *X. euvesicatoria* strain E3-1.

<sup>b</sup> Values followed by the same letter are not significantly different based on Waller-Duncan multiple range test ( $P = 0.05$ ).

Table 4-4. Incidence and recovery of *X. euvesicatoria* strain E3-1 in the field when treated with *X. perforans* mutant strain 91-118:: $\Delta$ *opgH* $\Delta$ *bcnB*. Incidence and recovery were measured by area under the incidence progress curve (AUIPC) and percent recovery, respectively

Treatment	2004		2005			
	Citra		Citra		Quincy	
	AUIPC <sup>a</sup>	% Recovery <sup>b</sup>	AUIPC	% Recovery	AUIPC	% Recovery
Uninoculated control	0 c <sup>c</sup>	0	425 b	13	157 b	7
Grower standard <sup>d</sup>	563.8 a	21	1273 a	37	757 ab	15
E3-1 alone	614.5 a	26	1435 a	54	1430 a	30
91 118:: $\Delta$ <i>opgH</i> $\Delta$ <i>bcnB</i> alone	0 c	0	446 b	5	92 b	2
91 118:: $\Delta$ <i>opgH</i> $\Delta$ <i>bcnB</i> (2) + E3-1 <sup>e</sup>	181.0 b	5	263 b	12	890 ab	25
91 118:: $\Delta$ <i>opgH</i> $\Delta$ <i>bcnB</i> (1) + E3-1 <sup>f</sup>	158.2 b	4	260 b	8	477 b	11

<sup>a</sup> AUIPC from fields evaluating E3-1 populations for each season for each treatment.

<sup>b</sup> The % recovery is the average percent recovery of *E3-1* populations for the season for each treatment.

<sup>c</sup> Values followed by the same letter are not significantly different based on Waller-Duncan multiple range test (P = 0.05).

<sup>d</sup> Grower standard plots were treated with Copper + Manzate and Actigard biweekly throughout the season.

<sup>e</sup> The 91 118:: $\Delta$ *opgH* $\Delta$ *bcnB* (2) + T1 plots were treated with  $\Delta$ *opgH* $\Delta$ *bcnB* every two weeks throughout the season.

<sup>f</sup> The 91 118:: $\Delta$ *opgH* $\Delta$ *bcnB* (1) + T1 plots were treated with  $\Delta$ *opgH* $\Delta$ *bcnB* weekly throughout the season.

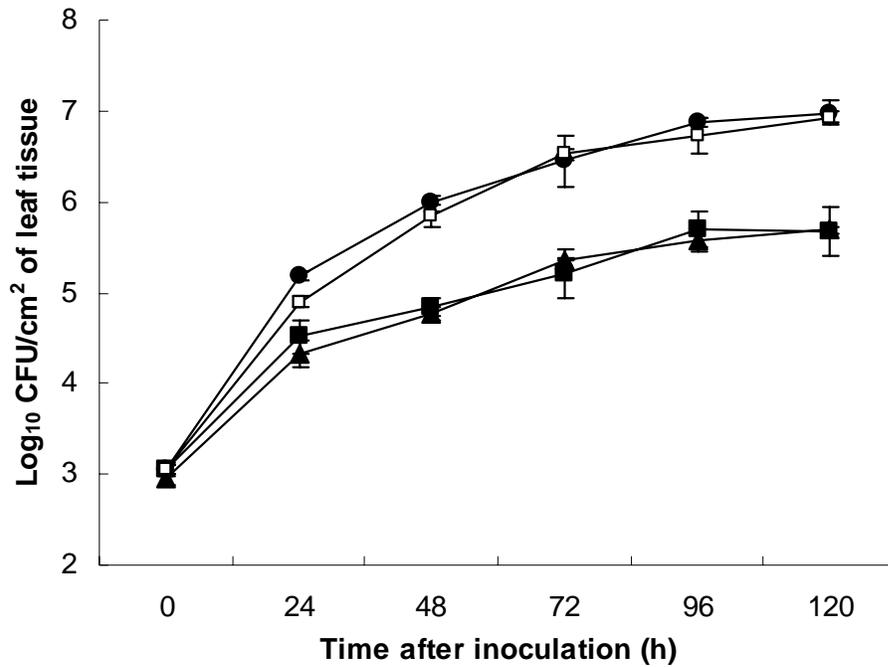


Figure 4-1. *In planta* growth of wild-type and mutant *X. perforans* strains. Plants were infiltrated with  $5 \times 10^5$  CFU/mL of 91-118:: $\Delta bcnB$  (□), 91-118 $\Delta opgH$  (■), 91-118:: $\Delta opgH\Delta bcnB$  (▲) and wt 91-118 (●) in tomato genotype Bonny Best. Error bars indicate the standard error.



Figure 4-2. Disease severity on Bonny Best leaflets 2 weeks after dip inoculation  $5 \times 10^6$  CFU/mL + 0.025% Silwet L-77) with *X. perforans* strains wild-type 91-118 (left), 91-118 $\Delta$ opgH (center) and 91-118:: $\Delta$ opgH $\Delta$ bcnB (right).

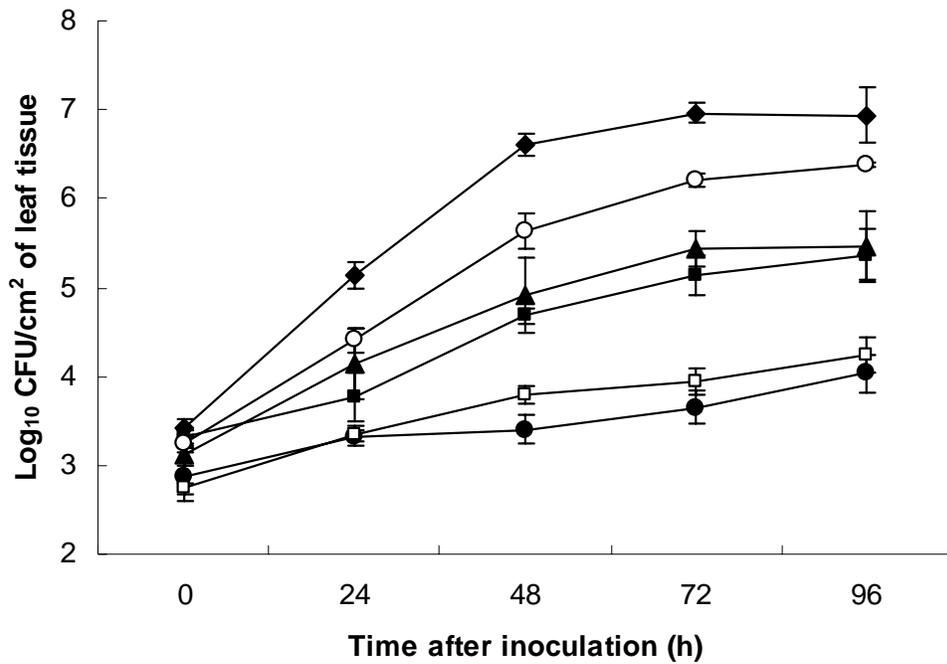


Figure 4-3. Growth room internal antagonism assay measuring *X. euvesicatoria* strain E3-1 in leaflets. Plants were infiltrated with  $5 \times 10^7$  CFU/mL of 91-118Δ*bcnB* (□), 91-118Δ*opgH* (■), 91-118::Δ*opgH*Δ*bcnB* (▲), wild-type 91-118 (●), wt *X. euvesicatoria* strain 91-106 (○) and water (◆) followed 18 h later by  $5 \times 10^6$  CFU/mL of E3-1 in tomato genotype Bonny Best. Error bars indicate the standard error.

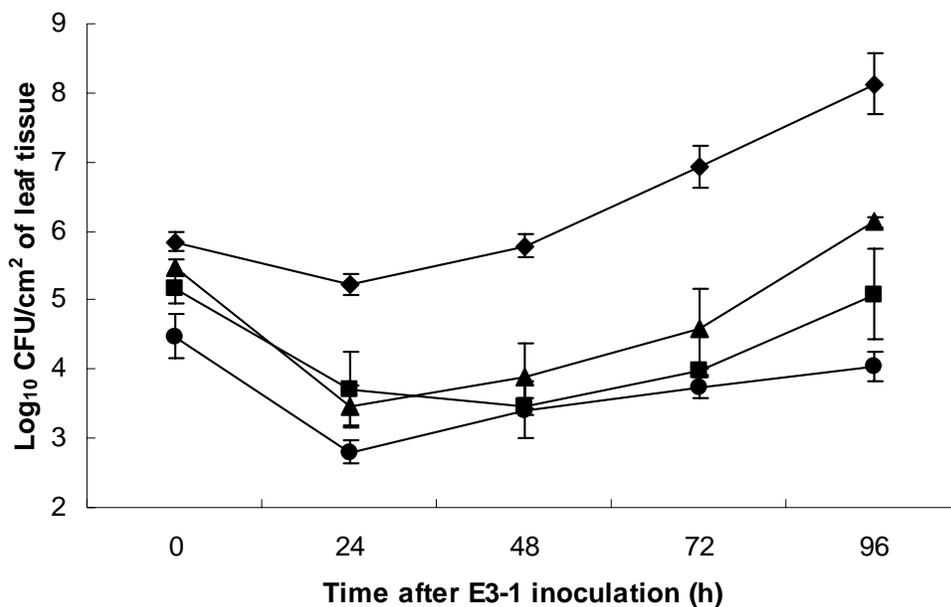


Figure 4-4. Growth room phyllosphere antagonism assay measuring *X. euvesicatoria* strain E3-1 in leaflets. Plants were dip inoculated with suspensions of  $5 \times 10^7$  CFU/mL (amended with 0.025% Silwet L-77) of 91-118 $\Delta$ opgH (■), 91-118:: $\Delta$ opgH $\Delta$ bcnB (▲), wild-type 91-118 (●), or *X. euvesicatoria* strain 91-106 (◆) followed 7 d later by spray inoculation of  $5 \times 10^7$  CFU/mL of E3-1 in tomato genotype Bonny Best. Error bars indicate the standard error.

