

PHYTOPATHOGENICITY, VIRULENCE FACTORS,
AND *IN PLANTA* MOVEMENT OF
SERRATIA MARCESCENS

By

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CHAPTER I

LITERATURE REVIEW

I. Introduction to cucurbit yellow vine disease

Economic value of cucurbits

Cucurbits, including squash (*Cucurbita maxima*), pumpkin (*C. moschata*), watermelon (*Citrullus lanatus* var. *lanatus*), cucumber (*Cucumis sativus*), zucchini (*C. lanatus*), gourd (*Lagenaria spp.*), and cantaloupe (*Cucumis melo*), are important crops in several regions of the US. For instance, in 2005 in the U.S, about 1.21 million tons of cantaloupe were produced and valued at about 789.1 million dollars; about 0.57 million tons of cucumber were harvested worth 148 million dollars; about 1.89 million tons of watermelon were grown, at about 409.2 million dollars (United State Department of Agriculture National Agriculture Statistics Services).

Cucurbit anatomy

The anatomy of cucurbitaceous plants is somewhat unusual because their phloem is bicollateral (Esau, 1965), and they are good subjects for metabolomic analysis because of the ease with which relatively pure phloem exudates can be obtained (Richardson et al., 1984). As shown in Fig. 1 (modified from Crafts, 1932), each vascular bundle in a stem of squash (*C. maxima*) consists of xylem, internal and external cambium with the internal cambium incompletely developed (Fahn 1990), and fascicular bundle-associated phloem. Fascicular phloem consists of internal central phloem and external central phloem, separated by xylem. Isolated strands of extrafascicular (outside the bundles) phloem occur scattered in

the parenchyma near both internal and external central phloem (Crafts, 1932). In addition, entocyclic and ectocyclic extrafascicular phloem strands occur in the tissue on either side of a peripheral ring of sclerenchyma, and extrafascicular phloem strands are scattered amongst cortex parenchyma cells. Extrafascicular commissural sieve tubes form lateral connections between the longitudinal phloem strands (Kempers et al., 1993).

Cucurbit yellow vine disease

Cucurbits are affected by a variety of insect and disease complexes. Prominent among these is a destructive vine decline, cucurbit yellow vine disease (CYVD), which was first observed in central Texas and Oklahoma in 1988 (Bruton et al., 1995). Since then, it has been detected in Missouri (Kabrick, 2002), Arkansas, Colorado, Connecticut, Kansas, Nebraska, Massachusetts (Bruton et al., 2003), Tennessee (Bost et al., 1999), Kentucky (Bessin, 2003), Ohio, and Michigan (Fletcher J., unpublished data). The first and most obvious symptom of yellow vine in several cucurbit crops is that the lower canopy leaves turn bright yellow, followed by gradual or rapid decline and death of the vine, especially in early-planted fields (Bruton et al., 1998). Older plants show a bright yellowing of the leaves but do not usually wilt. The diagnostic character of this disease is honey-brown phloem discoloration in the crown and primary root (Bruton et al., 1998). The susceptibility level among cucurbits is in the following descending order: squash, pumpkin, watermelon, and cantaloupe. Other wild cucurbits, various gourds and cucumbers have not been found to be naturally affected by CYVD (Pair et al., 2000). Losses range from less than 5% to 100% in affected fields of watermelon, squash, pumpkin, and cantaloupe (Bruton et al., 2003).

For some time after CYVD was recognized, the pathogen was described as a bacteria-like organism (BLO) because, although its morphology resembled that of walled bacteria, the microbe had not yet been cultured and properly characterized. Prominent phloem discoloration in the crown and primary root of diseased plants suggested that the causal agent was a phloem colonizing microbe, and indeed, transmission electron microscopy revealed bacteria in the sieve tubes of affected

cucurbits, but absent in healthy plants (Bruton et al., 1998). Dienes' stain, developed as a specific stain for animal mycoplasma colonies on agar by Dienes et al. (1948) and first reported to stain mollicutes in plant phloem sieve elements by Deeley et al. (1979), showed consistent staining in the phloem of symptomatic squash plants (Bextine et al., 2001). In 1997, the disease was associated with Gram-negative, rod-shaped and phloem-associated bacteria (Bruton et al., 1998), and bacteria isolated in pure cultures from diseased zucchini and watermelon were designated strains Z01 and W01, respectively (Bruton et al., 1998). Avila et al. (1998) found that the CYVD associated bacteria were detectable by polymerase chain reaction (PCR) using nonspecific primers designed from prokaryotic 16S rDNA. The deduced nucleotide sequence for 16S ribosomal DNA placed this organism within the gamma-3 proteobacteria, with the closest database relative being the genus *Serratia*. Rascoe et al. (2003), after extensive analysis on the 16S rDNA and *groE* sequences, concluded that the cucurbit isolates Z01 and W01 were *Serratia marcescens*, though significant differences in their biological functions and characteristics from those of non-cucurbit strains of the same species were demonstrated by fatty acid methyl ester profiles and substrate utilization assays. Koch's postulates were completed using strain Z01, confirming that *S. marcescens* was the causal agent of CYVD (Bruton et al., 2003). In rep-PCR and DNA-DNA hybridization comparison of *S. marcescens* strains, all 29 tested CYVD strains had identical banding patterns even though they had been isolated from several different cucurbit hosts, in different geographic locations, and in various years and were obviously differentiated from other strains of the species (Zhang et al., 2003).

Several lines of evidence pointed to the involvement of insects in CYVD. Bextine et al. (2001) showed that yellow vine did not develop in field-grown squash plants that were covered with insect-proof mesh, but plants exposed to the environment had a typical CYVD incidence rate. In other experiments (Pair et al., 2003), the use of insecticides lowered the incidence of yellow vine. The presence of high populations of squash bugs (*Anasa tristis*) in and near affected cucurbit fields made the insect a suspect for involvement in CYVD (Bextine et al., 2001;

Pair et al., 2000). The squash bug's piercing-sucking mouthparts are inserted into the host plant as it feeds in a variety of plant cell types, including collenchyma, parenchyma, phloem and xylem (Neal, 1993). Leaf feeding causes injuries to epidermal cells and mesophyll, but stylet insertions can reach the phloem (Beard, 1940; Neal, 1993). Light microscopy showed that squash bug stylets penetrate intracellularly toward the vascular bundles (Beard, 1940), sometimes penetrating xylem vessels and interrupting xylem transport (Neal, 1993). It is unclear how bacteria overcome: 1) the high sugar (often sucrose) concentrations and associated high osmotic pressure, and 2) unbalanced composition of amino acids, the dominant nitrogenous compounds in the phloem sap (Douglas, 2006).

The completion of Koch's postulates, using squash bugs as vectors, confirmed that this insect can transmit CYVD strains of *S. marcescens* from plant to plant (Bruton et al., 2003). The insect also can harbor the bacterium through the winter and transmit it to young cucurbit plants in the following spring (Pair et al., 2004). The ability of *A. tristis* to transmit *S. marcescens* after molting (Wayadande et al., 2005) is consistent with a circulative mode of transmission, in which the insect retains the ability to transmit after molting (Nault, 1997). Circulative transmission of *S. marcescens* implies that the bacteria transverse the squash bug gut, enter the hemocoel and possibly other tissues, move into the salivary glands and ultimately the salivary ducts. Once in the salivary duct, *S. marcescens* may be inoculated into plant hosts as the insect salivates during feeding. Other insects, such as the leafhoppers *Exitianus extiosus* and *Endria inimica* and the true bug *Lygus hesperus*, were shown to be experimental vectors, but whether species other than *A. tristis* are vectors in nature is not known (Al-Zadjali, 2002).

II. *S. marcescens* in diverse niches

S. marcescens, the first species identified in the genus *Serratia*, is a motile, non-sporulating, gram-negative, facultatively anaerobic bacillus belonging to the family Enterobacteriaceae. Most *S. marcescens* isolates produce a red pigment, prodigiosin, which may have been the cause of a condition known as "bloody

discoloration” of food, described as early as the 6th century BC by Pythagoras (Yu, 1979). Because of its bright red color and its reputation as a benign bacterium, *S. marcescens* was used in experiments to track microbes. For instance, it was released by the US military in 1950 as a biological marker for mock biowarfare in the Pacific Ocean near San Francisco, and was subsequently monitored in the environment (Richards, 1979). However, *S. marcescens* has since been found to be a pathogen of humans, especially those immunocompromised by other conditions.

The first report of *S. marcescens* as a cause of nosocomial human infection, transmitted predominantly through patient to patient contact, was at Stanford University Hospital (Wheat et al., 1951). Since then, many types of human infections have been attributed to *S. marcescens*, including urinary tract infection (Cox, 1985), meningitis (Sautter et al., 1984), endocarditis (Korner et al., 1994), septicaemia (Heltberg et al., 1993), peritonitis (Connacher et al., 1988), bacteremias (Volkow-Fernandez et al., 1993), arthritis septica (Nakashima et al., 1987), pneumonia, osteomyelitis, ocular and skin infections (Yu, 1979). *S. marcescens* also was reported to cause community-acquired soft tissue infection (Bonner and Meharg, 1983) and necrotizing fasciitis (Liangpunsakul and Pursell, 2001). Hospital-acquired *Serratia spp.* infection was more frequent than community-acquired infection (Manfredi et al., 2000).

The bacterium’s opportunistic behavior became more evident when hospitalization and instrumentation were involved. For instance, in 1987, pigmented *S. marcescens* was isolated from the hands and equipment of patient caretakers in an outbreak involving 10 male patients in a cardiac surgery unit (Wilhelmi et al., 1987). *S. marcescens* might play an important role in appreciable morbidity among patients with HIV disease, especially when associated with a low titer of CD4+ cells (a type of lymphocyte leading the attack against infections), neutropaenia and hospitalization (Manfredi et al., 2000). These effects may result from the secretion by *S. marcescens* of several exoenzymes, such as gelatinase, lecithinase, proteinase and chitinase. However, almost all strains of *S. marcescens* also secrete a cytotoxin, responsible for hemolysis of human and

animal erythrocytes and the release of inflammatory mediators from leukocytes (Braun et al., 1993). Hemolytic activity is determined by two proteins: ShlA and ShlB. *ShlA* encodes the hemolysin and *shlB* encodes an outer membrane protein required for the secretion of hemolytic ShlA protein across the outer membrane into the culture medium (Poole et al., 1988).

S. marcescens infections in humans are difficult to treat because of high resistance to a wide variety of antibiotics, including ampicillin and cephalosporins (Ball et al., 1977), aztreonam and imipenem (Ito et al., 1995), and fluoroquinolones (Hejazi and Falkiner, 1997). In various bacterial species fluoroquinolone resistance is the result of mutations in DNA gyrase and/or overexpression of multidrug resistance efflux pumps (Mortimer and Piddock, 1991). Multidrug resistance efflux pumps of bacteria play a major role in the intrinsic and acquired resistance against various human pathogens (Tseng et al., 1999). An efflux mechanism for fluoroquinolones was postulated and identified in *S. marcescens* (Berlanga et al., 2000), and a resistance nodulation cell division pump-encoding gene in this organism was suggested as a possible candidate responsible for drug efflux (Kumar et al., 2005).

S. marcescens is also a pathogen to insects, having been reported in Coleoptera, Hymenoptera, Lepidoptera, and Diptera (Krieg, 1987). Although most *S. marcescens* strains isolated from humans are nonpigmented, most of those isolated from insects produce prodigiosin. Red strains of *S. marcescens* have frequently been isolated from healthy, diseased and dead field-collected insects (Steinhaus, 1959; Bucher, 1963; Bell, 1969) and are commonly reported as pathogens of insectary-reared insects (Grimont and Grimont, 1978). *S. marcescens* was isolated by Lapesme (1937) from rearing colonies of the grasshopper *Schistocerca gregaria* Forsk (Orthoptera), by Bell (1969) from eggs of insectary-reared *Helicoverpa zea* (Boddie) and by Lynch et al. (1976) from field collected egg masses of the European corn borer (*Ostrinia nubilalis* Hubner). *S. marcescens* also caused disease and reduced adult longevity in *Heliothis zea* and *H. virescens* (F.), *Diatraea grandiosella* (Dyar), *Microplitis croceipes* (Cresson), *Dendroctonus frontalis* (Zimmermann), *Anthonomus grandis*

(Boheman), and *Curculio caryae* (Horn) (Sikorowski and Laurence, 1998). Inglis and Laurence (2001) reported that egg production from *H. virescens* (Lepidoptera: Noctuidae) adults inoculated as larvae was reduced by 30%, and egg hatch was reduced by 12% over a 10 day-period due to infection by *S. marcescens*. Although the bacterium is not usually pathogenic to insects when present only in the digestive tract in small numbers (Sikorowski and Goodwin, 1985), once it enters the hemocoel it reproduces rapidly and causes death in 1 to 3 days (Tanada and Kaya, 1993). A unique unpigmented, highly virulent strain of *S. marcescens* was identified; fewer than 10 of these bacteria were necessary to kill a corn earworm larva (Farrar et al., 2000). Heppler et al. (2005) showed that *S. marcescens* was pathogenic to the pea aphid (*Acyrtosiphon pisum* (Harris)). Recently, a study demonstrated that different *S. marcescens* strains were also pathogenic to the squash bug (*A. tristis*) (Heppler, unpublished data).

In addition to colonizing insects and humans, *S. marcescens* plays various roles in plants. For example, this species occurs in several rice varieties as a diazotroph (nitrogen-fixing bacterium) (Gyaneshwar et al., 2001). Strain IRBG500 endophytically colonized rice roots, stems and leaves, where, by using microscopy allied with marker genes and immunogold labeling, it was observed to colonize intercellular spaces, senescing root cortical cells, aerenchyma and xylem vessels. The bacterium was not seen within intact living host cells, such as phloem sieve tubes or parenchyma, however.

Other *S. marcescens* strains are characterized as plant growth-promoting rhizobacteria (PGPR). For example, the rhizobacterial *S. marcescens* strain 90-166 induced systemic resistance of cucumber against several pathogens, including fungi (*Colletotrichum orbiculare*), bacteria (*Pseudomonas syringae* pv. *tabaci* and pv. *lachrymans*) (Wei et al., 1996), and viruses (cucumber mosaic cucumovirus) (Raupach et al., 1996). Some *S. marcescens* strains were actually evaluated as potential biocontrol agents. *S. marcescens* strain MSU-97, an epiphyte on *Rhynholacis pedicillata* (an aquatic plant native to the Carrao River of the Venezuelan-Guyanese region of South America) produced oocydin A, a unique, chlorinated macrocyclic lactone, which showed activity against oomycetes such

as *Pythium ultimum*, *Phytophthora parasitica*, *Phytophthora cinnamomi* and *Phytophthora citriphora* (Strobel et al., 1999). Strain B2 suppressed fungal diseases caused by *Rhizoctonia solani* and *Fusarium oxysporum* f.sp. *cyclaminis* (Someya et al., 2000). *S. marcescens* 90-166 induced systemic protection to fusiform rust and significantly reduced the number of galls over a two year study (Enebak and Carey, 2000). *S. marcescens* NBRI1213 induced phenylalanine ammonia-lyase, peroxidase, and polyphenoloxidase activities in leaf and root of betelvine which increased the plant resistance to *Phytophthora nicotianae* infection (Lavania et al., 2006).