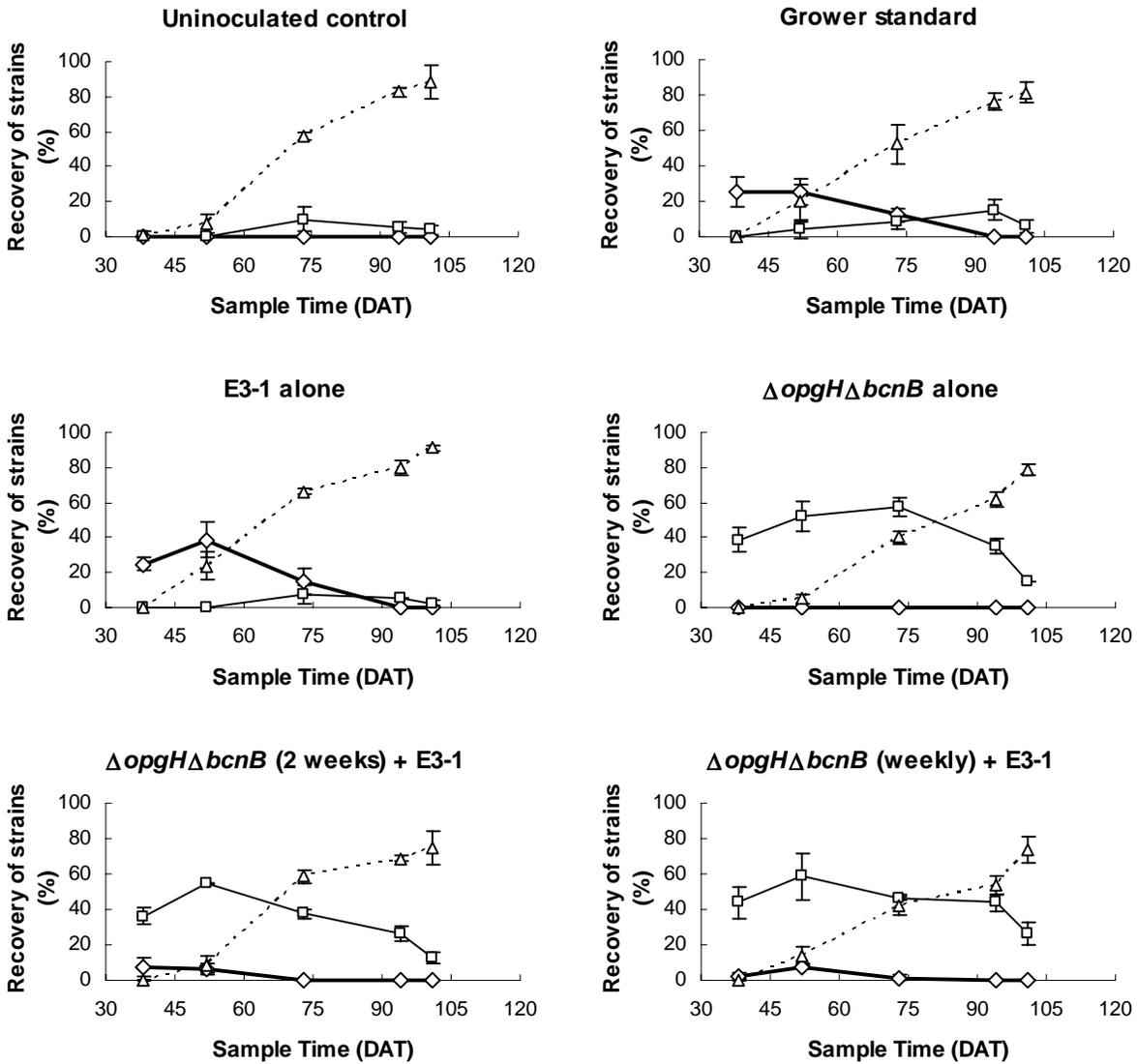


Figure 4-5. Quincy 2004 field experiment: Percent recovery of *X. perforans* strain 91-118:: $\Delta opgH\Delta bcnB$  (□), native wild-type strains (Δ) and *X. euvesicatoria* strain E3-1 (◇) from lesions from plants from the following treatments: (1) uninoculated control; (2) E3-1 followed by grower standard (copper + manzate & actigard); (3) E3-1 alone; (4) 91-118:: $\Delta opgH\Delta bcnB$  alone; (5) E3-1 followed by 91-118:: $\Delta opgH\Delta bcnB$  applied every two weeks; and (6) E3-1 followed by 91-118:: $\Delta opgH\Delta bcnB$  applied weekly. Sample times are indicated as days after transplanting (DAT). Error bars indicate the standard error.

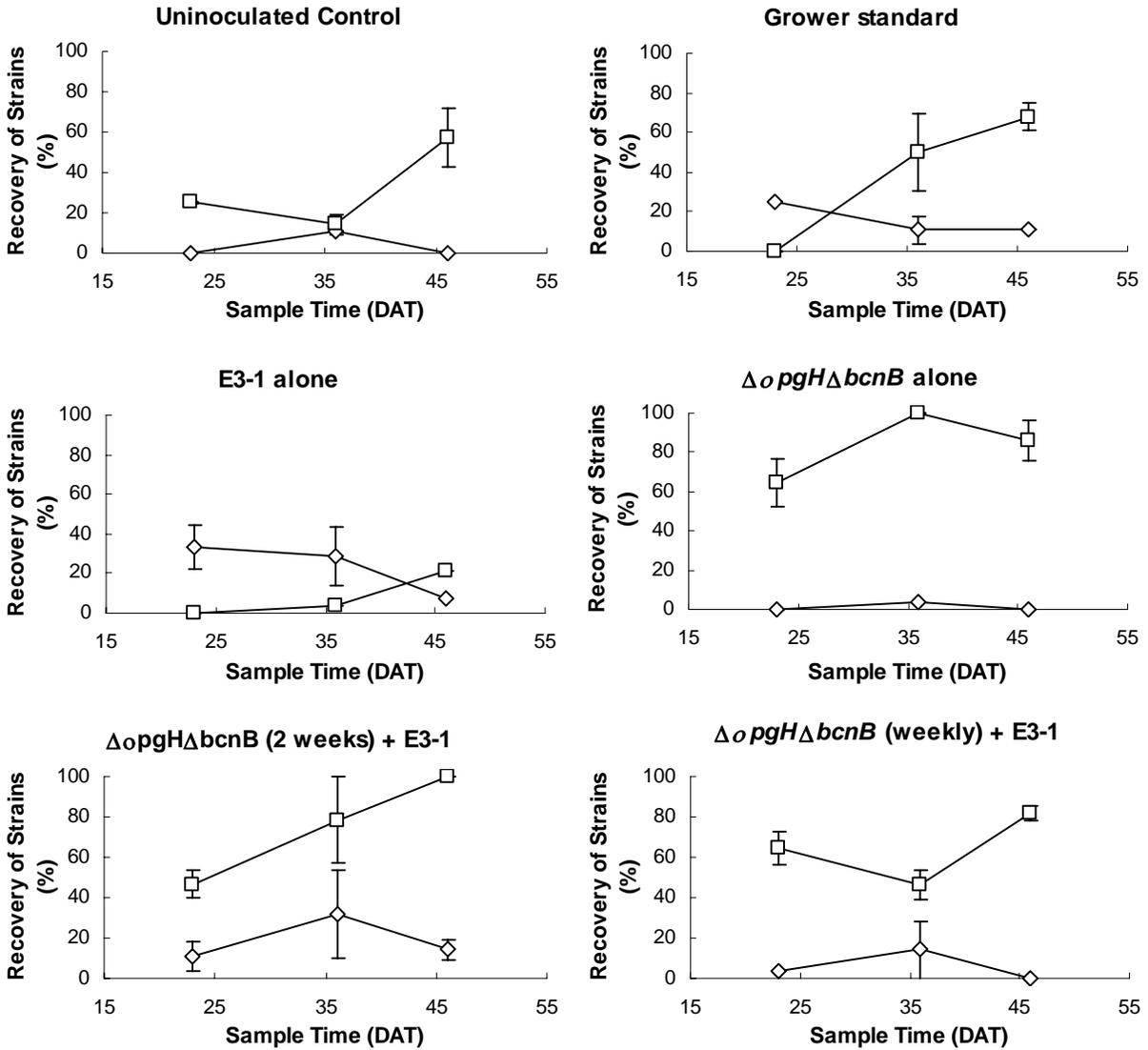


Figure 4-6. Quincy 2005 field experiment: Percent recovery of *X. perforans* strain 91-118:: $\Delta opgH\Delta bcnB$  (□) and *X. euvesicatoria* strain E3-1 (◇) from asymptomatic leaves from plants that received the following treatments: (1) uninoculated control; (2) E3-1 followed by grower standard (copper + manzate & actigard); (3) E3-1 alone; (4) 91-118:: $\Delta opgH\Delta bcnB$  alone; (5) E3-1 followed by 91-118:: $\Delta opgH\Delta bcnB$  applied every two weeks; and (6) E3-1 followed by 91-118:: $\Delta opgH\Delta bcnB$  applied weekly. Sample times are indicated as days after transplanting (DAT). Error bars indicate the standard error.

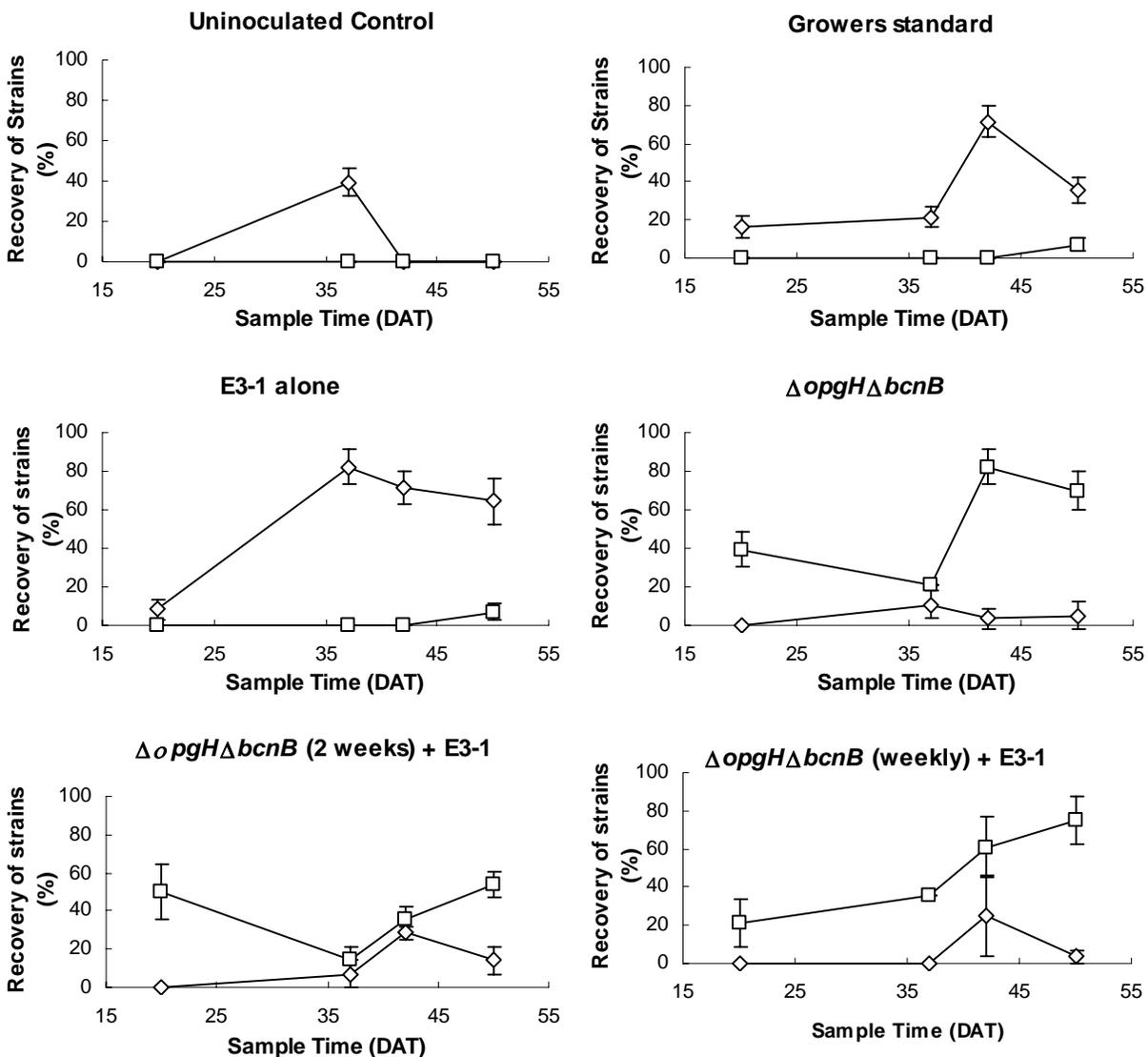


Figure 4-7. Citra 2005 field experiment: Percent recovery of *X. perforans* strain 91-118:: $\Delta opgH\Delta bcnB$  (□) and *X. euvesicatoria* strain E3-1 ( $\diamond$ ) from asymptomatic leaves from plants that received the following treatments: (1) uninoculated control; (2) E3-1 followed by grower standard (copper + manzate & actigard); (3) E3-1 alone; (4) 91-118:: $\Delta opgH\Delta bcnB$  alone; (5) E3-1 followed by 91-118:: $\Delta opgH\Delta bcnB$  applied every two weeks; and (6) E3-1 followed by 91-118:: $\Delta opgH\Delta bcnB$  applied weekly. Sample times are indicated as days after transplanting (DAT). Error bars indicate the standard error.

## CHAPTER 5 SUMMARY AND DISCUSSION

*Xanthomonas perforans* strain 91-118 produces at least three different bacteriocin-like compounds (BcnA, BcnB, BcnC), antagonistic toward *X. euvesicatoria* strains (155). Hert *et al.* previously (61) demonstrated that two of these bacteriocins previously discovered in *X. perforans* (BcnA and BcnC) (154) could effectively control *X. euvesicatoria* populations in greenhouse and field experiments. The goal of this study was to evaluate a new biological control strategy utilizing pathogenicity-attenuated, bacteriocin-producing *X. perforans* strains for control of bacteriocin-sensitive strains of *X. euvesicatoria*. The objectives of this study were: (I) to further characterize the bacteriocins associated with *X. perforans*, (II) to identify and individually delete pathogenicity genes to create partially pathogenic mutants of *X. perforans*; and (III) to determine the ability for these pathogenicity-attenuated mutant strains to antagonize *X. euvesicatoria* *in vitro*, *in planta* in the greenhouse and under field conditions.

For the first objective bacteriocins of *X. perforans* partially characterized by Tudor-Nelson *et al.* (155) were further characterized to determine their activities and possible functions. Disruption analysis has shown that BcnA is part of a multiple component family of bacteriocins. The toxin and immunity function to BcnA were localized to ORFA and ORF5, respectively. Using predicted localization, based on bioinformatics software SOSUI and PSORT, a model was created to represent the possible positions and roles of each ORF. The model suggests four steps: (I): pre-BcnA delivery into the periplasm chaperoned by ORF4; (II) Processing of pre-BcnA and delivery of the active BcnA outside of the cell by ORF2 and ORF3; (III) Entry of active BcnA into *Xanthomonas* cells (unknown); (IVa) BcnA suppressed by ORF5; and (IVb) BcnA inhibition (either in the periplasm or in the cytoplasm). The type 2 secretion system (T2SS) was also shown to be involved in the activity of BcnA. A *X. euvesicatoria* strain

transconjugant containing a T2SS mutant (91:106:: $\Delta xpsD$ ) and a BcnA expressing plasmid (pXV12.1) did not produce any detectible BcnA activity in plate assays. Further research is needed to confirm the involvement of the T2SS in BcnA secretion.

Previously BcnB and BcnC were localized to a 5.9 kb and 1.7 kb fragment, respectively (61, 154). In this study BcnB was shown to be produced by an ORF with endoproteinase Arg-C homology, while BcnC was found to have homology toward the extracellular metalloprotease family of genes. Disruption mutagenesis and protease assays confirmed that BcnB is in the endoproteinase Arg-C family of serine endoproteases and BcnC to be a metalloprotease. There is preliminary information that BcnB and BcnC are secreted via the T2SS. A *X. euvesicatoria* strain transconjugant containing a T2SS mutant (91:106:: $\Delta xpsD$ ) and a BcnB or BcnC expressing plasmid (pL5.8, and pL5.1, respectively) did not produce any detectible BcnB or BcnC activity in plate assays. Further research is needed to confirm the involvement of the T2SS in BcnB and BcnC secretion.

The second objective was to identify pathogenicity-related genes for disruption to create mutants of *X. perforans* with reduced virulence. The goal of this objective was to identify genes that could be mutated that would provide a dramatic reduction in disease symptoms while still maintaining the significant expression levels of BcnA and BcnC. Two mutants, 91-118:: $\Delta opgH$  and 91-118:: $\Delta gumD$ , stood out as strong candidates for further study. Both mutants had a pathogenicity-attenuated phenotype in disease severity and growth rate experiments, while exhibiting high levels of antagonism toward the bacteriocin-sensitive *X. euvesicatoria* strains.

Mutant 91-118:: $\Delta xopA$  was the only mutant to exhibit intermediate levels of disease severity and reduced growth rate, while 91-118:: $\Delta hpaB$ , 91-118:: $\Delta hpaC$  and 91-118:: $\Delta avrBs2$  were only slightly reduced. Antagonism assays revealed that these mutants (91-118:: $\Delta xopA$ ,

91-118:: $\Delta hpaB$ , 91-118:: $\Delta hpaC$  and 91-118:: $\Delta avrBs2$ ) were affected in antagonism toward *X. euvesicatoria* below what was observed by wild-type (wt) 91-118. Overall disease levels caused by these mutants were still too high to be a viable pathogenicity-attenuated mutant for use as a biocontrol agent.

In the final objective one pathogenically attenuated *X. perforans* mutant was selected as the model attenuated mutant for further studies. An osmoregulation gene, *opgHXcv*, was chosen (101) and, based on previous data (61), combined with an additional mutation ( $\Delta bcnB$ ) to create 91-118:: $\Delta opgH\Delta bcnB$ . In greenhouse experiments the growth rate and disease severity of both attenuated mutants 91-118:: $\Delta opgH$  and 91-118:: $\Delta opgH\Delta bcnB$  exhibited reduced growth rates compared to 91-118. The 91-118:: $\Delta opgH$  still maintained significant levels of antagonism toward E3-1 populations seen in the antagonism assay. Overall, mutants 91-118:: $\Delta opgH$  and 91-118:: $\Delta opgH\Delta bcnB$  were very effective in reducing *X. euvesicatoria* populations in the greenhouse. Interestingly, 91-118:: $\Delta opgH$  and 91-118:: $\Delta opgH\Delta bcnB$  were more effective at reducing external E3-1 populations than internal populations. Lindemann *et al.* (86) previously concluded that there is a direct correlation between phyllosphere populations and occurrence of disease. The existence of this correlation together with the reduction in *X. euvesicatoria* phyllosphere populations during antagonism experiments suggest that a biological control strategy for *X. euvesicatoria* by 91-118:: $\Delta opgH\Delta bcnB$  may be effective at reducing disease by reducing phyllosphere populations below the threshold level necessary to cause lesion development.

Field experiments using 91-118:: $\Delta opgH\Delta bcnB$  as a potential biological control agent were quite successful in reducing *X. euvesicatoria* populations. Weekly application of 91-118:: $\Delta opgH\Delta bcnB$  consistently reduced *X. euvesicatoria* population by up to 85 percent and

in two out of three experiments application every two weeks was also found to significantly reduce E3-1 populations. This reduction in *X. euvesicatoria* populations is similar to previous field data using 91-118:: $\Delta bcnB$  (61). Hert *et al.* (61) found co-inoculation of 91-118:: $\Delta bcnB$  and an *X. euvesicatoria* in the field, yielded less than 5 percent recovery of the *X. euvesicatoria* strain over all seasons tested. The 91-118:: $\Delta opgH\Delta bcnB$  mutant appears to colonize within the leaf tissue more effectively than non-pathogenic strains, similarly to *R. solanacearum hrcV* mutants (41), resulting in more effective biological control. These results suggest that the 91-118:: $\Delta opgH\Delta bcnB$  attenuated mutant has potential as a biological control agent for reducing *X. euvesicatoria* populations.

Further research is needed to optimize this system. Recent information from related bacteria and genomic sequences and functional genomics can be used to provide opportunities to improve our understanding of how pathogenic bacteria colonize and subsequently infect the host. Continued exploration of new innovative ideas will help us to utilize this knowledge in effective ways.

Bacterial spot disease is one of the most severe bacterial diseases affecting production of peppers and tomatoes in regions where they are grown (123). No single strategy has been successful for controlling this disease. The findings of this study warrant further investigation into the development of a biological control strategy for bacterial spot disease utilizing attenuated, bacteriocin expressing *X. perforans* strains.

Further research needs to be done to definitively determine the function and role of each bacteriocin. BcnA has been shown to need at least four proteins in order to produce a functional bacteriocin and we have a model for how those proteins may function. However more research is needed to determine the accuracy of the model.

APPENDIX A  
SEQUENCE AND ALIGNMENT OF BCNB AND PATHOGENICITY-RELATED GENES  
CHOSEN FOR DELETION

CACTAAAGGGAACAAAAGCTGGTACCGGTGGTTGCCAGCAGATGACATCCAGCATGGAAA - 60  
 ACCTAGCACTTTTACCAATGGATGGGCTTGTTCGGCCACATTATCGTCACAATGCCGGTCA - 120  
 CATGACGGCAGCACGGGACGGGCGTTTCAGGTTCGTCCGGGCGCTTGCCTGCGTGCCGGCA - 180  
 TTCCTTGCAACGGACACCTGCATGACCGACACGCCCCGCCGCGACGATGCGGCCACGCTG - 240  
 CAACGCTTCGGCTACGCACAGGAGCTCAAGCGGCAGCTCACGCTGAAGGATCTGTTGATC - 300  
 TACGGGCTGGTGTGCATGGTGGCCACGGCGCCGTTCTCGATCTTCGGCGGCGTCTTCGAC - 360  
 ATCAGTGCCGGGATGGTGGCGCTGACCTACCTGGTTCGGCTTCGTGGCAATGCTGTTACC - 420  
 GCACTGAGCTACCAGCAGATGTCGCAGGCATTTCCGGTGGCCGGTTCGGTATATGCCTAT - 480  
 GTCGGGCGCGGGCTGAGCAGCGGCATGGGATTTCTGGCTGGCTGGGCGATTCTGCTCGAC - 540  
 TACCTGCTGGTGGCGACGCTGTTGTACGTGGTTCGGCGCCAATGCGATGCAGACCGTATTG - 600  
 CCGGCGATACCGCAGCCGGCGTGGATTGGGTTCTTCGTGGTGGCTCAACACGGTGGTGAAC - 660  
 CTGCGTGGCATCGAAACCACCGCACGCGCAATCGCTTTTTCTGCTTGGCGAGTTGCTG - 720  
 GTCCTGGCGATGTTTCGTGGTGGTGGCGACGCTGGCGATCCAGCGCGGCGTCAATGGCGCG - 780  
 CACTGGAGCTGGCGCCCCCTGTATAACCCGCGAGGCGTTTTCTCCGCGAGCTGATCTTCAGC - 840  
 GCGTTGTCGGTGGCGGTGGTGTTCGTTCTCGGCTTCGATGCGATCTCCACCATGTCCGAG - 900  
 GAAGCGCGCGGGCGCAACCGCGTGGTGGGGCGCGAACCTTGCTGGCCTTGCTGATCGTG - 960  
 GCAGGGCTGTTTCATCTGCAAACCTGGCTGGCGGCATTGCTGCAGCCTACCCTGCAGCGC - 1020  
 TACCCAAGCCCGCAGGCATCCAACGATGCGTTCTTCGAGATCGGGCGGTTGATCGCCGGA - 1080  
 CCATGGCTGCAAATTGTCATTGCGCTCACGGTGGCGATCAGCGCGGCAATCGCCAATTCA - 1140  
 TTGGTTGCACAGGCGGCCACGTTCGCGACTGTTGTTTCGCCATGGCGCGCGACCGGCAATTG - 1200  
 CCGGGCTTTCTGAGTTACATCCACCCGCGTACCGGCGTACCGCAGCGCGGATCCTGCTG - 1260  
 GTGGCGGGCCTGAGCCTGGTACTGGGCGAAGTCTTCGTTCGGGCGAGATCGCGCTGCTGTCG - 1320  
 TCCTTGTGCAACGTTCGGCGCACTGACCGCCTTCGTGTTGCTGCATATCGCAGTGCTGTGG - 1380  
 CACTTCCGCACCCACGGCCGGCGTGTGCTGCATGGCGTGGTGGCCCTGATCGGGATCGTC - 1440  
 ATCCTGGCCTACGTCTGCTCAATGCCGACCTGCATGCACAACCTGGGCGGCGCTGCGTGG - 1500  
 ATGGCAGTGGGGCTGACGGTACTGGCGTTTTCTGAAACTCAGCGGACGCTCCACCGAATTG - 1560  
 CGCGCCAGCGACGCGCTGGAATAGCCTCAACAGCAGCACCGTCTGTTGTGCCGGCGTGCT - 1620  
 CCTGGCCCACCCGTGCTGCGGTGTGCGACGGATCAATCTCGTGCTGGGCAACCTCAAACA - 1680  
 CGTCATCAAGGTGGTGCATCACGCCATCAGCGCAAATACACAGGGCTTGACTTGAGAGA - 1740  
 TGGACGATCGCTCACCGTCGATCCGCTTCACGATGCGCTGCCACGACTTGCCACGGGCAT - 1800  
 GTTGAGTCCAAGCAGTGCCTGGAGCCGTTTTGACGTGCAGCAGCGATCTTCGTGGCCAG - 1860  
 GAGTCAGGATCAATCAAAAATTTAGAAACTCTCTCTTGGCCTGGAAAACCTCATTGCAAT - 1920

Figure A-1. BcnB sequence. Nucleotide sequence of a 5968 bp *KpnI* and *EcoRI* fragment containing *bcnB*. The deduced amino acid sequence is given for BcnB.