In the mid 1970's, *S. marcescens* was associated with disease in plant hosts when it was implicated in crown rot diseases on grain legumes including sainfoin in Utah (Sears et al., 1975) and alfalfa in Pennsylvania (Lukezic et al., 1982) and Utah (Turner and Van Alfen, 1983). Although in the latter two plants *S. marcescens* was considered to be part of a pathogen complex (including *Fusarium solani* and *Pseudomonas marginalis* var. *alfalfae*), *S. marcescens* was, equally to *F. solani* and *P. marginalis*, capable of inciting typically dark, necrotic crowns in Utah alfalfa.

III. *S. marcescens* Pathogenicity and Virulence Factors

Adherence

Adherence or attachment to a host tissue surface is normally the initial step of bacterial infection, and related activities are among the common components for bacterial pathogenesis in different hosts. Piliation is a determinant of microbial adherence to host epithelial surfaces (Reid and Sobel, 1987); for example, a nosocomial isolate of *S. marcescens* possesses pili and adheres to human uroepithelial cells (Yamamoto et al., 1985). Two classes of adhesins have been suggested for *S. marcescens* (Reid and Sobel, 1987). Those in one class, designated mannose-resistance (MR) pili, agglutinate chicken erythrocytes in the presence of D-mannose; those in the other class, mannose-sensitive (MS) pili,

exhibit mannose sensitive haemagglutination of guinea pig and chicken erythrocytes. The MS-piliated strain stimulates superoxide production by human polymorphonuclear leukocytes (PMNLs) associated with phagocytosis, causing tissue damage in infected organs. *S. marcescens* strain US46, a human urinary tract isolate, possesses both MS and MR pili (Mizunoe, et al., 1995). Previously, Zhang et al. (2005) identified a cluster of type I fimbrial genes in the chromosome of CYVD-causing *S. marcescens* strains that was absent in the non-CYVD strains examined. It is unclear what role fimbriae play in the virulence or pathogenicity of *S. marcescens* CYVD strains to cucurbit plants. Since these bacteria colonize the vascular system (Bruton et al., 2003), fimbriae might help bacteria attach to plant cell walls, aggregate and form biofilms, possibly inhibiting bacterial movement through the plant.

Another membrane component of the Gram-negative bacterial cell wall, the O-antigen, may also be involved in adherence. O-antigen is the exposed part of lipopolysaccharides (LPS) (see page 14) and its makeup contributes to cell wall variation (Hejazi and Falkiner, 1997). Some LPS molecules of Gram-negative bacteria have the potential structural diversity to mediate specific adherence and probably function as adhesins. The O-antigen may enhance the binding capacity of these ‘adhesins’. A comparison of the adhesion efficiency of wild type and O-antigen deficient spontaneous mutants of *S. marcescens* to plastic, glass and Foley catheters, and to human uroepithelial cells, showed that the O-antigen has a strong effect on the adhesion of *S. marcescens* to both inert and biological surfaces (Palomar et al., 1995). O-antigen also has the ability to bypass or overcome host defense mechanisms (Lerouge and Vanderleyden, 2002).

Protease

S. marcescens is an important opportunistic pathogen capable of causing several kinds of infectious diseases in human beings (Miyata et al., 1970). A number of extracellular proteases contribute to its pathogenesis. Three, designated 56K, 60K, and 73K proteases, were purified from culture filtrates of *S. marcescens* kums 3958, an isolate from a patient with a severe corneal ulcer

(Matsumoto et al., 1984). Those proteases play various roles in human disease development: 1) enhance vascular permeability caused by bradykinin (which forms from a blood plasma globulin and mediates the inflammatory response, increases vasodilation, and causes contraction of smooth muscle) through the activation of the Hageman factor (factor XII), a plasma protein (glycoprotein) (Matsumoto et al., 1986); 2) destroy plasma fibronectin (a high molecular weight molecule that spans the cell membrane and binds extracellular matrix components) (Molla et al., 1988) and transferrin (an iron ion delivery molecule) (Molla et al., 1986); 3) inactivate the complement system and generate chemotactic factors (Ward et al., 1973); and 4) cleave the α_1 -protease inhibitor (α_1 -PI), which plays an important role in the regulation of a protease cascade in clotting or fibrinolysis, thus enhancing the activation of the blood coagulation system, inflammatory response, vascular permeability, fibrinolysis, loss of blood volume and disseminated intravascular coagulation (Carrell and Bowswell, 1986).

Nuclease

Serratia nuclease can catalyze the hydrolytic cleavage of DNA and RNA between the 5'-phosphate and the 3'-oxygen of the sugar moiety in the presence of Mg^{2+} or several other divalent metal ions (Nestle and Roberts, 1969). The enzyme is able to cleave both single- and double-stranded substrates with similar efficiency (Meiss et al., 1995). The nuclease of *S. marcescens*, an extracellular protein encoded by the *nucA* gene (Chen et al., 1992), is initially synthesized as a pre-protein of 266 amino acids with a signal peptide consisting of 21 amino acid residues. Nuclease secretion is a two-step process in *S. marcescens*, in which the pre-enzyme is first rapidly exported to the periplasm, the result of typical Sec-dependent signal sequence processing, and then is translocated slowly across the outer membrane (Benedik and Strych, 1998; Suh et al., 1996). Nuclease expression and secretion are regulated by growth phase (Suh et al., 1996). Transcription of the *nucA* gene is also SOS-regulated (Chen et al., 1992) and requires, in addition, the transcriptional activator NucC (Jin et al., 1996). NucC is a member of the bacteriophage P2 Ogr family of transcriptional activators and

likely interacts with the α subunit of RNA polymerase. NucC binds to a region between -82 and -51 upstream of the transcriptional start of *nucA*, which includes a copy of the TGT±N12±ACA activator recognition motif, whose deletion obliterates activation (Winslow et al., 1998). *NucC* lies in an operon with two other genes, *nucD* and *nucE*, which bear strong resemblance to genes encoding phage proteins. NucE shows homology to phage holin proteins involved in releasing lysozyme to the peptidoglycan of Gram-negative bacteria. NucD shows significant homology to the type V lysozymes (Berkmen et al., 1997; Fastrez, 1996); its closest homologue is the lysozyme of bacteriophage P1 (Schmidt et al., 1996). Though the expression of NucE and NucD is coordinated with expression of NucA, they have no effect on the secretion of the *S. marcescens* extracellular nuclease (Strych et al., 1999).

Cytotoxin

An extracellular, heat-labile cytotoxin is an important virulence factor of at least some *S. marcescens* strains (Carbonell et al., 1996). Clinical isolates of *S. marcescens* had a cytotoxic effect on Vero (African green monkey kidney) cells (Carbonell et al., 1997), inducing cell rounding and detachment after 30 min incubation on Chinese hamster ovary and human larynx cell lines (Escobar et al., 2001). Escobar et al. (2001) also reported that cytotoxin from the same clinical strains caused morphological changes, such as shriveling and decay, on lettuce plantlets and onion bulbs, respectively. The cytotoxin is distinct from *S. marcescens* hemolysin and had no hemolytic activity (Carbonell et al., 2003).

Hemolysin

Almost all strains of *S. marcescens* secrete a lysin, responsible for hemolysis of human and animal erythrocytes and the release of inflammatory mediators from leukocytes (Braun et al., 1993). Hemolytic activity is determined by two proteins: ShlA and ShlB. *ShlA* encodes the hemolysin and *shlB* encodes an outer membrane protein required for the secretion of hemolytic ShlA across the outer

membrane (Poole et al., 1988). In the absence of ShlB, inactive ShlA (ShIA*) accumulates in the periplasm (Schiebel, et al., 1989). ShlA* can be activated *in vitro*, after sonic disruption of the cells, by incubation with a lysate of cells expressing the ShlB protein. ShlA* is activated by ShlB through a covalent modification. ShlB is required not only for activation of ShlA* but also for the secretion of active ShlA into the culture medium (Braun et al., 1993). ShlA and ShlB are synthesized as precursor proteins with typical amino-terminal signal sequences that are removed to yield the mature forms (Poole et al., 1988). ShlA is secreted into the culture medium while ShlB is found in the outer membrane (Schiebel, et al., 1989). Export of both proteins through the cytoplasmic membrane probably occurs via the Sec system, since inhibition of SecA ATPase activity by sodium azide causes accumulation of unprocessed ShlA and ShlB precursors.

Chitinases

Because it produces various chitinases, *S. marcescens* is very efficient at the biological degradation of chitin (Monreal and Reese, 1969). Chitinases are of biotechnological importance because they assist environmental microbes in roles as plant biocontrol agents against fungal infection. *S. marcescens* strain 2170 produces at least three chitinases (ChiA, ChiB and ChiC), a chitobiase and a chitin-binding protein (CBP21) (Suzuki, et al., 1998). These five proteins may represent the complete chitinolytic machinery of the bacterium. The *chiC* gene gives rise to various variants of ChiC in *S. marcescens* and two variants (ChiC1 and ChiC2) in recombinant *E. coli*. The primary sequences of ChiA, ChiB and ChiC1, as well as comparisons of available three-dimensional structures, show that these enzymes have a modular structure, which is very common for enzymes degrading insoluble biopolymers such as chitin and cellulose. All three *Serratia* chitinases contain a catalytic domain with the characteristics of family 18 glycosyl hydrolases (Herissat and Davis, 1997). Each of the enzymes contains domains that are putatively involved in substrate binding, and the catalytic domain of family 18 chitinases has a TIM-barrel fold $[(\alpha\beta)_8]$ and includes a

conserved glutamate residue that acts as an acid during catalysis (Tews et al., 1997). Perrakis et al. (1994) determined the complete structure of ChiA and revealed the location of a 114 residue domain with a fibronectin III-like fold that likely participates in chitin binding. ChiA has at least six well-defined subsites, one for each of the glucopyranose units of the polysaccharide substrate. The role of chitinase B is to digest the shorter GlcNAc (N-acetyl-D-glucosamine) oligomers, and render them capable of entering the periplasm (Brurberg et al., 1994). The most prominent difference between ChiC and the other two *S. marcescens* chitinases is the lack of the so-called $\alpha+\beta$ domain in the former. This domain makes up one of the walls of the substrate binding groove in ChiA and ChiB.

S. marcescens chitinase genes have been investigated as possible tools for the biocontrol of plant pathogens. Two chromosomal fragments encoding chitinolytic (and antifungal) activity from *S. marcescens* strain B JL200 were cloned (Sundheim et al., 1988). Watanabe et al. (1997) analysed the genetic chitinase system of *S. marcescens* 2170 by Tn5 transposon mutagenesis and obtained five classes of mutants that could be distinguished by the presence, size, appearance and timing of chitin clearing zones. The active site regions of chitinase genes contain many residues that are fully or highly conserved. For instance, alanine substitution of the conserved Trp167 at the -3 subsite in chitinase A enhanced transglycosylation (Aronson et al., 2006). ChiB hydrolyzes crystalline beta-chitin via a mechanism in which four exposed aromatic residues play important roles (Katouno et al., 2004). The *chiA* and *chiB* genes from *S. marcescens* have been transformed into other bacterial species such as *Pseudomonas fluorescens* and *E. coli* in an attempt to improve their ability to control fungal plant pathogens (Downing and Thomas, 2000) or to create new biocontrol agents. Furthermore, chitinase ChiA, partially purified after cloning into *E. coli*, reduced disease caused by *Sclerotium rolfsii* in beans and by *Rhizoctonia solani* in cotton (Shapira et al., 1989). When ChiA was combined with *Bacillus thuringiensis* or with low concentrations of the *B. thuringiensis* delta endotoxin, a synergistic toxic effect was seen on insect larvae (Regev et al., 1996). A similar synergistic effect was

also found between transgenic *P. fluorescens* carrying the *chiA* gene from *S. marcescens* (Downing and Thomas, 2000).

CBP21, secreted by *S. marcescens* when induced by chitin, is a 18.8-kDa protein which belongs to a family 33 carbohydrate-binding module and is known to bind to β -chitin (Suzuki et al., 1998). Its three-dimensional structure has been reported (Vaaje-Kolstad et al., 2005a). The *cbp21* gene sequence is highly conserved among most microorganisms that possess chitinase genes. Binding by CBP21 occurs on the surface, and also within the chitin structure (Zeltins and Schrempf, 1995). It has been hypothesized that CBP21-like proteins play a role in microbial attachment to chitin or enhance substrate availability by disruption of crystalline chitin (Schnellmann et al., 1994). Vaaje-Kolstad et al. (2005b) showed that CBP21 facilitates efficient chitin degradation by binding to the insoluble crystalline substrate, leading to structural changes and increasing substrate accessibility. CBP21 greatly increased hydrolysis of crystalline beta-chitin by chitinases A and C, while it was important for full degradation by chitinase B. These authors demonstrated that binding alone is not sufficient for CBP21 function by single mutations of CBP21 on the largely polar binding surface, which seems to depend on specific, mostly polar interactions between the protein and crystalline chitin.

Siderophores

Siderophores are low molecular weight, essentially ferric-specific ligands hyperexcreted by microorganisms grown in iron deficient conditions, which, together with membrane receptor proteins, form part of a high-affinity iron transport system (Neilands, 1981). Over 200 siderophores have been identified in various bacteria, the majority of which are attached either to catechols or hydroxamates (Hider, 1984). *S. marcescens* W225 expresses an iron (III) transport system that is considered a unique pathway because its uptake of Fe³⁺ occurs in the absence of an iron (III)-solubilizing siderophore, of an outer membrane receptor protein, and of the TonB and ExbBD proteins involved in outer membrane transport. Instead, three SfuABC proteins catalyze iron (III)

transport, exhibiting the typical features of periplasmic binding protein-dependent systems for transport across the cytoplasmic membrane (Angerer et al., 1992). *S. marcescens* strain SM365 imports heme and hemoproteins as iron sources independent of siderophore production, by mechanisms involving outer membrane heme-binding proteins (HasA) and heme transport systems (Letoffe et al., 1994). Siderophores may even play a role in biocontrol. Cucumber seeds treated with the rhizosphere bacterium *S. marcescens* strain 90-166 sustained significantly lower levels of anthracnose, caused by *Colletotrichum orbiculare*, when an iron chelator was provided in an iron-deficient environment (Press et al., 2001).