TTATTGCCGGCTATCTAATCCGGCCGGCGAGTGGCCGGCGGCTGGCGGTACGGCAGCCAC - 1980  
 GAGGTGAACAATTGCCTGCGGGGAAGACGCCGGTCTTCGGATGTTCCCTGCGACTTCC - 2040  
 ATGGGAGAAAAGATGCGCTGCAGATCGCCCGGCTCGATCTTGACAGGAAATAAGAGCGC - 2100  
 CACAACGTCAATTTCGTGATTGTCCGCCAGGTTGACAGTGTGGCCGGGCGTCCGTACG - 2160  
 GTGATGAACTGAGCGAAGCGATCACCGCTGGTGTGCCGGCGCGCTCGTCAGCAGCCCCG - 2220  
 GGGTGTATCCCAGTGCGGGTGATGCATGAAAAAGGGCACC GACTGGATCGGATCCAGGGCG - 2280  
 CGCGTAAACTGGCGCAGCGTGAATCGCAGTTCGCTGTGCTTCGATCTTAAGCTTTGCATA - 2340  
 TAGTGTGTGTGCGCGCCCCGCTGGCCCCGAGGCGCGGTGGATTGAATGAATAAATGCTGATC - 2400  
 TTGCCCGGGGCTCCGGCCGCGCGCAGAAAAGTGCGTGATGTGGAAGCCGGCGAGCGTGGCC - 2460  
 GCTGCTGCGCCCAGACTGTGTCCCCTCAACGACATCGACAACGCCTTGCCGCTCTCCCGC - 2520  
 GCGCGATTTCGTGTAGCGTTTTCAACACGTCTTTCATGACGGTTCGTCGGCGCGCGGCAAC - 2580  
 TGAGCTTCGGACTGCCACCATTCTGCCAGCCTGCTCCAACCTTGCCCAAGGTCGGAAGG - 2640  
 CGCTCGTCAAATATGTTGACGTGTACCTGTGCGATCTGGCTTTTTCATATCGCATAACAGA - 2700  
 TCGTCGGTTCGCATTTCAGGCATGTGCCGAAAAACCCAGCACCGCCACAATCACATCGTCG - 2760  
 GTCTCTCGCTCCCAGAGTTTTCAACCGCCGCGTGGACATCGGCACATTTCAGATGCAACCCA - 2820  
 CCGATGTCAGCCACCGTGCCTGGATCGCCCTTGTAGGATTTCGCTGCGCATGCGCGCATG - 2880  
 CTTTCGAGCACAGTGCGGCAGTGTATTTCCATTGCGGATCCTGTCCGTGCGGCTGCGCC - 2940  
 GATGCACGATCCACTTGGGCAATCGCGCTGACGAGATCGCATGCCTGGGTATCTACCTGC - 3000  
 GGCAGACGCATGCCAGATGACCGCCAGGCACCTGCCGTGATCGATCGTGTGCGGTTACGT - 3060  
 CCGCGTTTTGACCGGCGCAGCACTGAGACCTGGAACGC ACTGGCGCTGTCACGCGCGTGC - 3120  
 CAGGGGAAAGCGGTGCGGAGGCAGGCACGTGCTATCGCCGTGCGCGGATTGCGCGTGC - 3180  
 GGCAGCCTTGAAAAGAAGTTGAGGTGGGTGCGATGCGACATGTTTTAGGTGAGTGCGCA - 3240  
 GAGATTCTGTTTTATATCCCCACCTGGCACGCAGCCAGAGATGTGCTCCACCGCCGTGAAA - 3300  
 ACGAACGAAGGATTCACTGGCTCCACCATCGCTGTGCATCCCTCAAGGCCGGGTTGAGGT - 3360  
 CGCCCGAGGCCCTAGGAGCGCTGATCGCCACCACGACTATGACGTGGATTGAACACACCA - 3420  
 AGCTGCCACTGCGCAGCTGGATGCTGGACTTGACCTGCTGACTAGAACCAACATGGCCG - 3480  
 CCCTGGGAGCCTATGCACCGCCCGGGCCGACGGCATGACGGCCAGTGGATGACAGACG - 3540  
 CGTGCGGGCACTATATCGATCACGACCTTTTCATTCCCGAACGAGATTTTTAGCACATATT - 3600  
 CAGCTTTAAAGTTTTGCTAGGCTCGTTCCGGCGCCAACGATGGCGCCCGCTTGTTGCAGCC - 3660  
 CCCGCTGCAGCCTGACGATCCCTGACGAGAAGGACTACCCATGAATCGCAAGAACGCGAT - 3720  
  
 M N R K N A M  
 GTATCTGGCGTTGTTTTCCGCTGTTTTCCGGCACCGCAGCGGCCCGCCCGACCGAGAT - 3780  
 Y L A L F S A V S G T A A A A P P T E M  
 GGATGCAGCGCCGGTACCACCGCGCCGAGGCGGCCAAGCTTGGTGCGGCCACGTTGCA - 3840  
 D A A P V T T A P Q A A K L G A A T L Q  
 GTCGGCAAGTTTTACGCGGCGGCGTTCTGCCCACGCGCGTGGTGCAGCTCACCGCACCTAC - 3900  
 S A S L R G G V L P T R V V Q L T A P T

Figure A-1 continued.

CAGCGCCGAAATGGGTCTGTGTGCGCGAACGTCGTATTGCACAGGTCAAGCATGGGCAGCC - 3960  
S A E M G R V R E R R I A Q V K H G Q P  
GTTGCAGATCGGTTTTTTCGCGTGCGGTGACACAGCCGACGGTGAACCTGTCCAAGCTGGA - 4020  
L Q I G F S R A V T Q P T V N L S K L D  
TTGGCAGATGGCTCCCGATGGCTCGCGCGTGGCCAGTCTCAAAGTCAGCTCCGCACAGGC - 4080  
W Q M A P D G S R V A S L K V S S A Q A  
GGCGTCTGCTGCGTGCATCGCTGGTACTGCGTGGCGCCGGTGAACGCCTGGCGACCCATC - 4140  
A S L R A S L V L R G A G A T P G D P S  
CAAGGTGACGCTGCGCTTTGCCGGCAACGATGGTTCGCGTGTTCGAGCAATCCGGCGCCAG - 4200  
K V T L R F A G N D G R V F E Q S G A S  
CTTTGCTGCCAGCGGCAACGATATCGGTTGGTTCGCCGACGGTGTAGTGGCGAGGATCTGCT - 4260  
F A A S G N D I G W S P T V S G E D L L  
GGTGCAGCTGTGCTGCCGGCCGGGTTGTATCCGGAAAACCTTCAGCCTCAGCATTCCGCA - 4320  
V E L S L P A G L Y P E N F S L S I P Q  
GTTGTGCGCATCTGGATATCAGCCCCACTGCCAGCCCGCGGACATGATGACCATTGCCAT - 4380  
L S H L D I S P T A S P R D M M T I A I  
CGGCGAAAGCGATTCTCTGTCAGAACGACATCGTCTGCCGTGCCAACCCACGACCGGCTT - 4440  
G E S D S C Q N D I V C R A N P T T G F  
CACCAGCGCCGCAAAGGCGGTGGCACGCATGGTGTTCACCACCAGCCAGGGCTCGTTCCT - 4500  
T S A A K A V A R M V F T T S Q G S F L  
GTGCACCGGCACGCTGCTCAACAATACCAATTCGCCCAAGCGCAACCTGTTCTGGACCGC - 4560  
C T G T L L N N T N S P K R N L F W T A  
GGCGCATTGCATCAGCACGCAGACGGTGGCCAACACGCTGCAGACCTACTGGTTCTACGA - 4620  
A H C I S T Q T V A N T L Q T Y W F Y D  
TGCGGCCAGCTGCAATGGCAGTACGGTTCAGCTCGCAGGCAACCACATTGACGGGCGGGCGC - 4680  
A A S C N G S T V S S Q A T T L T G G A  
GTTCTGCGGCATGCCAACACCACGCGCGACACCGCATTGCTGGAGCTGAAGACCGCACC - 4740  
F L R H A N T T R D T A L L E L K T A P  
GCCAAGCGGCGCGTTCTATGCAGCGTGAATAGCGCGGCGATCGGTTCCACCGGCACCTC - 4800  
P S G A F Y A A W N S A A I G S T G T S  
GATTGTGCGCATCCATCACCCATCGGGCGACGTCAAGAAGTATTCGCTGGGCACGGTGAA - 4860  
I V G I H H P S G D V K K Y S L G T V N  
TGCACTGAGCAGCTCCATCGATGGCAAGAGCCCGCTCTATCGCGTGGTGTGGAGCGACGG - 4920  
A L S S S I D G K S P L Y R V V W S D G  
TGTGACCGAAGGCGGCTCGTCCGGCTCGGGCCTGTTACCGTTGCCAGCGGTGGCGCCTA - 4980  
V T E G G S S G S G L F T V A S G G A Y

Figure A-1 continued.

CCAGCTGCGTGGCGGCCTGTATGGCGGCTATTCGTTCTGTAGCGCGCAGACCGATCCGGA - 5040  
 Q L R G G L Y G G Y S F C S A Q T D P D  
 TTACTACTCGCGTTTCTCGGACGTGTATTCCACCATTTCCACCTACTTCGGCCAGTAAGC - 5100  
 Y Y S R F S D V Y S T I S T Y F G Q \*  
 GGTAAGCCGCGTGGCAGGCGACCTTGTGCCTGCCACGCTTGATCGCGCATCGCCGGGCAA - 5160  
 TCCGTTTGGCGGGCGATGCGTCTTTCGGCTCAGCGACCGTGCATGCGTTGATCCGTGCACT - 5220  
 GAAGTGTCCGCAGGCTGTCCAATGCCACCACCATCTTCTTGCCACGCACTCAAGGCAACG - 5280  
 GCGTGACGTGCTAGCGCGAGACATCGCTCCACGGCTGTTTTTTGCAGCCTCGCTCAACAC - 5340  
 ATTGATCGGTGTGCGGTTGCCGGCGCCATAGGTGTACCAGATCCACTTCGGCCAATCGGG - 5400  
 GAATGCCTGATTGACGTGCACTGCACCAACGGCGCACTAGCGGGCGGAGCGGGCGCAATG - 5460  
 ATGCAGACTGATGGGGCCTGTTCCCTTACAACGGTCGCCCATGGTTCGCGCACGTGCCCC - 5520  
 ACTGCTGGTCATCATCCTTGTGGCGTGGCGCTGCAAGCCTCCGCCCTGGCGGCCGGCAA - 5580  
 GCGGGCCGATCGCGAGGACATCAAGGCCTATGCGCTGCAGCGGTGCCTGGATAACAATA - 5640  
 CACGCGTAGCAGCAAGTACGCACCCGATCAGCTACGCGACCGCTCGTATCTGCTCACCAC - 5700  
 ATATGCGATGGACAACGCCAAGGTCGGCGCGACCGACCGCCTGCATCGCTTTGTGCGACGC - 5760  
 CAGCACGGCGGGGTTTGACAAGCGCGAAGTCCCGATGAAGGACGAAGCGCGCCGCGGCC - 5820  
 GTTCAATCGCATCTTCGCCCAATGCATGGCGTTCTATCGTTCCGCCGGCCTTGGATGAATT - 5880  
 CCTGCAGCCCCGGGGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAATT - 5940  
 CGCCCTATAGTGAGTCGTATTACAATTC - 5968

Figure A-1 continued.

$\xrightarrow{\text{hpaAF}}$   
GCTCAAGCTGGTGGTGGTGGTGGCCGCCATTGCCGTGACGGCGCCGTGGGGCGCCTCGGC - 60  
CATCATGCAGTTCGGCCAGGCATTGATGCAGGCGGCGTTCCCAATGATCCGTGCATCTCG - 120  
M I R R I S  
CCCGGCACCTTGGCGCCCTCGGTTTTCCATCGAGCATGGCGGCTCGCATCACCATGGCGAT - 180  
P G T L R P S V S I E H G G S H H H G D  
CATCATTCCGATGCGGCAGGCACCAGCGCGGGTTCGCTACCGACATTGCGGCACGAGCA - 240  
H H S D A A G T S A G S P T D I A A R A  
CCGCGTCTGCGCCCTGCTCCACCGCGCAGGCGACGCCGCGGGATCCGGTTCGCTGGACGGC - 300  
P R L R P A P P R R R R R G I R S L D G  
  
 $\xrightarrow{\text{hpaA2F}}$   $\xleftarrow{\text{hpaADF}}$   
CAGGAGGATGAGTTCGACGCCAACGAGCAGGAAGAGATCGAAGCCAAACGCGAGTGCGCA - 360  
Q E D E F D A N E Q E E I E A K R E C A  
CTGCGCGGACGGGTGAGCGTGGCGATCACGCCGGCCAGAGCCGGGAGCATGGCCAGGGC - 420  
L R G R V S V A I T P A Q S R E H G Q G  
GACCAGCACGGCGGTGACCGCAGCACGCATACCGACGATCCGCAGGCCGGTCCGTGGCAC - 480  
D Q H G G D R S T H T D D P Q A G P W H  
AGCGCCGCTCCAGCGCAATTGAGCGACAGCATCCGCACGTGCATCGACGCGATTGTCGAT - 540  
S A A P A Q L S D S I R T C I D A I V D  
CGCTACGTGACCAGGCGGACGCCGATCCAGTGGCCAAGCGACACGCGCTGGCTGCTGCG - 600  
R Y V T R R D A D P V A K R H A L A A A  
CTTGTGCGAGCTGCGCGCCGTGCGGTTTTCGCATCCTGCCGTGCCCCCTTGACCGCAACG - 660  
L V E L R A V G V S H P A V A P L T A T  
ATCTGGAGGTTGATGCGCGAGCATCTACGCGGTACGACAAGGCCACTGCGGCGGAAAAC - 720  
I W R L M R E H L R A Y D K A T A A E N  
CTGCTGGCCTTGGGACCCGATTGCTGGAGTTGATGCCATCGGAGTTGGAGCCGGTGCCC - 780  
L L A L R T R L L E L M P S E L E P V P  
  
 $\xleftarrow{\text{hpaA2R}}$   $\xrightarrow{\text{hpaADR}}$   
GCGCTACGCAATTTTCATCTCCTGCTTCCACTGATCCTGCTGAATGCGGAAAAACCGCGC - 840  
A L R N F H L L L P L I L L N A E K P R  
AGACGCATCGACCGTACACATGCCATCACACGTCTGAACACACTGCTGATTGAGCAACCA - 900  
R R I D R T H A I T R L N T L L I E Q P  
CAACAGGCGGCTCAGGAGGTTGCGCCAT**TGA**CCATGCAGCTTCGCGTACTGACAGGAACCC - 960  
Q Q A A Q E V R P \*  
  
 $\xleftarrow{\text{hpaAR}}$   
ACGCAGGCGCACGATTAGAT - 980

Figure A-2. Nucleotide sequence of a 980 bp fragment containing the *hpaA* ORF. The deduced amino acid sequence is given for HpaA. The positions of the annealing points of four primers (*hpaAF*, *hpaAR*, *hpaADF* and *hpaADR*) used for PCR are indicated.

hpaBF →

GCGACGCTGCGCGACACCCTGGCCGACCCCGCCCTCACCGTCCGTTACAAGGGCAATGGC - 60  
GTTTTCTCGGTTTCCGGCAGCAGCGACTACGCAGAACGCGCAAGCCGCCGATTGCCGAC - 120  
GTCCGCAGCGACCTGGGCCCCGAGGTCCGCGCGCTGCACGTAGAGATCAGCCAGCAAGAC - 180  
CCGTCCATCAAACCACCGACAATTACGACGCGGCATTGCTCGCCGATGGCCTGCACTAT - 240  
GTCGAAACGCCGGATGGCACCAAGCATATGACATCCCTGCCGCAGCAAGCGGCGCAATGA - 300  
GAGATCACACCATGTTTCGATGCAATGACCGATGCGGTCACTCAGGATATGAGCAAGATTC - 360  
TGCAGGCCAAGGCGATGGATCTGTCCGGCGAGCGGTGCGCAACGTGGAGACAGCGCTGG - 420  
ATGCCACTGCGCAACAGATTTCGCGTGCCTGGTCTGCAGCGAGCGACCAGGTTCGCGCGCA - 480  
ACGACTTCAACGTCTTGTATGACGGCATCACGGCCGCGCGCAACATCGTCGCGCACATCG - 540  
CAAGCATGCCGTAATCGATTTCTTGGTTCGCTCGGACCAAGTAAAACGCGCCGATAGGCG - 600  
CACCATTACATGCTCACTACGAAGGAGTACTGGTATGCAAATTTTTCTGAAGTAAGCTC - 660  
GTGGAGGTCCCGTGTGGCCAATGCATGGATGGCTTCACCGGTGGTTTGTCCAATGGAAT - 720  
TTCCGGCGCTGCTGCACTCTCTGGTGCAAACGGCCAGATGGATTTCGCTACTCGGCGACAT - 780

hpaB2F →

GTCAGCCTCGGACGACGCTCAGAAGTCCATGAACAACAAGATCACGATGCTCAAAAACGA - 840  
TCTGGACTTCAACGTGGCACTCAACAAGTTCATCGGCAAGGCGGGCGACAACGCTAAGCA - 900  
GCTCGTTGGCCAGTAATGGGCTCGGGCATGCGCAGGACCTGTAGGGGGTCTGTGCATGT - 960  
CTCACACAGATGAGCAGCGCGGATTTCGAAACCATCGTTTCGACAGATGTGCGAGGCACT - 1020

M S S A R F E T I V R Q M C E A L

GGACTTGCCGGATGTGGAGTCGGTGTGATCGGCGTGTGCTTTGGGTCGAGGGCTTTGA - 1080  
D L P D V E S V L D R R V L W V E G F E  
GGTCTATCTGCACCTGCCGACCCACAGCCCGAAGACGACGTAAAGGAAGAAGCACTGTA - 1140  
V Y L H L P T P Q P E D D V K E E A L Y  
TCTGCGCATCGCCTACGGGTTACCACCTGCCGGGCGGACATTGACCGTTTTTCAGGCTTCT - 1200  
L R I A Y G L P P A G R T L T V F R L L  
GCTCGAAGCCAATCTCTCGGTGTATGCGCAGGACCAGGCCAGCTTGGACTGAACGATGA - 1260  
L E A N L S V Y A Q D Q A Q L G L N D D  
CGGCGTTATCGTGTGATTGTGCGGGTACCCTGCGGACGACGACGTGGATGGAGCGTGGAT - 1320  
G V I V L I V R V P L D D D V D G A W I  
CTGCGATCTGCTTGCCCACTACGCCGAGCATGGCCGCTACTGGAACAACAATATCTTCGT - 1380  
C D L L A H Y A E H G R Y W N N N I F V  
GGCGCACGACGAGATGTTTCGAAGGAATCGCGACGGGCAACTATCTGTGGTTACGCGCCTG - 1440  
A H D E M F E G I A T G N Y L W L R A \*

Figure A-3. Nucleotide sequence of a 2345 bp fragment containing the *hpaB* ORF. The deduced amino acid sequence is given for HpaB. The positions of the annealing points of two primers (*hpaBF*, *hpaBR*, *hpaB2F* and *hpaB2R*) used for PCR are indicated. The *KpnI* restriction site used for disruption is underlined.

ATACAGGCTTGTTGGCAGCTTGCCGAAGCCTCAATCACGGCCGCAGCGGCGCCCATTCGC - 1500  
 CATGGCACAGCAACCTAGCTTGCCGGCCAACTCCACCGGCCTGGCACTCCTCTGCCCGAT - 1560  
 GCGCGCGGTGGCGCTGAGTGAAACCCACTTGCCATGTGGAAGCCTGACACGCCTGCATCT - 1620  
  

← hpaB2R

  
 GCGCCGGCTTGCTCCCCGATATAGGTTTGGCGGGAGATCGCAGGTCTGTTCGTGCCGGCTC - 1680  
 ACCGCGATGGCCGACACTGCACAGAACAAAGGCAGGCGCAGACGCATGGTCTACACCGTG - 1740  
 CACATCCGCGACGGCAGCTGATGCACCGGTGCTGTTGGCGCTGACGAGGGACACGACCAC - 1800  
 TTTGCCGGCGAGTTCTTTCCGGTGTGTTGACGTGCGCGTCAAGGTCGACGCGTTCCGGAGC - 1860  
 GACGGCGCCATCGAGTCGAGATGCAATGCCTGCCTCGTGCAGTGAGCGACCTGCGCAGA - 1920  
 TCGCGGCATGAGGCCGTGGTGTTCATGATTGCTGGTAATGGCTAAGCTGCCAGACACCA - 1980  
 CCAAGCCACAGACGCGACGAACGCGACGTCTGTGGCGGCGGTGTACAACACTCCTGAGTA - 2040  
 TCAACGCGGCAGCTGAGCGCTCATGCTCGCCCCTCTGCCACTGGCGAAGTGCTTCGCTG - 2100  
 TCATGCAGTGACGCTTGAGCGTTTGACGCTGGCCATCCAGTGCCTGCGTGGCCGCGTCG - 2160  
 CGGGGGACCGCGCTAAAGGTTTGACTCCACCCCTCGCGTAGTGCAGTGCGTACCGCATCG - 2220  
 ATGGTCGCGATCGAAACGGAAGTGCCTGCGGATCTCCGTACGCAATGTCTGCAGCGCAGTA - 2280  
  

← hpaBR

  
 CGCAGTGTCTGGCCAAGCGGCCAGGGCCAGGCGCGGCGCGCATCCCCTCCTCCATGGCA - 2340  
GCCTT - 2345

Figure A-3, continued.