Lipopolysaccharide

Lipopolysaccharide (LPS), a compound responsible for the biological activity of certain endotoxins, is located in the outer membrane of Gram-negative bacteria. It is comprised of three regions, lipid A, the O-antigen and the core. The O-antigen, a repetitive saccharide chain, is the most important immunogenic component determining the O-serotype of bacteria. O-antigen affected the adherence capability, the antibiotic susceptibility and the efficiency of transformation of *S. marcescens* (Palomar et al., 1995). Palomar et al. (1993) showed that bactericidal activity against *S. marcescens* depends upon the O-side chain length. Both the classical pathway (the activation of complement by an antigen-antibody complex) and an alternate pathway are activated by *S. marcescens* strains ATCC 274, 2170 and NIMA, and both pathways play an active role in the serum killing of strains of *Serratia* with defective O-side chains. Two *S. marcescens* genes involved in core LPS biosynthesis were cloned and characterized. These, when introduced into *E. coli* NM554, conferred resistance to bacteriocin 28b (which is produced by *S. marcescens* N28b) (Enfedaque et al., 1996).

LPS from Gram-negative bacteria is well recognized to be a potent microbial toxin. It has been postulated to play a critical role in the initiation of the proinflammatory events that contribute to a pathogenesis of human sepsis

(Rangel-Frausto, 1999). However, there is a poor correlation between serum lipopolysaccharide levels and mortality in septic patients. Luchi and Morrison (2000) compared the chemical, structural, and biological differences among LPSs from clinical isolates of *S. marcescens* and other bacteria. The relatively minor differences in LPS activity seemed unlikely to explain the discrepancy between serum endotoxin levels and mortality in patients with Gram-negative bacteria-induced sepsis.

IV. Host response to *S. marcescens* infection

There is a complex interaction between a microbial pathogen and a plant host. When a host plant is infected, it may respond in a variety of ways. Some virulence-associated microbial gene products trigger host-defense genes.

The nematode *Caenorhabditis elegans*, a genetically tractable organism, is a useful model for studying the animal response to *S. marcescens* infection. It has been hypothesized that a *C. elegans* defense response would share some of the features of the defense response pathways found in insects and in mammals (De Gregorio et al., 2001). Like *P. aeruginosa*, *S. marcescens* Db11 is capable of killing *C. elegans* either by a toxin-based mechanism or by establishing an infection. After being ingested by the nematode, the bacteria survive within the usually hostile environment of the nematode's intestine and proliferate and spread, leading to a systemic infection that kills the host (Kurz and Embank, 2000). For instance, strain Db11, a spontaneous streptomycin resistant derivative of strain Db10, which was originally isolated from moribund *Drosophila* flies, establishes an infection that kills *C. elegans*. In contrast, a pleiotropic, protease-deficient derivative of Db11 is less pathogenic in both *Drosophila* and *C. elegans* (Flyg and Xanthopoulos, 1983; Pujol et al., 2001). Interestingly, protease is also an important virulence factor in *S. marcescens* strain BG induction of mammalian keratitis and is responsible for corneal damage in a rabbit eye model (Lyerly et al., 1981). Screens of a *S. marcescens* transposon insertion library in *C. elegans* suggested that a significant number of genes are involved in virulence (Kurz and

Embank, 2000), and it is likely that many of these are important virulence determinants also in mammalian infections (Tan and Ausubel, 2000). Recently, Mallo et al. (2002) used an expression screen to look for *C. elegans* genes upregulated in response to infection by *S. marcescens*. Of 7,500 cDNAs surveyed, several were induced over twofold; most of these encoded lectins, which function in innate immunity in other organisms. Also upregulated was lysozyme 1. As lysozymes have been implicated in innate-immune responses, Mallo and coworkers (2002) also overexpressed *lys-1* in worms and demonstrated that the gene enhanced the worms' resistance to *S. marcescens*. *C. elegans* also was used to screen transposon Tn5 transformed *S. marcescens* ZO1-A mutants (Zhang, 2004), but *C. elegans* proved to be unsuitable as a model host because it survived and reproduced well in the presence of ZO1-A. The different responses of *C. elegans* to various strains of *S. marcescens* may result from differential bacterial pathogenicity determinants in the strains.

S. marcescens' pathogenicity may be influenced by its LPS structure. For example, strain Db1140 increased *Drosophila*'s sensitivity to immune hemolymph because, compared to strain Db11, it had a structural change in the LPS (Carlsson et al., 1998). Db1140 also elicited *Arabidopsis* defense responses via activation of an LRR receptor-like kinase, FLS2 (Gomez-Gomez and Boller, 2000).

Only a few bacteria have been identified to be multi-kingdom pathogens (*i.e.*, able to infect hosts in more than one kingdom). Among them, *Pseudomonas aeruginosa* has been more studied than *S. marcescens*. Like *S. marcescens*, *P. aeruginosa* infects humans (Britigan et al., 1997), plants (Kominos et al., 1972) and nematodes (Tan et al., 1999). The *P. aeruginosa* infection process in *Arabidopsis* includes tissue penetration, movement along the vascular parenchyma, and distribution in the leaf intercellular space. The effects of invading *P. aeruginosa* on plant cell walls and membrane structures include disruption of intercellular contacts and separation of mesophyll cells from each other (Plotnikova et al., 2000). Furthermore, by systematic mutagenesis, in *P. aeruginosa* strain PA14, of 13 pathogenicity related genes identified in

nematode screens, 10 were also required for pathogenicity in plants (Mahajan-Miklos et al., 1999; Tan et al., 1999). Moreover, out of a total of 21 genes identified in plant and nematode screens, 17 were required for mouse pathogenicity (Rahme et al., 1997; Mahajan-Miklos et al., 1999; Tan et al., 1999). Thus *P. aeruginosa* PA14 uses many common virulence factors in its interactions with diverse hosts including plants and animals.

Though Bruton et al. (1998) reported that CYVD strains of *S. marcescens* occurred in phloem, it is unclear how and where they enter the plant host and move from one location to another, whether CYVD strains are multi-host pathogens, and what mechanisms are involved in the infection and disease processes.

In this study, several plant species (squash, tobacco, carrot, and onion) were used to assess the pathogenicity of *S. marcescens* strains from different niches (Chapter II). The goal of the pathogenicity study were to understand: 1) whether *S. marcescens* strains isolated from various hosts can cause similar symptoms on the same plant species; 2) whether a single *S. marcescens* strain can affect different plant species; and 3) whether there is a difference in pathogenicity between CYVD-strains and non-CYVD strains on these test plant species. Answers to these questions might help us elucidate the disease epidemiology and facilitate control of this pathogen.

The second objective for this research was to understand how *S. marcescens* enters and moves in plants (Chapter III). Although CYVD-strains of *S. marcescens*, from field symptomatic squash plants, were detected in phloem using transmission electron microscopy, it is unknown how these bacteria enter or move from the inoculation site to other parts. So in these experiments, two strains of *S. marcescens* were labeled with green fluorescent protein and introduced into squash plants using different methods. The movement of labeled bacteria *in planta* was monitored by microscopy.

Why do some strains of *S. marcescens* cause disease on squash plants but others do not? It is possible that some bacterial genes play a key role in disease symptom development. To study bacterial gene function, one effective way is to

mutate the gene and to evaluate the mutants. In Chapter IV, two fimbrial genes, identified as being part of a fimbrial gene cluster present in CYVD-strains of *S. marcescens* but absent in all non-CYVD strains tested in our study, were separately disrupted using a group II intron. These gene-knockout effects were evaluated using transmission electron microscopy and pathogenicity tests on squash plants. Furthermore, some bacterial virulence factors may be highly conserved and shared by many bacteria. Known virulence factors were used as probes and their presence in CYVD-strains determined (Chapter V).

Overall, the objectives of this study were: 1) To evaluate relationships of *Serratia marcescens* strains with different plant species; 2) To characterize the entry, colonization and movement of *S. marcescens in planta*; 3) To characterize some virulence factors of *S. marcescens*.

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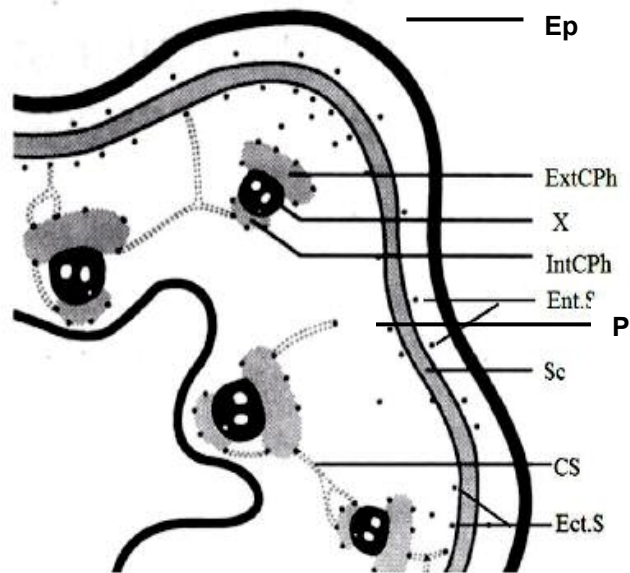


Fig.1.Schematic diagram of a transverse section of a *Cucurbita maxima* stem showing its complex vascular system with bicollateral vascular bundles. Abbreviations ExtPh: external central phloem; IntCPh, internal central phloem; X, xylem; CS, commissural sieve tube; Sc, sclerenchyma ring; Ect.S, ectocyclic tube; Ent.S, entocyclic sieve tube; Ep, epidermis; P, parenchyma. Modified from Crafts, 1932.

CHAPTER II

PHYTOPATHOGENICITY OF *SERRATIA MARCESCENS* STRAINS IN DIFFERENT PLANT HOST SPECIES

Abstract

Strains of *Serratia marcescens* colonize many niches such as water, soil, humans, animals, insects, and plants. Certain strains cause cucurbit yellow vine disease (CYVD). To assess whether phytopathogenicity is strain-specific, tobacco leaves were needle-inoculated with various *S. marcescens* strains and other bacterial species. A HR-like response was observed within 24 h after inoculation (HAI) with all *S. marcescens* strains inoculated at 1×10^9 cells/ml. Inoculated at 5×10^6 - 1×10^7 cells/ml, CYVD strains caused chlorotic lesions at 60 HAI, while non-CYVD strains did so at 48 or 60 HAI. The response to CYVD strains of *S. marcescens*, fork-inoculated to squash and carrot plant stems, and to onion bulbs (the latter two plant species are non-hosts of CYVD), differed. Squash seedlings were stunted, onion bulbs were water-soaked and rotten, and carrot seedlings wilted and died. Non-CYVD strains of *S. marcescens* caused symptoms similar to those caused by CYVD strains on tobacco, carrot and onion bulbs, but no symptoms on squash plants. Rates of infection also differed; 17% of squash plants inoculated with CYVD strains, but 0% of those receiving non-CYVD strains, showed symptoms. Only 0-33% of carrot plants and onion bulbs needle-inoculated with CYVD strains showed wilting or rotting, while 70-100% of those receiving non-CYVD strains did so. When *S. marcescens* inoculum was dropped onto the surface of peeled onion bulbs to assess whether *S. marcescens* could

enter these plants naturally, only non-CYVD strains caused symptoms. This is the first report that *S. marcescens* strains from non-plant niches can cause symptoms in plants and that their interactions with the plant host differ from those of CYVD strains.

Introduction

Cucurbit yellow vine disease (CYVD) was first observed in squash (*Cucurbita maxima*) and pumpkin (*C. pepo*) in 1988 in Oklahoma and Texas (Bruton et al., 1995), but is now known to occur in other cucurbits, including watermelon and cantaloupe. The disease has been reported in Missouri (Kabrick 2002), Arkansas, Colorado, Connecticut, Kansas, Nebraska, Tennessee, Massachusetts (Bruton et al., 2003), Kentucky (Bessin, 2003), Indiana, Ohio, and Michigan (Fletcher J., unpublished data). The first and most obvious symptom of yellow vine in several cucurbit crops is bright yellow color in the lower canopy leaves, gradual or rapid decline and death of the vine, and a honey-brown phloem discoloration in the crown and primary root, especially in early-planted fields (Bruton et al., 1998). Older plants show a bright yellowing of the leaves but do not usually wilt. The susceptibility level among cucurbits is in the following descending order: squash, pumpkin, watermelon, and cantaloupe. Other wild cucurbits, various gourds and cucumbers have not been found to be naturally affected by CYVD (Pair et al., 2000). Losses range from less than 5% to 100% in CYVD-affected fields of watermelon, squash, pumpkin, and cantaloupe (Bruton et al., 2003).

For some time after CYVD was described, the pathogen was described as a bacteria-like organism (BLO) because, although its morphology resembled that of walled bacteria, the microbe had not yet been cultured and properly characterized. In 1997, the disease was associated with Gram-negative, rod-shaped and phloem-associated bacteria (Bruton et al., 1998). In 1998, bacteria isolated in pure cultures from diseased zucchini and watermelon were designated strains Z01 and W01, respectively (Bruton et al., 1998). Avila et al. (1998) found that the CYVD organism was detectable by polymerase chain reaction (PCR) using nonspecific

primers designed from prokaryotic 16S rDNA. The deduced nucleotide sequence for 16S ribosomal DNA placed this organism within the gamma-3 proteobacteria, with the closest database relative being within the genus *Serratia*. Rascoe et al. (2003), after extensive analysis on the 16S rDNA and *groE* sequences, concluded that the cucurbit isolates Z01 and W01 were *S. marcescens*, though significant differences in their biological functions and characteristics from those of non-cucurbit strains were demonstrated by fatty acid methyl ester profiles and substrate utilization assays. Koch's postulates were completed using strain Z01, confirming that *S. marcescens* was the causal agent of CYVD (Bruton et al., 2003).

S. marcescens is a motile, non-sporulating, gram-negative, facultatively anaerobic bacillus belonging to the family *Enterobacteriaceae*. It can be isolated from diverse niches, such as soil (Pares, 1964), water (Ajithkumar et al., 2003), insects (Bucher 1960), plants (Lukezic et al., 1982; Wei et al., 1996; Gyaneshwar et al., 2001), invertebrates, and humans (Wheat, 1951).

Although yellow vine disease was first detected in 1988 (Bruton et al., 1995) and can be a yield-limiting factor for cultivated cucurbits, it was not known whether this bacterium could infect other plants, or whether infections of all plant hosts involve similar mechanisms. In the current study, we tested the pathogenicity of CYVD strains of *S. marcescens* to several plant species. Tobacco (*Nicotiana tabacum*), was chosen to assess the production of a hypersensitive reaction (HR) (Klement et al., 1964). HR, characterized by the rapid death of plant cells that contact pathogenic organisms, is generally associated with plant resistance to pathogens (Klement and Goodman, 1967).