$\xrightarrow{\text{hpaCF}}$   
CGCATACCGCAACCGCAGGCACCGGGCAGCTGCCCGCAGTGATGCTCGACCATGCTGTGCG - 60  
AACAACTGATCAGGCAATCGATTTCGCGCGACCGCGGCCGCAATTTCTGGCACTTCCGC - 120  
CCGAGCAGGCGAACCCAGCTTGTGCGAACAGGTGGAACGCATCGTCGGCGACCATGCGCAGC - 180  
ATCCGCTGGCGGTGGTTCGCGTCGATGGACGTGCGCAGGTATGTGCGCCGCATGATCGAAG - 240  
CACGGCTGACCTGGCTGCAGGTCTATTGTTCCAGGAGCTGGGTTTCGGAGGTGCAGCTGC - 300  
AGCCGATCGGTAGAGTGGTGGTGTGATGCGCAAGCCGCCCTCCGCCATGTGCGCATCCT - 360  
M R K P P L R H V R I L  
GCCGGTCAGCGGCGCACTGCAGCGGCCGGCGGCTCCAGCCACGCCGGCCCGGTTCGGCGCT - 420  
P V S G A L Q R P A A P A T P A R S A L  
 $\xrightarrow{\text{hpaC2F}}$   
CCGCTCCAGCTTCTGCAACTGCGCCAGCGCTTGCAGCAGCGCACAGCTCGCGCTTCCCTG - 480  
R S S F L Q L R Q R L R S A Q L A L P C  
CATGGTGTGCCACCGCAGTGCAGCAGGATCGGCCGGAGCCTGATGCCGAGGAGGGCTT - 540  
M V L P P Q C D E D R P E P D A E E G F  
 $\xleftarrow{\text{hpaCDF}}$   
CACCGAGGCGCATGACAGCGTGCCCGTGCAAACCGACCCACCCTTACGTATCGAGGGGAC - 600  
T E A H D S V P V Q T D P P L R I E G T  
 $\xrightarrow{\text{hpaCDR}}$   
GAAACACCAAGAGCCGTCCCAAGGCAACGGCGATGGCGCAGTGGGGCGACAGATTGCGAC - 660  
K H Q E P S Q G N G D G A V G R Q I A T  
 $\xrightarrow{\hspace{1.5cm}}$   
CGAGTGGATACGCACGCAACGCGCCACATGGCGATCGACCACATCGCGCTGCGGGTGGC - 720  
E W I R T Q R A H M A I D H I A L R V A  
 $\xleftarrow{\text{hpaC2R}}$   
CGAGTTCTGCAATGCCCAACCGGTGCGCAGCGCAGGGAGCTGGGAAGCGTGGCTGGCTAT - 780  
E F C N A Q P V R S A G S W E A W L A I  
CGACCAAGAGGTCGTGCGCACAGACGACGTTGTTTTTGGCGCTTTCGCCGGACCAGCTATC - 840  
D Q E V V A Q T T L F L R L S P D Q L S  
GCTTCGCTTCAATACCAGTTCGCCAGATGCGCGCGAGGTA CTTTGGTGCAGAAAGCAGCG - 900  
L R F N T S S P D A R E V L W C G K Q R  
CCTGGAGGCTGCACTGACGTCCACGCTGAGTAGCAGCTCCAGATCAGCATCGAGGTTGT - 960  
L E A A L T S T L S S T L Q I S I E V V

Figure A-4. Nucleotide sequence of a 1419 bp fragment containing the *hpaC* ORF. The deduced amino acid sequence is given for HpaC. The positions of the annealing points of four primers (*hpaCF*, *hpaCR*, *hpaC2F*, *hpaC2R*, *hpaCDF* and *hpaCDR*) used for PCR are indicated.

**CTAAC**CGGGCACTGGCGACAGAAAGCCATTTGGAGGAACTCCATCTTGCTAACCGAGCAG - 1020  
 \*  
 AGCCAGACTCCGCCGGCATGTGCACTCTCTCAGGCGTTGACGCGCGTTGCGGCCGAGCGC - 1080  
 GCCCAGCTTGGCAGAGTGTTGCGGCACCCACGCGCAGCACGCCAATGCGGCTTTGCCACG - 1140  
 CATTGCCGTCGCATCTCGCCCAATGATGCGGCCCGCCTGCGCCTGCAGCTGGATGCCGGC - 1200  
 CAGATGGAGTTGCGGATTGCCGCGCGACGGCTTGGCGCTGCTATTGAACGAGGACGAT - 1260  
 GACTCACTACGCGTTTTGATCGCGGGAATGCTACTGGCTGATCGCCTGGGCGCATTGCG - 1320  
 CCGCTCGGCCTGGGCGCTGCCGAGGTGATCGCCTTCGAGCGCGATGCAGAGCCAGACGAC - 1380  
  

←
hpaCR

 TGCCACGGCATCGGCATGACACTGGGCGATCTCGATGCC - 1419

Figure A-4. continued.

XopAF →

AATCCGCGCGTGC GAAAGGCGCCGCGCATGCCAGCGCTGCCGCAAACAAAATGCGCTGCG - 60  
 GACCAGCCCCTGCCCCGACGACCACGTCCGGACCATTGCGCGCGCATGCGCCGCTTCGGCG - 120  
 AAAAGCCCGGACGAGGCCTCCCACTGCCAATTGGATACTTTATCGACATACTTGAATGCG - 180  
 ATCGTTGAATTGATCATGTGTGCTTACCTCGATCTCGATTGAATACAGGTCTCCAGGTGA - 240  
 GTAGAGCGCCGGTACACAACGACCCACGACGCTCGACGGCGGCAACATAACGCCACCTGT - 300  
 ACGCGATTTA**CTCGGG**ACCTACGAAACACTTAATAGGCAAGCGAAAAAAGTTTTTCAACG - 360  
 ACACCCCGTGGATTTTCTTATCGACCCTAAGAATCTTTTTTATTTACCTCTTCGCTTGCA - 420  
 CAAGCGTAATTTTCGATACTCTATGGCGCCGATGATTTTCAGCTTCTACTGTTTCAGCGGG - 480

XopA3F →

GCGGAAAGCGACACAGCAGTCGTTGCCTCCTGGCCCCGGCAGTTGAGCGAAAGACAAATC - 540

XopA2F →

CTAGTTAAACCAGAGAGAAATCGCTATGATCAATTCATTGAATACGTGCGCACCTCGGCGT - 600  
 M I N S L N T S H L G V  
 CGACTCTTCCTTTATGCAAGTCAACCCGGACCAATTTCAAAAATTCGATTCAAATCAAAG - 660  
 D S S F M Q V N P D Q F Q K F D S N Q S  
 CAATCAAGGCATCTCGGAAAAGCAGCTGGACCAACTGCTGACCCAGTTCATCTTTTCAAT - 720  
 N Q G I S E K Q L D Q L L T Q F I F S M  
 GCTTCTGCAGGACGACAATGCTGATGATTCCCCGAACTCTGACAAGCCCACCGATTTTCC - 780  
 L L Q D D N A D D S P N S D K P T D F P  
 GTCGCCACGCACCCAGATGCTAATGAATGTCATCGGAGACATTTTACAGGCGAAGAATGG - 840  
 S P R T Q M L M N V I G D I L Q A K N G

XopA2R ←

CGGACGCCTCGGTGGTTTTATCCGATGGAGGGCTCAACACTAGCCTCAGCCTCTCGGGCGA - 900  
 G R L G G L S D G G L N T S L S L S G D  
 CACTGCATCGATGCAGTAAACAACCTGTTGGTGCGCCCTACCGACCACGGCCTGCAGGTTG - 960  
 T A S M Q \*

XopA3R ←

CGGCAAACCTACTGCCGTTATCCACTTTGATCGCTTCCGGCTTGCCTCGTTGAACGACCA - 1320

Figure A-5. Nucleotide sequence of a 1716 bp fragment containing the *xopA* ORF. The deduced amino acid sequence is given for XopA. The positions of the annealing points of four primers (XopAF, XopAR, XopA2F, XopA2R, XopA3F and XopA3R) used for PCR are indicated. Two *AvaI* restriction sites are in **bold**.

GCCGCACCACTGCGTCGGCGACATCGTCAGCGCCCAAGGACTGATCCACGAAGATCTCCA - 1380  
 GGCATCATGCGTGAAGTGGTCCAGCACCGGCAGCAGCCGGAAGCGACGGCCATCGAACA - 1440  
 GCGCGTCACTGACGAAGTCCATGCCCCACAGCGTGTTGGGCGCCGTGGCCACCTTGATCG - 1500  
 GTTGCCGGCGACGACTGCTGCGACTACGTGCGGGCGACAATGCCGCAACGACAGGCCTT - 1560  
 CTTCTTGTAGATGCGATGCACCCGCTTGTGGTTGTCCCGCCAGCCTTCGCGCCGAGCA - 1620  
 CCACCAATACGCGTTTCGAGCCATAGTGGATGCGCGTCTGCGTGATCTCGCGCATCCGCA - 1680  
 AGCGGATGGCGCTGCAGTCGCGCGCCTTGGCCTTGTACGAAAACGCCGAGCGCGACATCG - 1740  
 CAACGATCCGCAATGCGCGTCGTTTCGCTCACGCCGAAGCGCTCTCTCAAGCGGACAACCC - 1800  
 AGCTGCGCTTCTGGGGCGCCCTTAGAGTTTTTTTTGTGACCACCTCCTGGAGCATCGCCTT - 1860  
  
XopAR  
←  
 GTCCAGGCTCAGGTCGGCAACCAGCTGCTTGAGCTTGCGGTTTTCTCTTCCAGCA - 1716

Figure A-5. continued.


  
**XopDF**

TGCTGCCTTTTTGATGGACAGTGGCTTGTGCGCATGTAAATGGCAGGCAGATGCTTCAGGA - 60  
 ACTGAATGAAGATCAACGCGACCAAGTCATACATCAAATAATAAGACGAATTGAGTATTG - 120  
 TCGGATCCTGAATATCGAGAAGTTGCGCTGAGCCGACTGGAGTCGGATTGCAGTGGAAA - 180  
 AATTACGCTAAGTCAACGGACTTTGGATCGCATTGACAAAGCCAAAGCCAAAGCCGAAGC - 240  
 CAAAGCCAAAGCCAAAGCCAAAGCCAAAGCCAAAGCCAAAGTCCGAGTCGAAGCCGGAGC - 300  
 CCAATGCAAGATCAACGAAATTATGGAATATATACCAAGATATGAAGCATTAGAGAAAAGT - 360  
   M E Y I P R Y E A L E K V  
 GCCAGTGCCTGTGGGATTCCATGCTTACCTGCGTGGTGATGGCTCATTTCGGCCCAGGGCT - 420  
   P V R V G F H A Y L R G D G S F G P G L  
 ATCTGGCATCCTTCGATACATGACCCAGATCAGAAGAAAAGATTGTATCTAGCAAGTGA - 480  
   S G I L R Y M T P D Q K K R L Y L A S E


  
**XopD2F**

GAGACGCAAACCTGGCCTTGGCCGCTCCAAAAAGCAAGCCTCTAAAAGGCGTATTCCGGAC - 540  
   R R K L A L A A P K S K P L K G V F R T  
 CCTCCATCAAAAACCAATTTGCTTCTTGAGATTTTCGAGCAAATTCAGCAATAGAGCGTA - 600  
   L H Q K P N L L L E I S S K F S N R A Y  
 CAGCATCAATGATTCAAGCAGCGCATATTTATACAAGCAGACCTGGAAGAAATGGTCGA - 660  
   S I N D S S S A Y L S Q A D L E E M V D


  
**XopDDF**

CGAGGAAACCGGCGAACTGACTCGTCTGGGTGAAGCAGTGATTTTCAGGAGCATCCCAAGG - 720  
   E E T G E L T R L G E A V I S G A S Q G


  
**XopD3F**

CATCCAGACGGCAATTCGAGCCAACCTCAGAATGCGTTATCAACAACCGGATCTGCCTCC - 780  
   I Q T A I R A N F R M R Y Q Q P D L P P  
 ATACAGTCCCCCTCAGGCCTTCCATCGGCCAGAAGAAACGTGGAATCCCATACTCCGGC - 840  
   Y S P P Q A F H R P E E T W N P H T P A


  
**XopDDR**

GGGTTCTTCTATTTCGTCCTGTTCCCGCCACCCCTTCTGGCGGTTGGCCGAGAACGC - 900  
   G S S Y S S L F P P T P S G G W P Q N A  
 ATCAGGTGAGTGGCATCCCGATACTCCGGCGGATTCTTCTATTTCGTCCTGTTCCCGCC - 960  
   S G E W H P D T P A D S S Y S S L F P P  
 CACCCCTTCTGGCGGTTGGCCGAGAACGCATCAGGTGAGTGGCATCCCGATACTCCGGC - 1020  
   T P S G G W P Q N A S G E W H P D T P A

Figure A-6. Nucleotide sequence of a 2173 bp fragment containing the ORF. The deduced amino acid sequence is given for XopD. The positions of the annealing points of four primers (XopDF, XopDR, XopD2F, XopD2R, XopD3F, XopDDF and XopDDR) used for PCR are indicated.

GGATTCTTCCTATTTCGTCCCTGTTCCCGCCCACCCCTTCTGGCGGTTGGCCGCAGAACGC - 1080  
D S S Y S S L F P P T P S G G W P Q N A  
ATCAGGTGAGTGGCATCCCGATACTCCGGCGGGTTATTCCCATCGTGCATGGCCAGCCCA - 1140  
S G E W H P D T P A G Y S H R A W P A Q  
GCCCCAAGCGTTCGAGTTCCACCTTCGATGATCTTGAGTCCTTGGACTATAGGCAGAACTA - 1200  
P E A S S S T F D D L E S L D Y R Q N Y  
TGTTTATCGCGAATTCGACCTTAACACCCCCCAGGAAATCGAGCAGCCAGGGTGGTGGCA - 1260  
G Y R E F D L N T P Q E I E Q P G W W Q  
GCAAGCCACGCCCCGCCAAAGCACGGACTCGACCTTCGATGGCCTCTCCTCCATGAGCCA - 1320  
Q A T P A Q S T D S T F D G L S S M S H  
TTACGGTAGCGAATTCGACCTCAACATCCCCCAGCAAGAAGAGTACCCTAATAACCATGG - 1380  
Y G S E F D L N I P Q Q E E Y P N N H G  
CACGCAGACCCCCATGGGATATTTCGGCCATGACTCCTGAAAGGATCGATGTGGACAATCT - 1440  
T Q T P M G Y S A M T P E R I D V D N L  
GCCGTGCCCCCAGGACGTGCGAGACCCCCGAACTTCTCCAGTGAGGGCCACTTCGTGGCT - 1500  
P S P Q D V A D P E L P P V R A T S W L  
GCTGGATGGACATTTGCGCGCCTACACCGATGACCTAGCTCGCCGATTGCGAGGGGAGCC - 1560  
L D G H L R A Y T D D L A R R L R G E P  
CAACGCCCATTTACTCCACTTTGCCGACTCGCAGGTAGTGACCATGCTGAGCTCCGCAGA - 1620  
N A H L L H F A D S Q V V T M L S S A D  
TCCAGACCAACAGGCCCCGCGCACAGCGCCTTCTTGCCGGAGACGACATCCACCTATCGT - 1680  
P D Q Q A R A Q R L L A G D D I P P I V  
  
← XopD2R  
GTTCTGCCGATCAATCAGCCCAACGCTCATTGGTCATTGCACGTGTCGACCGGCGTAA - 1740  
F L P I N Q P N A H W S L H V V D R R N  
CAAGGACGCTGTTGCGGCCTACCACTATGATTCCATGGCACAGAAGGACCCACAGCAACG - 1800  
K D A V A A Y H Y D S M A Q K D P Q Q R  
CTACCTTGCTGATATGGCGGCCTATCACCTTGGCCTTGATTATCAACAAACTCATGAAAT - 1860  
Y L A D M A A Y H L G L D Y Q Q T H E M  
GCCCATCGGATAACAGTTCGGACGGTTATTCTGCGGCGATCATGTGCTGACCGGGATAGA - 1920  
P I A I Q S D G Y S C G D H V L T G I E  
GGTGTGGCCACAGGGTACTCGACGGCACCTTCGACTACGCAGGCGGCAGGGACCTGAC - 1980  
V L A H R V L D G T F D Y A G G R D L T  
TGATATCGAACCAGACCGGCCTCATCAGGGATCGTCTTGCCCAAGCGGAGCAAGCTCC - 2040  
D I E P D R G L I R D R L A Q A E Q A P

Figure A-5. continued.

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AGCAGAAAGCAGCATCAGGCAAGTTCCGCACGATCCAACGAACAGAAGAAAAAGAAAAGC - 2100
A E S S I R Q V P H D P T N R R K R K A
                                     XopDR
AAGTGGTGGAAAAAGTCCTAGCAGTTCGACCATCAGCGGTAAAGTAGGGTTGGCAGGATT - 2160
S G G K S P S S S T I S G K V G L A G L
AGGGAAGGTCTGA - 2173
G K V *

```

Figure A-6. continued.



GCTCGTGCACAACCACTACTTTCGAAGCACCCAGCACGCGTGCGTCCGGAAAGGACGTCAT - 1140  
L V H N H Y F E A P S T R A S G K D V M  
GGTGCATCGCGGGCTGTTTCGATAATCACGCCGGCATTCCGGAAAACCTCGCTGGCGTCCAT - 1200  
V H R G L F D N H A G I P E N S L A S I  
CGATCATGCCTACGAGGTCGACCAGGGCTACCGCAATCTGGAGCTGGACGTCGAAGTCAG - 1260  
D H A Y E V D Q G Y R N L E L D V E V S  
TTCCGATGGCGTGCCGGTGTGATGCACGATTTTCAGCATCGGCCGCATGGCAGGCGACCC - 1320  
S D G V P V L M H D F S I G R M A G D P  
GCAGAACCAGTTGGTGTGCGAGGTGCCGTTTCCGAGCTGCGTGAAATGCCGTTGGTGAT - 1380  
Q N Q L V S Q V P F A E L R E M P L V I  
TCGCAACCCGTCTGACGGAACTACGTCAAGACCCAGACCATCGCCGGTGTGGAGCA - 1440  
R N P S D G N Y V K T D Q T I A G V E Q  
GATGCTGGAGCACGTGCTCAAAAAGCCCCGAGCCGATGTCCGGTGGCGCTGGACTGCAAGGA - 1500  
M L E H V L K K P E P M S V A L D C K E  
AAACACCGGCGAAGCAGTGGCGATGCTGCTGATGCGCCGGCCGGACCTGCGCAAGGCTGC - 1560  
N T G E A V A M L L M R R P D L R K A A  
GGCGATCAAGGTCTATGCCAAGTACTACACGGGCGGCTTTGACCAATTCCTGTCCAATTT - 1620  
A I K V Y A K Y Y T G G F D Q F L S N L  
GTACAAGCACTACCAGATCAACCCGTTGCACTCGCAGGATGCGCCACGTGCGCCGCGCT - 1680  
Y K H Y Q I N P L H S Q D A P R R A A L  
GGATCGCTTGCTGGCCAAGATCAACGTGGTGCCGGTCTTGAGCCAGGGCATGTTGAACGA - 1740  
D R L L A K I N V V P V L S Q G M L N D  
CGAGCGCTTGCGCGGCTTCTTCCGAAGCAATGAGCAGGGCGCCGCGGGGCTCGCAGACAC - 1800  
E R L R G F F R S N E Q G A A G L A D T  
CGCAATGCAGTGGCTGGACAGCTGGACCAAGATGCGCCCGGTGATCGTGGAGGCGGTGGC - 1860  
A M Q W L D S W T K M R P V I V E A V A  
CACCGACGACAGCGATGCCGGCAAGGCCATGGAATGGCTCGGACGCGGATGCGCCAGCC - 1920  
T D D S D A G K A M E M A R T R M R Q P  
GGACTCGGCCTACGCGAAGGCCGCGTATTCCGGTGGAGCTACCGGTATGAGGACTTTTCCGT - 1980  
D S A Y A K A A Y S V S Y R Y E D F S V  
GCCGCGCGCCAATCACGACAAGGACTACTACGTTTACCGCAACTTCGGTGGAGCTCCAAAA - 2040  
P R A N H D K D Y Y V Y R N F G E L Q K  
GCTCACCAACGAGGCCTTCGGCGTCAAGCGCACCCACGGCCGGCGGTTTTTCGCGACGATGG - 2100  
L T N E A F G V K R T T A G A F R D D G  
CGAAAGCCTGTTGACCGATCAGCCCCGAGGCCGAATTGCTCGCCATCCTGGAAAACCGCAC - 2160  
E S L L T D Q P E A E L L A I L E N R T

Figure A-7. continued



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X.p.  MIRRI SPGTLRPSVSI EHGGSHHHGDHHS DAAGTSAGSPTDIAARAPRLRPAPPPRRRRRG
X.e.  MIRRI SPGTLRPSVSI EHGGSHHHGDHHDASGTSAGSPTDIAARAPRLRPAPPPRRRRRG
X.o.  MIRRI SPGPLQPSVSTE HGDSSHNNADHHADAAGTSAGSPANIAARATRLRPAPPPRRRRRG
X.o.2 MIRRI SPGPLQPSVSTE HGGSHHHADHHADAASTSAGSPADIAARATRLRPAPPPRRRRRG
      * * * * * * * *
X.p.  IRSLDGQEDEFDANEQEEIEAKRECALRGRVSVAITPAQSREHGQGDQHGGDRSTHTDDP
X.e.  IRSLDGQEDEFDANEQEEIEAKRECALRGRVSVAITPAQSREHGQGDQHGGDRSTHTDDP
X.o.  NRSLEGQEDEFDANEQEEIEAKRECALRGRVSVAIAPAQ SREHGQSDQHGGDRNTHTDDT
X.o.2 NRSLDGHEDEFDANEQEEIEAKRECALRGRVSVAIAPAQ SREHGQSDQHGGDRNTHTDDT
      * * * * * * * *
X.p.  QAGPWHSAAPAQLSDSIRTCIDAI VDRYVTRRDADPVAKRHALAAALVELRAVGVSHPAV
X.e.  QAGPWHSAAPAQLSDSIRTCIDAI VDRYVTRRDADPVAKRHALAAALVELRAVGVSHPAV
X.o.  QAGPWRGAAPAQVTDSIRTCIDAILDRYVARRDADPVAKRNALAAALVELRAIGVSHPAV
X.o.2 QAGPWHGGAPRQVTDSIRTCIDAILNRYVARRDADPVAKRDALAAALVELRAIGVSHPAV
      *** * ** * * *
X.p.  APLTATIWRMLREHLRAYDKATAAENLLALRTRLLELMPSELEPVPALRNFHLLPLILL
X.e.  APLTATIWRMLREHLRAYDKATAAENLLALRTRLLELMPSELEPVPALRNFHLLPLILL
X.o.  APLTETVWRMLREHLSSYDRATAAENLQALRTRLLELMPSELEPVPALRNFHLLPLILL
X.o.2 APLTETVWRMLREHLSSYDKATAAENLQTLRTRLLELMPSELEPVPALRNFHLLPLILL
      * * * * * * *
X.p.  NAEKPRRRIDRTHAITRLNNTLLIEQPQQAQEV RP
X.e.  NAEKPRRRVDRTHAITRLNNTLLIEQPQQAQEV RP
X.o.  NAEKPRRQVDRRHAI TRLNNTLLIEQPQQAQEV RP
X.o.2 NAEKPRRQVDRRHAI TRLNNTLLIEQPEQAQEV RP
      ** * *

```

Figure A-8. Similarity of amino acid sequence of HpaA in *X. perforans* (X.p.) to homologs in *X. euvesicatoria* (X.e.), *X. oryzae* pv. *oryzicola* (X.o.) and *X. oryzae* pv. *oryzae* (X.o.2) using the multiple sequence alignment tool CLUSTALW. Mismatch AA sequences are indicated by an asterisk.

```

X.p.  MSSARFETIVRQMCEALDLPDVESVLDLRRVLWVEGFVYLHLPTPQPEDD
X.e.  MSSARFETIVRQMCEALDLPDVESVLDLRRVLWVEGFVYLHLPTPQPEDD
X.o.  MSSARFETIVRQMCEALDLPDVESVLSRRVLWVEGFVYLHLPTPQPGDD
X.o.2  MSTARFETIVRQMCEALDLPDVSVLSRRVLWVEGFVYLHLPTPQPGDD
      *           *           *           *

X.p.  VKEEALYLRIAYGLPPAGRTLTVFRLLLEANLSVYAQDQAQLGLNDDGVI
X.e.  VKEEALYLRIAYGLPPAGRTLTVFRLLLEANLSVYAQDQAQLGLNDDGVI
X.o.  AQQEALYLRIAYGLPPAGRTLTVFRLLLEANLSVYAQDQAQLGLNDNGVI
X.o.2  AQEEALYLRIAYGLPPAGRTLNVFRLLLEANLSVYAQDQAQLGLNDDGVI
      **                *                *

X.p.  VLIVRVPLDDDVDGAWICDLLAHYAEHGRYWNNNIFVAHDEMFEGIATGN
X.e.  VLIVRVPLDDDVDGAWICDLLAHYAEHGRYWNNNIFVAHDEMFEGIATGN
X.o.  VLIVRVPLDDDVDGAWICDLLAHYAEHGRYWNNNIFVAHDEMFEGIATGN
X.o.2  VLIVRVPLDNDVDGAWICDLLAHYAEHGRYWNNNIFVAHDEMFEGIATGN
      *

X.p.  YLWLRA
X.e.  YLWLRA
X.o.  YLWLRA
X.o.2 YLWLRA