Materials and Methods

Bacterial strains and growth conditions. Tested bacteria included type strains of several *Serratia* species, *S. marcescens* isolates from different ecological niches, *Escherichia coli* and two *Pseudomonas syringae* pathovars (Table 1). Bacteria were stored at -80°C in aliquots of 1.5 ml Luria-Bertani (LB)

broth containing 15% glycerol. For use in the experiments, bacteria were streaked onto LB or nutrient agar (Sambrook et al., 1989) and incubated overnight at 28°C (*S. marcescens* and *P. syringae*) or 37°C (*E. coli*).

Plants. Tobacco (*Nicotiana tabacum*), carrot (*Daucus carota*) and squash (*Cucurbita maxima*) plants were propagated and maintained in the greenhouse. Tobacco seeds were sown in a pot (5 x 5 x 8 cm) and transplanted, one seedling per pot, after 10 days. Four wk-old tobacco leaves were used for the hypersensitivity test. Squash seeds were sown in 128-cell plastic trays and placed in the greenhouse 4-7 days (23-35°C), to reach the closed cotyledon stage. Three to five carrot seeds per pot were sown and six wk-old carrot plants were used for inoculation. Onion (*Allium cepa*) bulbs were purchased at a grocery store.

Bacterial quantification. The relationship between bacterial titer and optical density at 600 nm (OD₆₀₀) was assessed. In one approach, the OD₆₀₀ of *S. marcescens* ZO1-A, WO1-A, HO1-A, and type strain ATCC13880 cell suspensions in phosphate buffered saline (PBS, 0.01M, pH 7.0, containing 0.15 M NaCl) at approximately 2-5 X 10⁷ cells per ml were determined. Bacterial cell numbers in a series of diluted solutions were determined by counting, using an Olympus BH2 microscope (Tokyo, Japan) and a hemocytometer. In a colony plating method, log-phase cultures of *S. marcescens* ZO1-A, WO1-A, HO1-A, and type strain ATCC13880 (approximately 2 X 10⁹ cells/ml) were serially diluted 10-fold to 10⁻⁷ in PBS. A volume of 0.1 ml of each of the serial dilutions was assessed for optical density at 600 nm and plated onto LB agar plates (3 plates/dilution) and incubated at 28°C overnight.

Inoculation of squash. *S. marcescens* strains (ZO1-A, WO1-A, HO1-A and 90-166) were streaked on nutrient agar plates, incubated 4 days at 28°C, then diluted to about 1 x 10¹¹ cells/ml (cell number determined using a UV/vis spectrometer at OD₄₂₀ (Bruton et al., 2003)) with sterile PBS. To inoculate, a multi-pronged “inoculating fork” (Bruton et al., 2003) was dipped into the bacterial suspension, picking up a drop of about 10 microliters. The junction between the cotyledon and stem was jabbed at least 15 times with the fork such that bacteria were introduced into the stem interior. Squash plants were

transferred to pots (5 x 5 x 8 cm) 7 days after inoculation. Each bacterial strain was inoculated into 40 plants, except ZO1-A, which was inoculated into 60 plants. The test was performed three times. After four weeks incubation in the greenhouse, the squash stems were sliced horizontally and examined visually for phloem necrosis, using Dienes' stain (Dienes et al., 1948), a reagent reported to stain phloem sieve tubes colonized by mollicutes (Deeley et al., 1979). Bacterial DNA was extracted from plant tissues as described previously (Aljanabi and Martinez, 1997), with slight modifications. Briefly, about 250 mg of squash stem, excised from a point about 3 cm below the inoculation site, was used for DNA extraction. The tissue was placed into a 1.5 ml microtube on ice for 1 h, then homogenized in 200 µl of sterile homogenizing buffer (0.1 M NaCl, 0.2 M sucrose, 0.5% sodium dodecyl sulfate (SDS), 0.1 M Tris-HCl pH 8.0 and 0.05 M EDTA (pH 8.0) using a plastic pestle. The pestle was washed with the same buffer and the homogenizer was kept on ice for 30 min. The homogenized samples were incubated at 65 °C for 1 h, then 90 µl 8 M potassium acetate was added and mixed well. Samples were again incubated on ice for 1 h then centrifuged at 16,060 g for 15 min at room temperature. The supernatant was transferred to a new microtube. An equal volume of isopropanol was added to each sample, mixed well, and samples were incubated at -20°C for 2 h before being centrifuged again as above. The pellet was washed with 70% ethanol, air-dried at room temperature for 10 min and finally resuspended in 50 µl sterile Tris-EDTA buffer (pH 8.0). About 1 µl extracted DNA was examined by PCR (YV1 and YV4 primers; 95°C 2 min, {94°C 30 sec, 55°C 30 sec, 72°C 2 min}, 34 cycles; 72°C 10min, 4°C 2h) to confirm the bacterial presence in the plants.

Inoculation of tobacco. Several strains of *S. marcescens*, including three CYVD strains (ZO1-A, WO1-A, CO1-A), one human pathogenic strain (HO1-A), one plant growth-promoting rhizobacterium (90-166) and two endophytic strains (RO1-A and RO2-A), *E. coli* OP50 and two *Pseudomonas syringae* pathovars (Table 1), were streaked from frozen aliquots onto nutrient agar (NA) (Sambrook et al., 1989) and incubated at 28°C for 24 h. Single colonies were then transferred to 5 ml of Luria Bertani (LB) (Sambrook et al., 1989) broth, (except for *P.*

syringae pathovars, which were grown in MG broth (Keane et al., 1970). The cultures were incubated at 28°C, with shaking at 220 rpm, for 18 h. Cell numbers were estimated by OD₆₀₀. *P. syringae* pv. *tomato* DC3000 was used as a positive control for hypersensitivity, *P. syringae* pv. *tabaci* as a positive control for pathogenicity, and distilled H₂O and LB medium as negative controls. Bacteria were grown to a titer of approximately 1 X 10⁹ cells/ml, centrifuged (5000 X g for 20 min at 4 °C) and resuspended in PBS to a titer of approximately 5 x 10⁶ – 1 x 10⁷ cells/ml. This suspension was injected, using a 26 1/2 gauge needle and puncturing the adaxial surface, into the intercellular spaces of four wk-old tobacco leaves. The liquid that accumulated in a “water-soaked” spot underneath the epidermis showed the location of the inoculum in each leaf (Klement et al., 1963). Each treatment was performed a total of four times. The four treatments were: 1) Inoculation of each tobacco leaf with three different bacterial species; 2) Inoculation of each tobacco leaf with a single *S. marcescens* strain, with two strains per plant; 3) Inoculation of each tobacco leaf with bacterial culture filtrate (0.20 micron); 4) Inoculation of each tobacco leaf with bacterial suspensions heated (100°C, 30 min) to kill bacteria. Inoculated plants were incubated in the greenhouse at 23-35°C and examined every 12 h for symptom development.

Inoculation of onions. Bacteria were prepared as above and centrifuged at 5000 X g (4 °C) for 20 min. The supernatant and pellets were separated. Pellets were resuspended in PBS to a titer of 1 X 10⁸ cells/ml. With a sterilized scalpel, onion bulbs were spliced vertically into wedges (~10 x 40 mm), and the supernatant and resuspended bacteria were either injected, using a 26 1/2 g needle, into the interior of 20 wedges or dropped (10 ul/drop) onto the adaxial surface of 20 wedges. The inoculated onion tissues were placed into a 90 mm Petri dish lined with moistened paper towels and incubated at 28°C for symptom development.

Inoculation of carrots. *S. marcescens* strains ZO1-A, WO1-A, HO1-A and RO2-A were inoculated to carrot plants in the same manner as to the squash plants. PBS buffer, the diluent for bacterial suspensions, was used as negative

control. The inoculated carrot plants were kept in the greenhouse for symptom development.

Results

Bacterial quantification. There was no significant difference in the accuracy of titer determination by hemocytometer counts and colony counts. As shown in Fig. 1, within the range of 10^7 - 10^9 cells/ml, cell numbers of all the *S. marcescens* strains were directly proportional to the OD₆₀₀ value. Below 10^7 or above 10^9 cells/ml the linear relationships were lost. The relationship between cell number and OD₆₀₀ value differed among the strains.

Pathogenicity on squash. Only CYVD strains of *S. marcescens* caused disease on squash plants. After 4 wk, no symptoms were detected in plants inoculated with non-CYVD strains, RO2-A, 90-166 or HO1-A (Table 2). About $17 \pm 5\%$ and $19 \pm 7\%$ of squash plants inoculated with ZO1-A and WO1-A, respectively, showed stunting and leaf cupping. Other symptoms considered typical for CYVD, such as bright chlorosis and gradual or rapid decline and death of the vine, were not observed. When the diseased plants' crowns were hand-sectioned and stained with Dienes agent, blue patches in the parenchyma and phloem were observed, indicating bacterial infection (Fig. 4). PCR results showed that about $48 \pm 7\%$, $44 \pm 3\%$ of the plants inoculated with ZO1-A or WO1-A, respectively, contained detectable titers of bacteria, although fewer than 20% had symptoms. Interestingly, $41 \pm 5\%$, $36 \pm 3\%$, or $21 \pm 7\%$ of plants inoculated with non-CYVD strains RO2-A, 90-166, or HO1-A, respectively, were PCR positive despite the lack of symptoms.

Hypersensitivity on tobacco. None of the *S. marcescens* strains tested, regardless of origin, produced a typical hypersensitive reaction (HR), generally defined as a localized necrotic lesion within 24 h on tobacco leaves when inoculated at a titer of 5×10^6 to 1×10^7 cells/ml. The appearance of symptoms on leaves was related to the inoculation titer. When tobacco leaves were inoculated with *S. marcescens* ZO1-A, *E. coli* OP50, or *P. syringae* pv. *tomato* DC3000, a

typical HR was seen within 3 h after inoculation (HAI) only at the site inoculated with the latter (Table 2). No HR-like reaction was seen, as long as 48 HAI, at sites inoculated with the other bacteria tested. At 60 HAI, some sites receiving inoculum of *S. marcescens* ZO1-A became chlorotic, then necrotic (Fig. 2). However, at this titer *S. marcescens* 90-166, a cotton endophyte, caused chlorotic lesions 48 HAI, and WO1-A and HO1-A did so at 72 HAI (Table 2). When bacteria were inoculated at a much higher titer (10^9 cells/ml), however, all strains (ZO1-A, W01-A, C01-A, 90-166, H01-A, *E. coli* OP50, and *P. syringae* pv. *tabaci*) produced indistinguishable necrotic reactions within 24 h, and *P. syringae* pv. *tomato* DC3000 did so within 3 HAI.

After heating (100°C, 30 min) or filtering (0.20 micron) the bacterial cultures, no bacteria grew on LB plates. When tobacco leaves were inoculated with such preparations, no symptoms appeared for over one wk. However, chlorotic lesions appeared 8 days after inoculation (DAI) on leaves inoculated with CYVD strains or with HO1-A, and 9 DAI on leaves receiving 90-166. No symptoms appeared on leaves inoculated with *E. coli* OP50 (Fig. 2) or with PBS (Table 2).

Pathogenicity on onion. The ability of *S. marcescens* strains to cause CYVD in cucurbit hosts was negatively correlated with their ability to cause symptoms when inoculated as bacterial culture suspensions into onion bulbs. All tested *S. marcescens* strains except CO1-A caused water-soaking and rotting of the onion bulb tissues, but the incidence of symptom development differed among strains. As shown in Table 3 and Fig. 3, cultures of *S. marcescens* strains RO1-A and RO2-A (rice endophytes), 90-166 (a cotton endophyte) and HO1-A (a human pathogen) produced water-soaking, tissue browning and necrosis in most or all (93-100%) of the onion bulbs inoculated. In contrast, cultures of CYVD strains ZO1-A and WO1-A caused development of similar symptoms in only 18 ± 2 % or 17 ± 4 %, respectively, of inoculated bulbs, and CO1-A produced symptoms in none of the inoculated bulbs. With only one exception, the presence of intact bacterial cells was necessary for symptom development; culture supernatants from which bacteria were removed by centrifugation or filtration caused no symptoms in the onion bulbs, while bacterial pellets, resuspended in PBS,

produced necrosis similar to that caused by bacterial cultures. The exception was the rice endophytic strain RO2-A, the supernatant of which caused symptoms in approximately half of the onion bulbs inoculated with it.

Pathogenicity on carrot. All inoculated carrot plants wilted immediately after inoculation, probably due to wounding. Control plants inoculated with PBS recovered after two days, while plants inoculated with *S. marcescens* strains continued to show wilting symptoms. At 4-5 DAI, all the carrot plants inoculated with *S. marcescens* RO2-A and 70 ± 5 % of those inoculated with 90-166 died, while only 35 ± 3 % and 32 ± 2 % of those inoculated with ZO1-A and WO1-A, respectively, did so (Table 3 and Fig. 5).

Discussion

The relationships between bacterial cell number and OD₆₀₀ value differed among different *S. marcescens* strains. At the same cell number, CYVD strains of *S. marcescens* had a lower optical density than non-CYVD strains (Fig. 1). It is possible that gene expression in these strains differed. For instance, HO1-A, a human isolate, produces a characteristic red pigment, prodigiosin, which may cause a higher optical density. Fimbriae, a hair-like appendage on cell surface, present in CYVD strains but absent in non-CYVD strains, may also affect bacterial optical density. A difference in bacterial cell size, morphology or aggregation propensity could cause differences in light reflection/refraction. Difference of cell numbers between colony plating and counting was observed but not significant. The sensitivity of optical density and cell count methods may vary, or it may be that some dead bacteria were detected by spectrophotometer but not in the cell count on plates.

Tobacco is widely used to test for the production of a hypersensitive reaction (HR) since Klement et al. (1964) first demonstrated that infiltration of bacteria pathogenic to non-tobacco plant hosts caused tissue collapse on tobacco leaves. HR on tobacco, which is one indication of bacterial isolate's pathogenicity, is characterized by rapid cell collapse at the infection site, generally within 24 h

after inoculation (Popham et al., 1993). Cell death is visible only microscopically if the inoculum concentration is lower than a threshold level (a bacterial inoculum titer of $< 10^6$ cells/ml or $< 10^4$ cells/cm² in the plant) (Turner and Novacky, 1974). However, if nonhost plant tissue is inoculated with a sufficient concentration of pathogenic bacteria, the entire area of inoculated tissue rapidly collapses and becomes necrotic (Turner and Novacky, 1974). If *S. marcescens* strains caused a HR on tobacco, we may use this test to screen for pathogenicity of experimental mutants. In our study, when inoculated onto tobacco leaves at a titer typical for hypersensitivity testing (about 5×10^6 cells/ml), HR was seen about 3 hours after inoculation (HAI) on tobacco leaves receiving *P. syringae* pv. *tomato* DC3000 but not on tobacco leaves receiving *S. marcescens*. *S. marcescens* strains did cause necrosis about 12 HAI, but only when inoculated at very high titer (1×10^9 cells/ml). At such high titers, even the non-pathogen, *E. coli* OP50, produced necrosis, indicating that inoculation at this titer is unreliable for evaluating HR. No HR occurred on leaves inoculated with *E. coli* OP50 at the lower titer of 5×10^6 cells/ml, even at 108 HAI (Fig. 2). Chlorotic lesions appearing 60 HAI on tobacco leaves inoculated with ZO1-A at 5×10^6 cells/ml were not considered to be a HR because they were not localized, but enlarged over time, and persisted at least two days without causing cell collapse (Fig. 2, panels E to G). By 72 HAI, except for 90-166, all tested *S. marcescens* strains caused chlorotic lesions that became necrotic, enlarging over time.

When *S. marcescens* cultures were heated or filtered before being inoculated into tobacco leaves, the chlorotic lesions produced were similar to those caused by living cells but took a longer time to develop (8 DAI rather than 3 DAI) (Table 2). Since no bacteria grew on medium inoculated with such preparations, it is possible that *S. marcescens* produces one or more heat-labile components, such as cytotoxins (Carbonell et al., 1996; Escobar et al., 2001), which might contribute to the reaction of tobacco leaves.

Inoculation of onion bulbs and carrot and squash plants also showed differential pathogenicity among the bacteria tested. Inoculation of CYVD strains ZO1-A and WO1-A resulted in a low rate (18% and 17%, respectively) of

infection on onion bulbs, while CO1-A caused no symptoms. Symptoms produced by RO2-A, a beneficial rice endophyte (Gyaneshwar et al., 2001), on onions and carrots were more severe than those caused by the CYVD strains. Interestingly, the non-CYVD strains RO2-A, 90-166 and HO1-A caused no symptoms on squash (a natural host plant of CYVD strains), a result consistent with that of a previous study (B. Bruton, personal communication) in which no symptoms were observed in squash plants inoculated with RO2-A, though their presence in the host plant following inoculation was detected by PCR.

These experiments demonstrated that *S. marcescens* affects several plant species. For instance, CYVD strains, such as ZO1-A and WO1-A, caused chlorosis on tobacco leaves, wilting of carrots, rotting of onion bulbs, and stunting of squash. However, the infection rate on noncucurbit species was lower for CYVD strains than for strains from other niches. Many plant pathogens cause disease in a limited number of host plant species as a consequence of a long coevolutionary history (Rahme et al., 2000). The pathogenicity of 90-166, RO2-A, and HO1-A on our tested species may result from an ability of those plants to trigger the expression of bacterial virulence factors. Alternatively, the fact that bacteria were fork-inoculated into our test plants, rather than being introduced by squash bugs as they are in nature, may result in bacterial colonization of abnormal sites (see chapter III) or ability to overcome plant defense responses.

Previous work (Pair et al., 2003) showed that the CYVD strains of *S. marcescens* are transmitted in nature by insects. Symptoms in squash plants caused by *S. marcescens* after insect transmission were more severe than those that developed after the same strains were mechanically inoculated (Bruton et al., 2003). The insect vectors might assist the bacteria to enter and colonize particular tissues, such as the sieve tubes, either by direct placement or by secreting enzymes to dissolve plant cell wall components. Exactly how, and in which tissues, the bacteria colonize and translocate within the host plants remains unclear. Further work is needed to understand the movement of the bacteria in the host plants.

Differences among *S. marcescens* strains in disease incidence in various host species may result from differential gene expression. Previous work (Rascoe et al., 2003) demonstrated that *S. marcescens* CYVD strains lacked 30 of the 95 metabolic capabilities that were present in the cotton endophyte, 90-166, and the clinical isolate H01-A. Furthermore, although most *S. marcescens* strains, including CYVD strains, have chitinase and hemolysin genes, CYVD strains failed to degrade chitin or to hemolyze red blood cells while non-CYVD strains did so (Chapter V). Alternatively, differential pathogenicity among various *S. marcescens* strains may be caused by differential presence or absence of key genes. Zhang et al (2005) identified a type I fimbrial gene cluster in the chromosome of CYVD-causing *S. marcescens* strains that was absent in the non-CYVD strains examined. Targeted disruption of *fimA* and *fimH* in the CYVD strain ZO1-A reduced the virulence on squash (Chapter IV).

In summary, *S. marcescens* can cause several different types of symptom syndromes in different plant species. But only *S. marcescens* CYVD strains cause symptoms on squash plants. Further work is needed to identify the mechanisms by which different *S. marcescens* strains interact with host plants.

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Table 1. Bacteria used in these experiments.

Strain used in this study	Alternative name	Host	Source or Reference
<i>Serratia marcescens</i>			
ZO1-A		Zucchini	Bruton et al., 1998
WO1-A		Watermelon	Bruton et al., 1998
PO1-A		Pumpkin	Fletcher
CO1-A		Cantaloupe	Fletcher
RO1-A	IRBG 501	Rice	Gyaneshwar et al., 2001
RO2-A	IRBG 502	Rice	Gyaneshwar et al., 2001
HO1-A		Human	Rascoe et al., 2003
90-166	G01	Cotton	J. W. Kloepper
ATCC 13880	Type strain RF4738	Pond water	ATCC
<i>Pseudomonas syringae</i>			
pv. <i>tabaci</i>		Tobacco	Obukowicz et al., 1985
pv. <i>tomato</i> DC3000	PSESM, 223283	Tomato	Moore <i>et al.</i> , 1989
<i>E. coli</i> OP50			
			<i>Caenorhabditis</i> Genetics Center

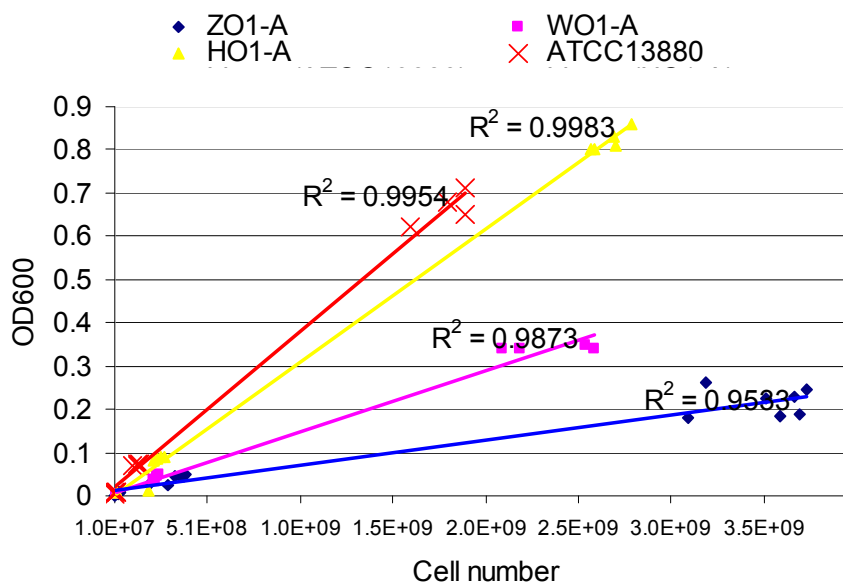


Fig. 1. Relationship between cell number and OD₆₀₀ of *Serratia marcescens* ZO1-A, WO1-A, HO1-A, and ATCC13880.

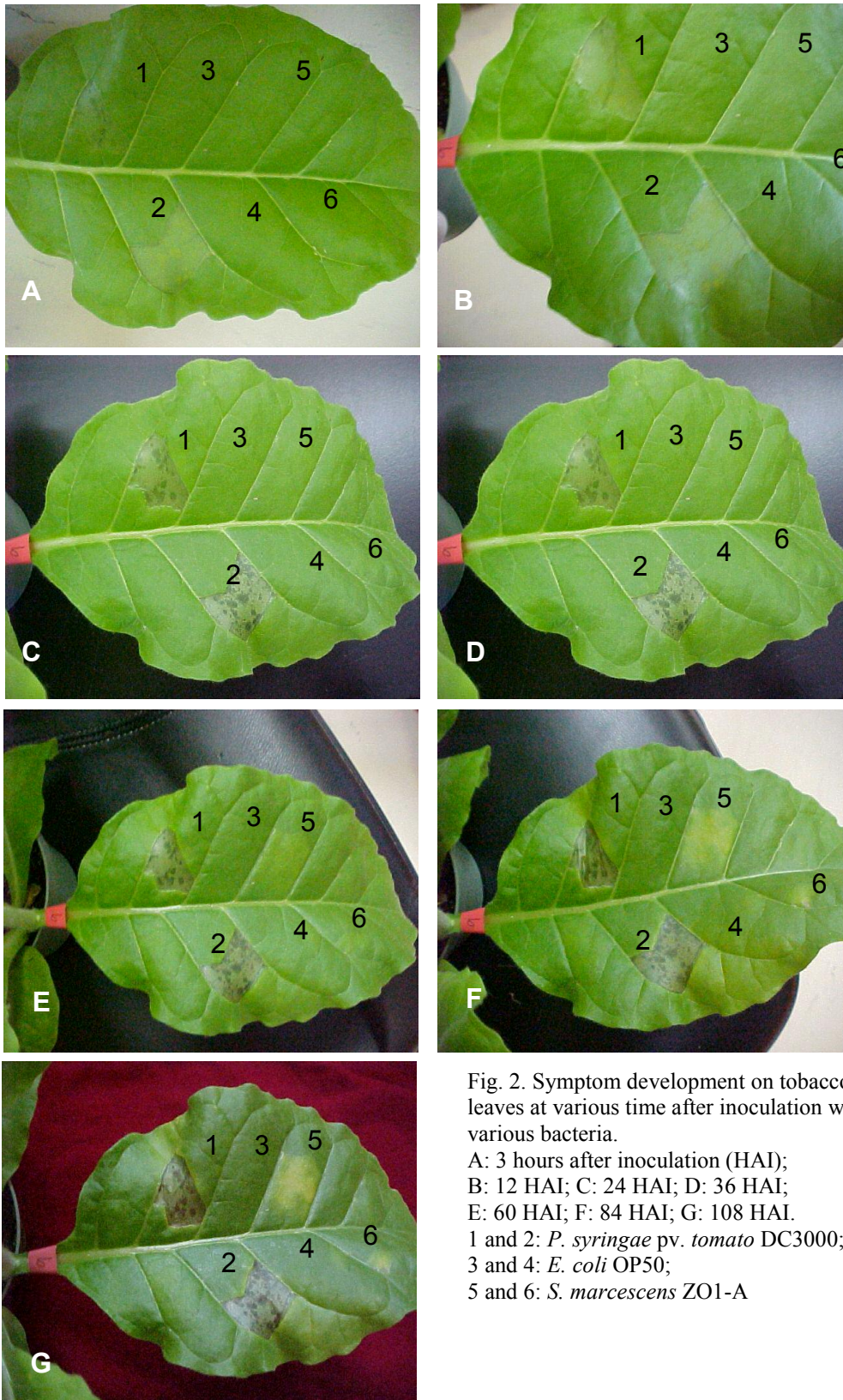


Fig. 2. Symptom development on tobacco leaves at various time after inoculation with various bacteria.

A: 3 hours after inoculation (HAI);

B: 12 HAI; C: 24 HAI; D: 36 HAI;

E: 60 HAI; F: 84 HAI; G: 108 HAI.

1 and 2: *P. syringae* pv. *tomato* DC3000;

3 and 4: *E. coli* OP50;

5 and 6: *S. marcescens* ZO1-A

Table 2. Reaction of tobacco leaves to inoculation of bacteria

Bacteria	Pathovars or strains	Treatment	Time (Hours after inoculation)										
			24	48	72	96	120	144	168	192	216	240	>240
<i>Pseudomonas syringae</i>	pv tomato DC3000	Culture	HR										
		Heated	-	-	-	-	-	-	-	-	C	C+N	N+
		Filtered	-	-	-	-	-	-	-	C	N	N+	
	pv tabaci	Culture	-	-C	C+	C+N		N+					
		Heated	-	-	-	-	-	-	-	C	C	C	N
		Filtered	-	-	-	-	-	-	-	C	C	C	N
<i>Serratia marcescens</i>	WO1-A	Culture	-	-	C	C+N		N+					
		Heated	-	-	-	-	-	-	-	C	C	C	N
		Filtered	-	-	-	-	-	-	-	C	C	C	N
	ZO1-A	Culture			C	C+N		N+					
		Heated	-	-	-	-	-	-	-	C	C	C	N
		Filtered	-	-	-	-	-	-	-	C	C	C	N
	CO1-A	Culture			C	Y+N		N+					
		Heated	-	-	-	-	-	-	-	C	C	C	N
		Filtered	-	-	-	-	-	-	-	C	C	C	N
	90-166	Culture	-	C	C+N	N+							
		Heated	-	-	-	-	-	-	-	-	C	C	N
		Filtered	-	-	-	-	-	-	-	-	C	C	N
	HO1-A	Culture	-	-	C	C+N		N+					
		Heated	-	-	-	-	-	-	-	C	C+N		
		Filtered	-	-	-	-	-	-	-	C	C+N		
	RO1-A	Culture	-	-	C	C+N		N+					
		Heated	-	-	-	-	-	-	-	C	C+N		
		Filtered	-	-	-	-	-	-	-	C	C+N		
RO2-A	Culture	-	-	C	C+N		N+						
	Heated	-	-	-	-	-	-	-	C	C+N			
	Filtered	-	-	-	-	-	-	-	C	C+N			
<i>E. coli</i>	OP50	Culture	-	-	-	-	-	-	-	-	-	-	
		Heated	-	-	-	-	-	-	-	-	-	-	
		Filtered	-	-	-	-	-	-	-	-	-	-	
	PBS	-	-	-	-	-	-	-	-	-	-	-	

Culture, diluted to 5×10^6 - 1×10^7 cells/ml; h: bacterial culture heated, 100°C, 30 min; f: bacterial culture filtered (0.20 micron); HR: hypersensitive reaction; C: chlorotic lesion; N: necrotic lesion; N+: area of necrosis increased in size.