```

Figure A-9. Similarity of amino acid sequence of HpaB in *X. perforans* (X.p.) to homologs in *X. euvesicatoria* (X.e.), *X. oryzae* pv. *oryzicola* (X.o.) and *X. oryzae* pv. *oryzicola* (X.o.2) using the multiple sequence alignment tool CLUSTALW. Mismatch AA sequences are indicated by an asterisk.

```

X.p. MRKPPLRHVRILPVSGALQRPAAPATPARSALRSSFLQLRQLRSLAQLALPCMVLPQQCD
X.e. MRKPPLRHVRILPVSGALQRPAAPATPARSALRSSFLQLRQLRSLAQLALPCMVLPQQCD
X.o. MRKLPLRHVRILPVSGALQRPATPATPARSAQRSSFLQLRQLRSLVELALPCMVLPQQCD
X.o.2 -----MRILPVSGALQRPATPATPARSAQRSSFLQLRQLRSLVELALPCMVLPQQCD
***** * * * *

X.p. -EDRPEPDAEEGFTEAHDSVPVQTDPPPLRIEGTKHQEPSQNGDGA VGRQIATEWIRTQR
X.e. -EDRPGPDAEEGFTEAHDSVPVQTDPPPLRIEGTKHQEPSQNGDDGA VGRQIATEWIRTQR
X.o. DEDRPEPDAEERFTEARDSAPVQTDPPPLRLDGPKHREPPQGNDNGA VGRQIATEWIRTQR
X.o.2 DEDRPEPDAEERFTEARDSAPVQTDPPPLRLDGPKHREPPQGNDNGA VGRQIATEWIRTQR
* * * * *

X.p. AHMAIDHIALRVAEFCNAQPVR SAGSWEAWLAIDQEVVAQTTLFLRLSPDQLSLRFNTSS
X.e. AHMAIDHIALRVAEFCNAQPVR SAGSWEAWLAIDQEVVAQTTLFLRLSPDQLSLRFNTSS
X.o. AQMAIDHIALRVADFCNAKPVR SAGSWEAWLDIDQEVVAQTTLFLRLSPHQLSLRFNTSS
X.o.2 AQMAIDHIALRVADFCNAKPVR SAGSWEAWLDIDQEVVAQTTLFLRLSPHQLSLRFNTSS
* * * * *

X.p. PDAREVLWCGKQRLEAALTSTLSSTLQISIEVV
X.e. PDAREVLWCGKQRLEAALTSTLSSTLQISIEVV
X.o. PDAREVLWCGRQRLEAALTSTLSSTLQISIEVV
X.o.2 PDAREVLWCGRQRLEAALTSTLSSTLQISIEVV
*
```

Figure A-10. Similarity of amino acid sequence of HpaC in *X. perforans* (X.p.) to homologs in *X. euvesicatoria* (X.e.), *X. oryzae* pv. *oryzicola* (X.o.) and *X. oryzae* pv. *oryzicola* (X.o.2) using the multiple sequence alignment tool CLUSTALW. Mismatch AA sequences are indicated by an asterisk.

```

X.p. MPRALLAARRVFEVIMRIGPLQPSIAHTAAPALPTHTSAISPTQVPHMPGNTPLLRERPR
X.e. -----MRIGPLQPSIAHTAAPALPTHTSAISPTQVPHMPGNTPLLRERPR
X.c. -----MRIGPLQPSVAHTAAPALPTHTSVVSPTQVPHMPGDIPPLLRERPR
X.o. -----MRSFMRIGPPQTSIAHTDALAIPTHTSASSPTQVPHVQGNTPLLRERAP
***** * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
X.p. RRATNMPPLVPLNDSAMTGKPALVALDSEFSEQRLAEVQARQITVQTLQAKLATHLAQAG
X.e. RRAGNMPPLVPLNDSAMTGKPALVALDSEFSEQRLAEVQARQITVQTLQGLATHLAQAG
X.c. RRADSAPPLVPLNDSAMTGKPALVALDSEFSEQRLAEVQARQITVQTLQTKLATHLAQAG
X.o. RRADSAPPLVPLNDSAMTGKPALVALDSEFSEQRLAEVQARQITVQTLQTKLATHLAQAG
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
X.p. TALKPDSIAARFAAGTLEPVYLDTAAFNAMSRGLPARARAAAGPVLIDAQQGRIIFNLQR
X.e. TALKPDSIAARFAAGTLEPVYLDTAAFNAMSRGLPARARAAAGPVLIDAQQGRIIFNLQR
X.c. TPLKPDZIAARFAAGTLEPVYLDTAAFNAMSRGLPARARAAAGPVLIDAQQGRIIFNLQR
X.o. TALTPDSIAARFAAGALEPVYLDTAAFNAMSRGLPARARAAAGPVLIDAQQCRIVFNLQR
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
X.p. AFAPGDTFSDAALAALGKQLNLSGHGLATPNWLQPAAGTPGRRKLQQAARYHGHEVPARD
X.e. AFAPGDTFSDAALAALGKQLNLSGHGLATPNWLQPAAGTPGRRKLQQAARYHGHEVPARD
X.c. AFAPGDSSDAALAALGKQLNLSGHGLATPNWLQPAARTPARRKLQQAARYHGHEVPARD
X.o. AFASGDTFSDAALTALGKALDLPGHGLATPDWLQPAARTPTRRKLQHAPRYHGHEVPARD
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
X.p. GGAGFFKANDHRLLEGGQVLLRNHQSLVHNHYFEAPSTRASGKDVMMVHRGLFDNHAGIP
X.e. GGAGFFKANDHRLLEGGQVLLRNHQSLVHNHYFEAPSTRAFSGKDVMMVHRGLFDNHAGIP
X.c. GGAGFFKANDHRLLEGGQALLRNHRQSLVHDHYFEAPSTRAFSGKDVMMVHRGLFDNHAGIP
X.o. GGAFFKANDHRLLEGGQALLRNHRKALVHDHYFEAPSTRALGKDVMMVHRGLFDNHAGIP
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
X.p. ENSLASIDHAYEVDQGYRNLELDVEVSSDGVVPLMHDFSIGRMAGDPQNQLVSQVPFAEL
X.e. ENSLASIDHAYE--QGYRNLELDVEVSSDGVVPLMHDFSIGRMAGDPQNRLVSQVPFAEL
X.c. ENSLASIDHAYE--QGYRNLELDVEVSADGVVPLMHDFSIGRMAGDPQNRLVSQVPFAEL
X.o. ENSLSSIDNAYA--KGYRNLELDVEVSADGVVPLMHDFSIGRMAGDPQNRLVSQVPFAEL
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
X.p. REMPLVIRNPSDGNVYKTDQTIAGVEQMLEHVLLKKEPMSVALDCKENTGEAVAMLLMRR
X.e. REMPLVIRNPSDGNVYKTDQTIAGVEQMLEHVLLKKEPMSVALDCKENTGEAVAMLLMRR
X.c. REMPLVIRNPSDGNVYKTDQTIAGVEQMLEHVLLKKEPMSVALDCKENTGEAVAMLLMRR
X.o. REMPLVIRNPSDGNVYKTDQTIAGVEQMLEHVLLKKEPMSVALDCKENTGEAVAMLLMRR
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
X.p. PDLRKA AAAIKVYAKYYTGGFDQFLSNLYKHYQINPLHSQDAPRRAALDRLLAKINVVPVL
X.e. PDLRKA AAAIKVYAKYYTGGFDQFLSNLYKHYQINPLHSQDAPRRAALDRLLAKINVVPVL
X.c. PDLRKA AAAIKVYAKYYTGGFDQFLSNLYKHYQINPLHSQDAPRRAALGRLLAKINVVPVL
X.o. PDLRKA AAAIKVYAKYYTGGFDQFLSNLYKHYQINPLHSQDAPRRAALDRLLAKINVVPVL
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
X.p. SQGMLNDRLRGFFRSNEQGAAGLADTAMQWLDSWTKMRPVIVEAVATDDSDAGKAMEMA
X.e. SQGMLNDRLRGFFRSNEQGAAGLADTAMQWLDSWTKMRPVIVEAVATDDSDAGKAMEMA
X.c. SQGMLNDRLRGFFRSNEQGAAGLADTAMQWLDSWTKMRPVIVEAVATDDSDAGKAMEAA
X.o. SQAMLNDEHLRGFFRSNDDGAEGGLADTAMQWLESWTRMRPVIVEAVATDDSDAGKAMAMA
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
X.p. RTRMRQPDSAYAKAAYS SVSYRYEDFSVPRANHDKDYVYRNFGE LQKLTNEAFGVKRTTA
X.e. RTRMRQPDSAYAKAAYS SVSYRYEDFSVPRANHDKDYVYRNFGE LQKLTNEAFGVKRTTA
X.c. RARMRQPDSAYAKAAYS SVSYRYEDFSVPRANHDKDYVYRNFGE LQKLTDEAFGVKRTTA
X.o. RERM RQPDSAYAKAAYS SVSYRYEDFSVPRANHDKDYVYRNFGE LQKLTNEAFGVKRTTA
* * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

```

Figure A-11. Similarity of amino acid sequence of AvrBs2 in *X. perforans* (X.p.) to homologs in *X. euvesicatoria* (X.e.), *X. axonopodis* pv. *citri* (X.c.) and *X. oryzae* pv. *oryzae* (X.o.) using the multiple sequence alignment tool CLUSTALW. Mismatch AA sequences are indicated by an asterisk.



```

X.p.  MINSLNTSHLGVDS S FMQVNP DQFQK FDSNQSNQGIS EKQLDQLLTQFIF SMLLQDDNAD
X.e.  MINSLNTSHLGVDS S FMQVNP DQFQK FDSNQSNQGIS EKQLDQLLTQFIF SMLLQDDNAD
X.c.  -MNSLNT-QIGANSS FLQVDPS QNTQF GPNQGNQGIS EKQLDQLLTQLI MALLQQSNNAD
X.g.  -MNSLNT-QLGANSS FFQVDPG QNTQSSPNQGNQGIS EKQLDQLLTQLI MALLQQSNNAE
      **      ***      *      *      *      *      *      *      *      *      *      *      *
X.p.  -----DSPNSDKPTDFPSPRTQMLMNVIGDILQAKNGGRLGGLSDGGLN
X.e.  -----DSPNSDKPTDFPSPRTQMLMNVIGDILQAKNGGRLGGLSDGGLN
X.c.  QGQG----GDSGGQGGNSRQAGQPNGSPSAYTQMLMNVIGDILQAQNGGGFGGGFGGGF
X.g.  QGQGGQGGDSGGQGGNPRQAGQSNQSPSOYTQALMNVIGDILQAQNGGGFGGGFGGGF
      ***** **      *      *      *      *      *      *      *
X.p.  -----TSLSLSGDTASMQ
X.e.  -----TSLSLSGDTASMQ
X.c.  GGLGTSLGSSLASDTGSMQ
X.g.  ILVTSLASDTGSMQ
      ***** **      *

```

Figure A-12. Similarity of amino acid sequence of XopA in *X. perforans* (X.p.) to homologs in *X. euvesicatoria* (X.e.), *X. citri* (X.c.) and *X. axonopodis* pv. *glycines* (X.g.) using the multiple sequence alignment tool CLUSTALW. Mismatch AA sequences are indicated by an asterisk.



```
X.p.      HDPTNRRKRKASGGKSPSSSTISGKVGLAGLGKV
X.e.      ARSNEQKKKKSKWWKKF-----
X.c.      ERSIEQKKKKSKWWKKF-----
          ***** * *****
```

Figure A-13. continued.

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## BIOGRAPHICAL SKETCH

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