Table 3. *Serratia marcescens* pathogenicity on different plant species

<i>Serratia marcescens</i> strains		Squash* N=180	Tobacco N=90	Onion N=60	Carrot N=60
ZO1-A	Incidence %	17 ± 5	100	18 ± 2	35 ± 3
	Symptoms	Yellowing, stunting, leaf cupping	Chlorotic lesion, localized necrosis	Bulb rot	Wilt
	PCR [▲]	48 ± 7	ND	ND	ND
WO1-A	Incidence %	16 ± 7	100	17 ± 4	32 ± 2
	Symptoms	Yellowing, stunting, leaf cupping	Chlorotic lesion, localized necrosis	Bulb rot	Wilt
	PCR	44 ± 3	ND	ND	ND
RO2-A	Incidence %	0	100	100	100
	Symptoms	No visible symptoms	Chlorotic lesion, localized necrosis	Bulb rot	Wilt
	PCR	41 ± 5	ND	ND	ND
90-166	Incidence %	0	100	100	70 ± 5
	Symptoms	No visible symptoms	Chlorotic lesion, localized necrosis	Bulb rot	Wilt
	PCR %	36 ± 3	ND	ND	ND
HO1-A	Incidence %	0	100	100	ND
	Symptoms	No visible symptoms	Chlorotic lesion, localized necrosis	Bulb rot	Wilt
	PCR	21 ± 7	ND	ND	ND
PBS	Incidence %	0	0	0	0
	Symptoms	N	N	N	N
	PCR	0	ND	ND	ND

* Bacteria were inoculated at a titer of 10^{11} cells/ml on squash plants; $5 \times 10^6 - 1 \times 10^7$ cells/ml on tobacco; 1×10^8 cells/ml on carrot or onion; N, number of tested plants.

▲ Percentage of samples PCR positive.

* Results are means ± SD of at least three experiments. N, no symptoms observed; ND, not done.

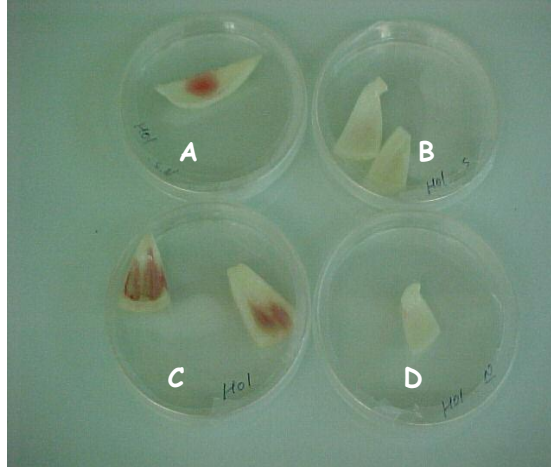


Fig. 3. Reaction of onion bulbs to inoculation with *Serratia marcescens* HO1-A. A: Bacterial culture dropped onto bulb surface; B: Heat-killed bacteria dropped on bulb surface; C: Bacterial culture injected into bulbs; D: Bacterial filtrate dropped on bulb surface.

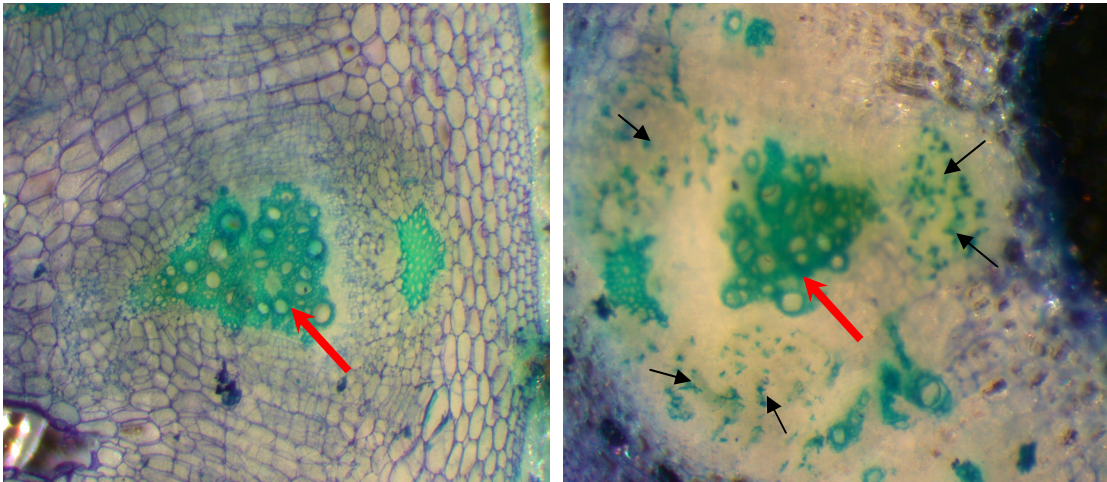


Fig. 4. Dienes' stain of transverse sections of CYVD symptomatic and healthy squash plant crowns. Left: Healthy plant with xylem stained green (red arrow); Right: Diseased plant – phloem and parenchyma were dark green (Black arrows).



Fig.5. Reaction of carrot plants 5 days after fork-inoculation with *Serratia marcescens*.
A: PBS; B, RO2-A.

CHAPTER III

COLONIZATION AND MOVEMENT OF *SERRATIA MARCESCENS* IN SQUASH PLANTS

Abstract

Serratia marcescens, a versatile gram-negative microbe, is the causal agent of cucurbit yellow vine disease (CYVD) in many cucurbit production areas of the U.S. Although previous studies demonstrated that CYVD strains of *S. marcescens* were transmitted from plant to plant by the squash bug (*A. tristis*), associated with phloem and leading to stunting and chlorosis, it is not known whether these bacteria also can enter plants through natural openings, such as stomata and hydathodes. It also is unknown what host tissues are colonized by the bacteria, or by what route they translocate within the plant host. In this study, plasmid pSMC21, carrying a gene encoding green fluorescent protein (GFP), was transformed into two strains of *S. marcescens* (the CYVD pathogenic ZO1-A and the non-pathogenic rice endophyte RO2-A), designated GFP-ZO1 and GFP-RO2, respectively. The labeled bacteria were confirmed by viewing GFP under UV radiation and by colony PCR using GFP specific primers. The labeled bacteria could be recovered from plants 5 wk after inoculation and their identity was confirmed by PCR, indicating that this plasmid was stable without selection *in planta*. GFP-ZO1 and GFP-RO2 were introduced to squash leaves by dropping onto the surface or by vacuum infiltration, or into stems by wounding. Fluorescence and confocal microscopy revealed that *S. marcescens* placed on the leaf surface did not move into the plant interior through the stomata. Labeled bacteria forced into the intercellular spaces by vacuum infiltration remained in the vicinity of the stomata through which they entered, and apparently did not move

to, or enter, the vascular bundles. In contrast, labeled bacteria introduced into squash stems via a multi-pronged inoculating fork were found within xylem vessels, at and below the inoculation sites. This is the first report of CYVD strains of *S. marcescens* colonizing and being translocated in xylem vessels. Our results showed that the colonization and translocation of *S. marcescens* in squash plants were apparently dependent upon how and where the bacteria were introduced.

Introduction

Cucurbit yellow vine disease (CYVD), a destructive vine decline, was first observed in central Texas and Oklahoma in 1988 (Bruton et al., 1998). Since then, it has been diagnosed in Missouri (Kabrick 2002), Arkansas, Colorado, Connecticut, Kansas, Nebraska, Tennessee, Massachusetts (Bruton et al., 2003), Kentucky (Bessin, 2003), Indiana, Ohio, and Michigan (Fletcher J., unpublished data). In several cucurbit species the lower canopy leaves turn bright yellow, followed by gradual or rapid decline and death of the vine, especially in early-planted fields (Bruton et al., 1998). Phloem discoloration and necrosis are considered key diagnostic features, and *S. marcescens* moved systemically throughout the host plant (Bruton et al., 2003).

No yellow vine disease developed in field-grown squash plants covered with insect-proof mesh, but plants exposed to the environment had a typical CYVD incidence rate (Bextine et al., 2001). When the use of insecticides was found to lower the incidence of yellow vine, squash bugs (*Anasa tristis*) were among the most common insects in the tested field (Pair et al., 2003). The piercing-sucking mouthparts of these insects are inserted into the host plant, where they can feed in a variety of plant cell types, such as collenchyma, parenchyma, phloem and xylem cells (Neal, 1993). Squash bug feeding on leaves injures epidermal cells and mesophyll, but their stylets are capable of reaching the phloem (Beard 1940, Neal 1993). Light microscopy showed that the stylets penetrate intracellularly toward the vascular bundles (Beard, 1940). *A. tristis* mouthparts also penetrate xylem vessels, interrupting xylem transport (Neal, 1993). It is unclear how bacteria

overcome: 1) the high sugar (often sucrose) concentrations and associated high osmotic pressure, and 2) the unbalanced composition of amino acids, the dominant nitrogenous compounds in the phloem sap (Douglas, 2006).

S. marcescens, a motile, non-sporulating, gram-negative microbe, has been isolated from many niches, such as water, soil, plants, insects, and even human beings. Among numerous isolates from diverse ecological niches that were characterized by Zhang and co-workers (2003), RO2-A (also called IBRG502), a beneficial rice endophyte, is most closely related to CYVD strains, such as the zucchini isolate ZO1-A. Pathogenicity tests demonstrated different host reactions to *S. marcescens* RO2-A and ZO1-A when they were introduced into tobacco, carrot, onion and squash (Chapter II). In a previous study (Gyaneshwar et al., 2001) RO2-A was observed within “intercellular spaces, senescing root cortical cells, aerenchyma, and xylem vessels” but not inside intact cells. In our experiments, RO2-A was detected by PCR in mechanically inoculated squash plants even though it did not cause visible symptoms (Chapter II).

Green fluorescent protein (GFP), a useful genetic marker from the jellyfish *Aequorea victoria*, has been widely used to facilitate biological research on plant-microbe interactions in living systems. It can be used to localize and identify GFP tagged bacteria in infected plant cells and tissues. Unlike conventional microscopic probes, GFP allows observation of the labeled protein in living cells. Bacterial cells tagged with GFP can be visualized and enumerated *in situ* and samples need not be fixed, hybridized or stained (Tombolini et al., 1997). The genetically altered GFP works in both eukaryotes and prokaryotes (Chalfie et al., 1994). For example, using a *gfp*-marked strain of *Xylophilus ampelinus*, which causes bacterial necrosis of grapevine, the relative importance of epiphytic and endophytic phases of plant colonization in disease development were evaluated (Grall et al., 2003). GFP also has been used to monitor the development of the human parasite, *Leishmanis donovani*, in the gut of phlebotomine sand flies, *Lutzomyia longipalpis*, *L. ovallesis* and *L. youngi* (Guevara et al., 2001) and the fate of *Serratia entomophila*, causal agent of amber disease, in the New Zealand grass grub, *Costelytra zealandica* (Hurst et al., 2002).

Although previous studies demonstrated that, in nature, CYVD strains of *S. marcescens* were introduced into host plants by the squash bug (*A. tristis*) (Bruton et al., 2001; 2003; Wayadande et al., 2005), leading to stunting symptoms, we wanted to know whether these bacteria also can enter plants through natural openings, such as stomata and hydathodes. We also sought to better understand what host tissues are colonized by the bacteria, and by what route (s) they translocate within the plant host. In this study, we introduced a plasmid construct containing the gene for GFP into CYVD pathogenic and non-pathogenic strains of *S. marcescens* and then visually monitored *S. marcescens*' entry and movement in the squash plant.

Materials and Methods

Bacterial isolates and growth conditions. Strains of *S. marcescens* used in this study included a CYVD isolate, ZO1-A, isolated from zucchini in Oklahoma (Bextine, 2001) and a non-CYVD isolate, RO2-A, a rice endophyte, also known as IBGR502, provided by P. Gyaneshwar (International Rice Research Institute, the Philippines). Bacteria were stored at -80°C in aliquots of 1.5 ml Luria-Bertani (LB) broth containing 15% glycerol. For use in experiments, bacteria were streaked onto LB agar (Sambrook et al., 1989) and incubated at 28°C for 24 h.

Labeling *S. marcescens* with green fluorescent protein (GFP). Plasmid pSMC21, carrying the gene for GFP from *A. victoria* (originally constructed by Bloemberg et al (1997) and obtained from Alejandro Penaloza-Vazquez, Oklahoma State University), was used to label *S. marcescens* ZO1-A and RO2-A by electroporation. To prepare competent cells for electroporation, a fresh colony of ZO1-A or RO2-A cells was inoculated into 5 ml of LB medium and grown with shaking (200 rpm) at 28 °C overnight. Two ml of the resulting suspensions were added to 200 ml of LB medium in 1 liter flasks and grown for 4-5 h with vigorous shaking at 28 °C, until the absorbance at 600 nm was ~0.6. Bacterial suspensions were transferred to 200 ml conical centrifuge tubes and incubated on ice for 60 min before pelleting at 2000 X g, at 4 °C, 15 min. The cell pellet was

washed twice in 160 ml sterilized, ice-cold water and centrifuged as before. The supernatant was discarded, the pellet washed in 20 ml 10% glycerol and the cell suspension transferred to a 30 ml tube. After centrifugation at 2000 X g for 15 min, all but 5 ml of the supernatant was removed and the remaining cells were resuspended in 10% glycerol. These competent cells were stored at -80 °C until used.

For transformation, 1 µl plasmid pSMC21 (100 ng/µl in nanopure water) was added to 50 µl competent cells and incubated on ice for 10 min. *S. marcescens* cells were transformed with a Bio-Rad gene pulser (200 Ohms/25µF/ 2.5 KV). One ml of SOB (0.5% yeast extract, 2.0% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, pH 7.0) at room temperature was added and the bacteria shaken at 28 °C for 1 h. The cells (100 µl) were plated onto LB-agar containing 50 µg/ml ampicillin and 30 µg/ml kanamycin and incubated overnight at 30°C. The plates were observed under UV radiation (312 nm, Fisherbiotech, Pittsburgh, PA) to detect fluorescent colonies (designated GFP-ZO1 and GFP-RO2, respectively).

The *gfp* gene was detected in the labeled cells by colony PCR using primers: GFPF (5'CTGGAGTTGTCCCAATTCTTG) and GFPR (5'TCCCAGCAGCTGT TACAAAC). PCR amplification was performed in 25 µl reaction mixtures containing a single bacterial colony as template, 1X Gitshier buffer (Gibco BRL, Gaithersburg, MD), 100 µM of each dNTP, 0.5 mM of each primer, 2 mM MgCl₂ (Gibco BRL), and 5 U *Taq* DNA polymerase (Gibco BRL) per reaction. This mixture was placed in a GeneAmp PCR system 9600 (Perkin-Elmer Cetus, Norwalk, CT) thermal cycler and subjected to a 5 min denaturation step at 95 °C followed by 35 cycles at 95 °C for 45 s, 50 °C (other primers at 56°C) for 30 s and 72 °C for 90 s. The reaction mixture was held at 72 °C for 10 min and then stored at 4 °C until analyzed on a 1.5% agarose gel.

The stability of plasmid pSMC21 in ZO1-A and RO2-A was tested as in a previous study (Bloemberg et al., 1997). Briefly, transformed bacteria were grown in the presence of the appropriate antibiotics, washed twice, diluted 1:1,000 in LB medium without antibiotics, and grown overnight. The procedure of dilution and

growth was repeated four times, resulting in at least 30 generations of growth in the absence of antibiotic selection pressure. Plasmid stability was subsequently evaluated by plating an aliquot of the final culture on LB plates and then streaking 100 single colonies on LB agar plates with and without the appropriate antibiotic.

Inoculation of the GFP labeled bacteria into squash plants.

(i) Inoculation by dropping onto the leaf surface. To determine whether *S. marcescens* enters squash plants (*Cucurbita maxima*) via natural openings, such as stomata and hydathodes, GFP-labeled bacteria were inoculated by dropping onto plant leaf surfaces. Two ml bacterial suspensions of GFP-ZO1 and GFP-RO2 ($\sim 3 \times 10^9$ cells/ml) were centrifuged (2 min at 4 °C, 900 X g) and re-suspended in 20 ml PBS buffer (0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl) to a final concentration of $\sim 3 \times 10^8$ cells/ml. Squash leaves detached from 3 wk old plants were surface-sterilized in 70% ethanol for 30 s and 10% bleach for 1 min, washed three times in sterile distilled water, and then air dried in a laminar flow hood for about 10 min. About 10 μ l re-suspended bacteria were dropped onto the surface (15 drops/leaf) of 45 leaves. PBS buffer was dropped similarly onto 45 control plant leaves. All treated leaves were placed onto the surface of 1% agar plates (3 leaves/plate) to maintain moisture and incubated in a growth chamber (23°C; light 12 h: dark 12 h). Three to five leaves from each treatment were examined every two days for one week. Each time, samples were viewed before and after washing with 10% bleach for 1 min and rinsing four times in sterile distilled water. Five to ten small pieces of leaves (10 to 15 mm long, 5-10mm wide) from each treated leaf were examined using a Nikon Eclipse E600 microscope (Nikon Corporation, Tokyo, Japan). Use of a B-2A filter (DM 505 and EX 450- 490) enabled clear distinction between plant tissue autofluorescence and the GFP present in labeled bacteria, as this filter causes autofluorescence to appear dark brown, red or yellow. Digital images using fluorescence microscopy were processed using software SPOT version 2.2 and Adobe Photoshop 6.0.

For confocal examination, samples washed as described above were processed in a method modified from Grall et al (2003). Briefly, five to ten small pieces of leaves (10 to 15 mm long, 5-10 mm wide) were fixed with 4% paraformaldehyde

in fixing phosphate buffer (20% solution A [2.4% Na₂HPO₄ · 12H₂O] plus 80% buffer B [0.9% NaH₂PO₄ · H₂O] [pH 7.2]) at 4°C for 24 h and washed three times in the same buffer (3 X 15 min). The samples were stored at 4°C and rinsed in fixing phosphate buffer every 3 days until used. A Leica TCS SP2 (Leica Microsystems, Bannockburn, IL) confocal imaging system, attached to a Leica DMRE microscope, was used at a 488 nm excitation wavelength to study the localization of the GFP labeled bacteria in squash plants. Fifteen to 30 optical sections of each sample were taken of each section at 0.2- to 2 μm intervals. The tests consisted of four replicates.

(ii) Inoculation by vacuum infiltration. To determine whether *S. marcescens*, introduced into host plant intercellular spaces, are able to move into other plant tissues, vacuum infiltration was used to inoculate squash leaves. Detached squash leaves (45 leaves/treatment) were surface-sterilized as in (i) and placed into a 500 ml beaker containing 300 ml (about ~3 X 10⁸ cells/ml) GFP-labeled *S. marcescens*. PBS was used for control plants (45 leaves). When a vacuum compressor was applied for 5 min to remove the air inside the beaker, bubbles forming on the leaf surfaces were an indication of the removal of air from the plant's interior spaces. As the vacuum was released, the bacterial suspension was forced through the open stomata into squash leaves. Immediately after applying the vacuum, leaves were washed with PBS to remove any bacteria not tightly attached to the leaf surface, then maintained and examined as in (i) every two days for a week. There were three replicates for this test.

(iii) Inoculation by stab wounding. This experiment was designed to determine whether *S. marcescens* strains can move from the inoculation site to other parts of the plant. Squash plants (*C. maxima*) were grown in the greenhouse 4-7 days, to the closed cotyledon stage. Bacteria (GFP-ZO1 and GFP-RO2) were streaked onto LB plates with appropriate antibiotics at room temperature for four days and then suspended in PBS to a concentration of 10¹¹ cells/ml, as estimated by OD₄₂₀. To inoculate squash seedlings, a multi-pronged “inoculating fork” (Bruton et al., 2003) was dipped into the bacterial suspension, picking up a drop of about 10 μl. The stem, just below the cotyledon, was jabbed about 15 times

with the fork, such that the prongs penetrated approximately 1/3-1/2 of the way through the stem. For each treatment, 45 squash plants were inoculated. The inoculated plants were maintained and examined as in (i) except that 3-5 plants/treatment were sacrificed after fork-inoculation of labeled *S. marcescens*. A small piece of stem (5 to 8 mm long, less than 4 mm diameter) from each plant, taken 30 mm below the point of inoculation, was excised every two days for the first week and weekly thereafter.

For long distance movement, 4 plants/treatment were fixed as described in (iii) except that the whole stem from each plant was cut upward and downward from the inoculation site into ~1.5 cm lengths and put into a 96-well ELISA plate in order, and then longitudinally sectioned and examined using fluorescence microscopy. The farthest point above or below the site of inoculation at which fluorescence was observed was recorded as the distance that the labeled bacteria moved. The test was performed four times.

Recovery and PCR identification of bacteria from inoculated plants.

Three squash plants, selected randomly from the fork-inoculated plant group, were sampled each week for one month for bacterial isolation. Stem pieces (~0.5 g, 1-2 cm long, beginning 3 cm below the inoculation site) were surface-sterilized (70% ethanol, 30 s; 10% bleach, 1 min), washed three times in sterile distilled water, air dried (10 min) and macerated in a sterile 1.5-ml microtube with a plastic pestle. Each macerate was serially diluted in PBS and plated onto LB medium containing 50 µg/ml ampicillin and 30 µg/ml kanamycin to select for plasmid pSMC21. Colonies were assessed for fluorescence using a UV trans-illuminator (312 nm, Fisher Biotech). To confirm the presence of GFP in the inoculated *S. marcescens* transformants, two pairs of primers were used for colony PCR: YV1 and YV4 (Melcher et al., 1999); and GFPF and GFPR as mentioned previously. PCR amplification was performed as described above.

Results

Labeling ZO1-A and RO2-A with GFP. To track *S. marcescens*' location in the squash plants, the bacteria were labeled with *gfp* from *A. victoria*, carried on plasmid pSMC21. Colonies of GFP-labeled *S. marcescens* CYVD strain ZO1-A and non-CYVD strain RO2-A, grown on LB agar, expressed typical green fluorescence when viewed using fluorescence microscopy, while wild type cells did not (Fig. 1). There was no visible difference in colony shape between the GFP-labeled and wild type cells on LB plates. When the stability of plasmid pSMC21 in ZO1-A and RO2-A was assessed under nonselective conditions, 98%, 91%, 84% and 79% of the bacterial cells still carried the plasmid after at least 5, 10, 20, and 30 generations of growth, respectively. There was no statistical difference (*t* test) in plasmid stability between GFP-ZO1 and GFP-RO2.

Inoculation by dropping on the leaf surface. To learn whether GFP-ZO1 and GFP-RO2 cells could enter squash leaves through natural openings such as stomata, bacterial suspensions were dropped onto surface-sterilized squash leaves. No symptoms were detected on the inoculated or control leaves up to five days after inoculation. Beginning on day six, all the leaves, regardless of whether they received bacteria or PBS, became chlorotic and were not suitable for examination thereafter. Fluorescence microscopy showed that labeled bacteria remained on the surface up to 7 days after inoculation and retained pSMC21 for the duration of the experiment (Fig. 1B). To determine whether the bacteria moved from the leaf surface into the leaf interior, treated leaves were washed by 10% bleach for 30 s to kill surface bacteria and washed to remove loosely attached bacteria from the leaf surface just before examination, and then fixed. Fluorescence microscopy showed that the majority of the labeled bacteria had been washed from the leaf surfaces (Fig. 1C).

Inoculation by vacuum infiltration. Dark green water-soaked spots developed on leaves during vacuum infiltration of *S. marcescens* or PBS into squash leaves. The water-soaking disappeared on control leaves after about one day, and on bacteria-treated leaves after two days. No symptoms were seen on the

leaves for the first four days after inoculation. Beginning on day five, all the leaves, regardless of whether they received bacteria or PBS, became chlorotic and were not suitable for examination thereafter. Fluorescence and confocal microscopy showed that many GFP-ZO1 and GFP-RO2 cells remained on the leaf surfaces. Confocal microscopy suggested that some entered, via the stomata, into the intercellular spaces reaching a depth of at least 150 μm , and moving laterally 10-30 μm away from the stomata, but there was no evidence of long distance movement or entry into the vascular system (Fig. 2).

Long distance movement of *S. marcescens* in squash plants. Four of 45 (8.9%) of cotyledon-stage squash seedlings, fork-inoculated at the junction of cotyledon and stem with *S. marcescens* GFP-ZO1, displayed wilted cotyledons, shriveled stems and premature death. Five control plants and 15% of those inoculated with GFP-RO2 showed similar symptoms. The remainder (41 and 38 plants, inoculated with GFP-ZO1 and GFP-RO2, respectively) wilted briefly but soon recovered and resembled the control plants for the remainder of the experiment. Immediately after inoculation, labeled GFP-ZO1 and GFP-RO2 were detected using the B-2A filter, in many plant tissues (including epidermis, parenchyma, phloem and xylem), in the immediate vicinity of the inoculation site (Fig. 3) but nowhere else in the plant. Because plant cells in the path of the fork inoculation sustained significant damage, it was not possible to determine whether the labeled bacteria were intercellular, intracellular or both. Two days after inoculation (DAI), GFP labeled ZO1 and RO2 were detected 2 cm below the inoculation site inside xylem vessels (Fig. 4 D and E), while the control plants (inoculated with PBS) showed only dark brown, red or yellow auto-fluorescence (Fig. 4 B). Though there were 6-8 xylem vessels in each vascular bundle, bacteria were generally detected in only one or two vessels (Fig. 4 D and E). In longitudinally sectioned stems, large aggregates or colonies of fluorescent bacteria were visible inside the xylem vessels in a pattern of discontinuous accumulation (Fig. 5 D). Single cells or small colonies were observed in discontinuous patches, apparently associated with secondary xylem wall thickenings (Fig. 5 E). These results are consistent with observations of the stem

cross sections, in which bacterial colonization was noted inside a single vessel of a vascular bundle containing 6-8 vessels. Both GFP-ZO1 and GFP-RO2 were seen inside xylem vessels. GFP-ZO1, but not GFP-RO2, was occasionally detected in the intercellular spaces of the parenchyma or associated with the parenchyma cell walls (Fig. 6). On one occasion a few bacteria were visible inside a parenchyma cell (Fig. 6C).

Paraformaldehyde fixed stems were optically sectioned, in either a transverse or longitudinal direction. Compared to images of physical sections, taken using fluorescence microscopy, serial visual images of a single stem slice using confocal microscopy provided more precise information about *S. marcescens* colonization sites. As before, fluorescent bacteria were visible inside the xylem vessels, often associated with the cell walls and not completely filling the vessels (Fig. 7, and Fig. 8 D and E). In our experiments, labeled bacteria were seen in only one or two of the 6-8 bundles in the stems (Fig. 8 D and E).

Labeled *S. marcescens* moved upward and downward from the inoculation site. Fluorescent cells were observed 2 ± 0.8 cm below the inoculation site 2-4 day after inoculation. Labeled bacteria moved about 2.5 ± 1.1 and 3.1 ± 1.6 cm downward from the inoculation site 2 and 4 wk after inoculation, respectively. No fluorescent cells were observed above the inoculation site until 3 wk after inoculation (1.8 ± 0.8 cm). The farthest distance traveled was about 7.8 cm downward, and 3.4 cm upward from the inoculation site.

Recovery and PCR identification of bacteria from inoculated plants. Samples from GFP-ZO1 and GFP-RO2 inoculated plants, spread onto LB plates with appropriate antibiotics, produced many fluorescent colonies as long as five wk after inoculation. Colony PCR confirmed that these colonies were GFP-labeled *S. marcescens* ZO1 and RO2 (Fig. 9).

Discussion

GFP is a valuable tool for study of plant-microbe interactions in living systems (Chalfie et al., 1994; Grall et al., 2003). In this study, the plasmid

pSMC21, which carries a bright mutant version of GFP (Cormack et al., 1996) and is maintained stably in *Pseudomonas spp* (Bloemberg et al., 1997), was transformed into two *S. marcescens* strains, the CYVD-causing ZO1-A and the rice endophyte RO2-A. The fluorescence was strongly expressed in *S. marcescens*, especially in ZO1-A, such that bacterial colonies appeared light green. The plasmid was stable in both of the tested *S. marcescens* strains over at least 30 generations of growth on artificial medium, or five weeks *in planta* (Fig. 8), both in nonselective conditions. Though we did not compare the pathogenicities of the GFP-labeled and wild type bacteria, it has been demonstrated that *gfp*-marked strains of other bacteria, such as *X. ampelinus*, were as pathogenic as their parent strain (Grall et al., 2003).

We applied the *gfp*-labeled *S. marcescens* to study bacterial entry, movement and colonization in plants. GFP-ZO1 and GFP-RO2, dropped onto the surface of detached squash leaves, were visible as fluorescing cells on the surface for at least 5 days. *S. marcescens* apparently did not enter the plant from the natural openings (such as stomata) after being dropped onto leaf surfaces. There were no visible symptoms on the leaves drop-inoculated with *gfp*-labeled *S. marcescens*. Fluorescence and confocal microscopy demonstrated that these cells remained on the leaf surfaces even after washing, but there was no evidence that any bacteria penetrated through stomatal openings. Though we did not assess the bacterial titer on the inoculated leaf surface, the number of fluorescing cells was lower than the number originally introduced. This could reflect an inability of *gfp*-labeled *S. marcescens* to multiply on the leaf surfaces, death of inoculated cells, and/or the loss of the *gfp* gene in a portion of the population. Other epiphytic bacteria, such as *P. syringae* (Hirano et al., 2000) and *X. ampelinus* (Grall et al., 2003) multiply on plant surfaces and enter their intercellular spaces through natural leaf openings. The fact that *S. marcescens* did not may indicate that it functions better as an endophyte than as an epiphytic bacterium. Indeed, RO2-A (*S. marcescens* IBRG502) is a rice endophyte (Gyaneshwar et al., 2001). Although endophytes have been reported in various plants and are postulated to play an important role in sustainable crop production (Sturz, et al., 2000), the mechanisms they apply to

enter the plant and establish the endophytic relationship are not well understood. Fissures created by the emergence of lateral roots or dissolution of cell wall components may facilitate bacterial entry in some cases (James et al., 1998).

In our study, *S. marcescens*, inoculated by vacuum infiltration, remained in the vicinity of the stomata and apparently did not move to, or enter, the vascular bundles (Fig. 2). Vacuum infiltration has been used by others for introducing pathogenic bacteria into the intercellular spaces to study the interactions between plants and pathogens (Buell et al., 1997; Wilson et al., 1999; Venisse et al., 2001). When *Xanthomonas campestris* pv *campestris*, a vascular pathogen that typically enters the plant via hydathodes (Alvarez, 2000), was vacuum infiltrated into *Arabidopsis thaliana* accession *Landsberg erecta* (Ler), a systemic necrosis developed.

In contrast to the vacuum-infiltrated *S. marcescens*, fork-inoculated bacteria were deposited directly into various tissues, including the epidermis, parenchyma and vascular bundles, all of which sustained some direct mechanical damage (Fig. 3). Within two days GFP-labeled bacteria had entered and colonized xylem vessels at and below the inoculation sites (Fig. 4, 5, 6, and 8). The “ladder rung” distribution of the fluorescing material suggests that bacteria, moving within the xylem sap flow, may lodge in crevasses or ridges created by the secondary wall thickenings characteristic of xylem vessels (Fig. 5, D and F; Fig. 8, D and F). Interestingly, GFP-labeled bacteria were observed above the inoculation sites, suggesting that *S. marcescens* translocation was predominantly downward. It is possible that the method of inoculation and site affect bacterial movement. For instance, a previous study (Grall et al., 2003) demonstrated that *X. ampelinus* moved downward when applied on a wound, but moved both upward and downward when sprayed on plant surfaces.

The fact that labeled bacteria were observed in the xylem of our test plants was unexpected. Previous transmission electron microscopy of field-collected CYVD-symptomatic cucurbit plants had revealed bacteria colonized the sieve tubes, and a diagnostic feature of this disease was honey-brown phloem discoloration in the crown and primary root (Bruton et al., 1998).

What could explain the apparent discrepancy between the findings of this study and those of Bruton et al. (1998)? It is possible that *S. marcescens* colonizes different squash plant tissues depending on how and where the bacteria are applied. In the field, CYVD strains of *S. marcescens* are transmitted by squash bugs (*A. tristis*) (Bruton et al., 1995). These insects feed in a variety of plant tissues, including phloem (Neal, 1993), and although the manner in which *A. tristis*-transmitted *S. marcescens* could invade phloem sieve tubes has not been described, it is clear from both electron microscopy evidence and systemic movement of the bacteria in CYVD-affected cucurbits (Bruton et al., 1998) that the vascular tissue is involved. Bacteria seen in phloem sieve tubes of CYVD affected but not healthy plants (Bruton et al., 1998) are circumstantial evidence of phloem colonization. In the present study, in contrast, *S. marcescens* was introduced into squash stems mechanically, without insect introduction. It is possible some feature related to the squash bug is required for phloem invasion. A chemical component of *A. tristis* saliva might compromise sieve tube cell walls, or might trigger *S. marcescens* to produce a plant cell wall degrading enzyme. Alternatively, perhaps some physical aspect of squash bug feeding could alter the integrity of the plant cell walls, allowing *S. marcescens* to transverse them into sieve cell lumens.

No visible symptoms occurred on squash plants inoculated with *S. marcescens* GFP-RO2, a finding consistent with previous studies (Bruton, personal communication, and Chapter 2). It is possible that RO2-A lacks some key genes involved in pathogenicity or virulence on squash plants. Zhang et al (2005) identified a type I fimbrial gene cluster in the chromosome of CYVD-causing *S. marcescens* strains that was absent in the non-CYVD strains examined, such as RO2-A. *fimA* or *fimH* knockout in the CYVD strain ZO1-A reduced the virulence on squash (Chapter IV). No significant difference in symptoms was observed between squash plants inoculated with GFP-ZO1 and those inoculated with wild type ZO1-A. These results were consistent with a previous study (Grall et al., 2003) in which GFP labeled *X. ampelinus* was as pathogenic as the parent strain.

Our results demonstrate that the nature and location (s) of squash tissue colonization by *S. marcescens* are influenced by the method of inoculation. Very few bacteria, after being dropped directly onto the leaf surface, passed through stomata into intercellular spaces of the underlying parenchyma, and those that did so remained in the immediate vicinity of the opening, suggesting that stomatal entry is not a natural pathway in CYVD development in the field. Bacteria that were forced by vacuum infiltration through stomata and into the leaf interior spread farther and produced a water-soaked spot, but still failed to enter the vascular tissue or to move systemically. Our third method of bacterial introduction, via an inoculating fork into squash seedling stems, indiscriminately damages plant cells while depositing bacteria in multiple spots. Thus, of the three inoculation methods tested in this study it most closely resembles the natural introduction of CYVD strains of *S. marcescens* via squash bug feeding. Why bacteria inoculated in this way colonized within the xylem (and not the phloem, as expected), and dominantly at sites below the site of inoculation remains unclear. The answers to these questions will undoubtedly provide further insight into the niche adaptation strategies of this cosmopolitan bacterial species.

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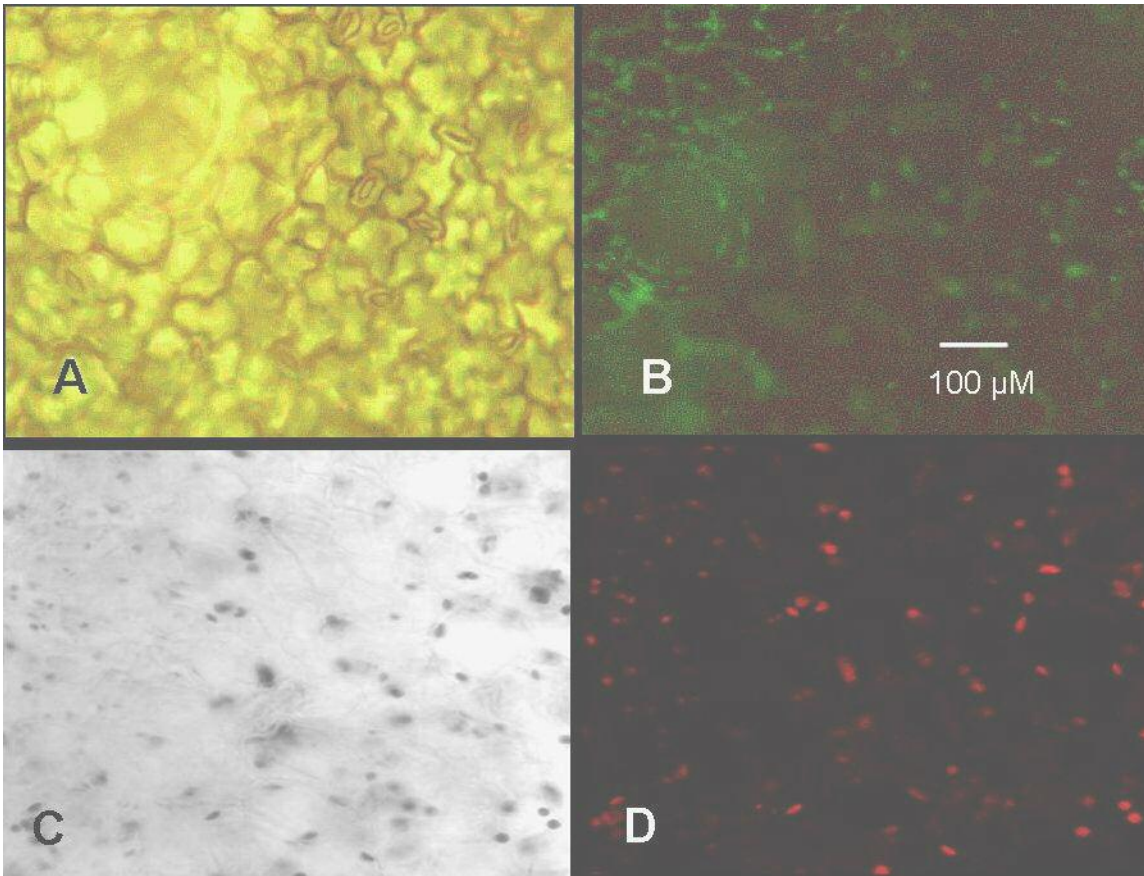


Fig.1. Representative squash leaf drop-inoculated with *Serratia marcescens* GFP-ZO1. A, B, viewed using fluorescence microscopy; bacteria apparently remained on leaf surface (A, bright field; B, fluorescence). C, D, viewed using confocal microscopy after washing and transverse sectioning (C, bright field; D, fluorescence).

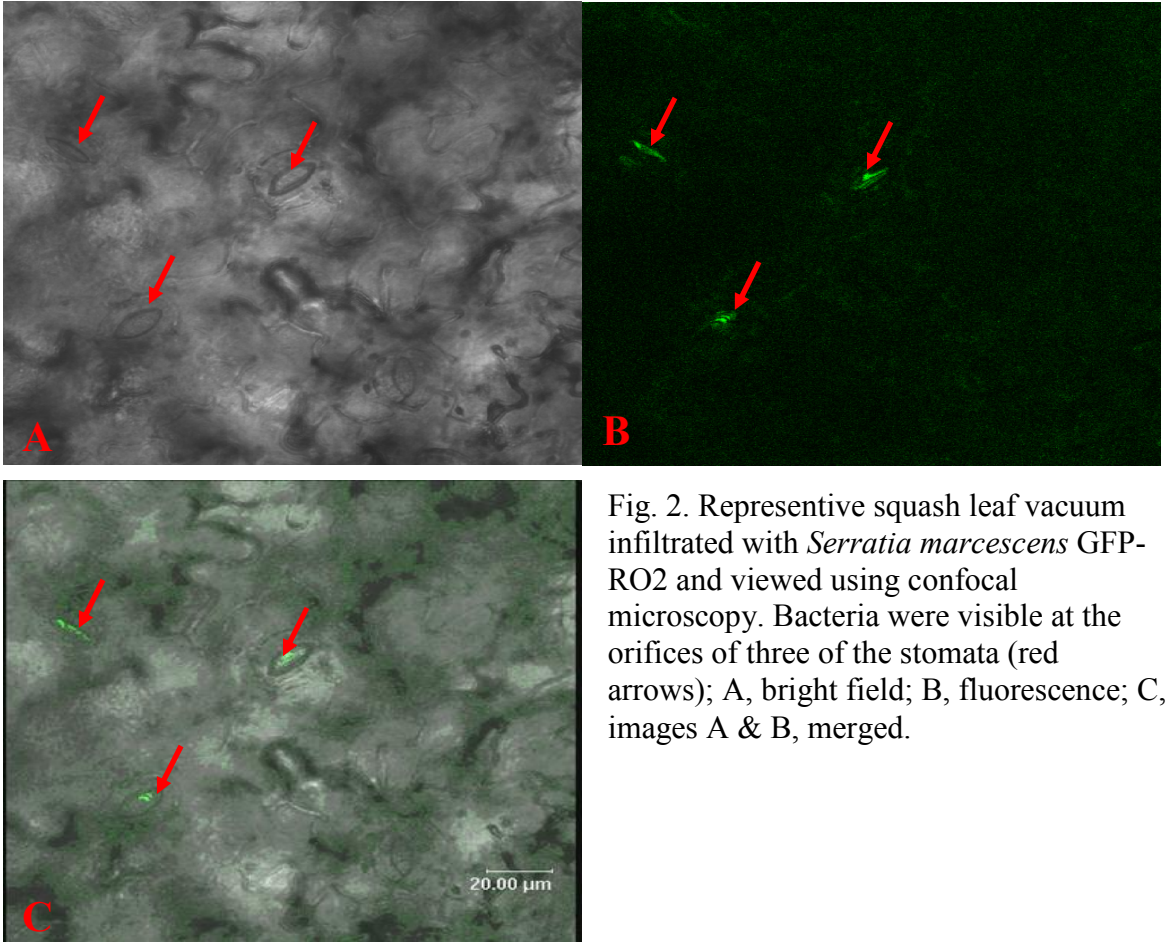


Fig. 2. Representative squash leaf vacuum infiltrated with *Serratia marcescens* GFP-RO2 and viewed using confocal microscopy. Bacteria were visible at the orifices of three of the stomata (red arrows); A, bright field; B, fluorescence; C, images A & B, merged.

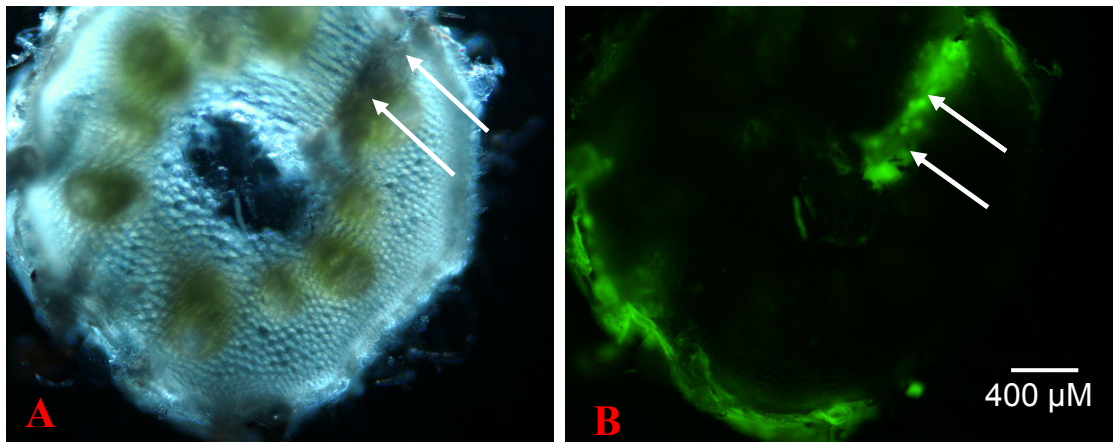


Fig. 3. Transverse section of a squash stem at the site of *Serratia marcescens* GFP-ZO1 introduction using an inoculating fork, viewed using light (A) or fluorescence microscopy (B). Arrows indicate the inoculation site.

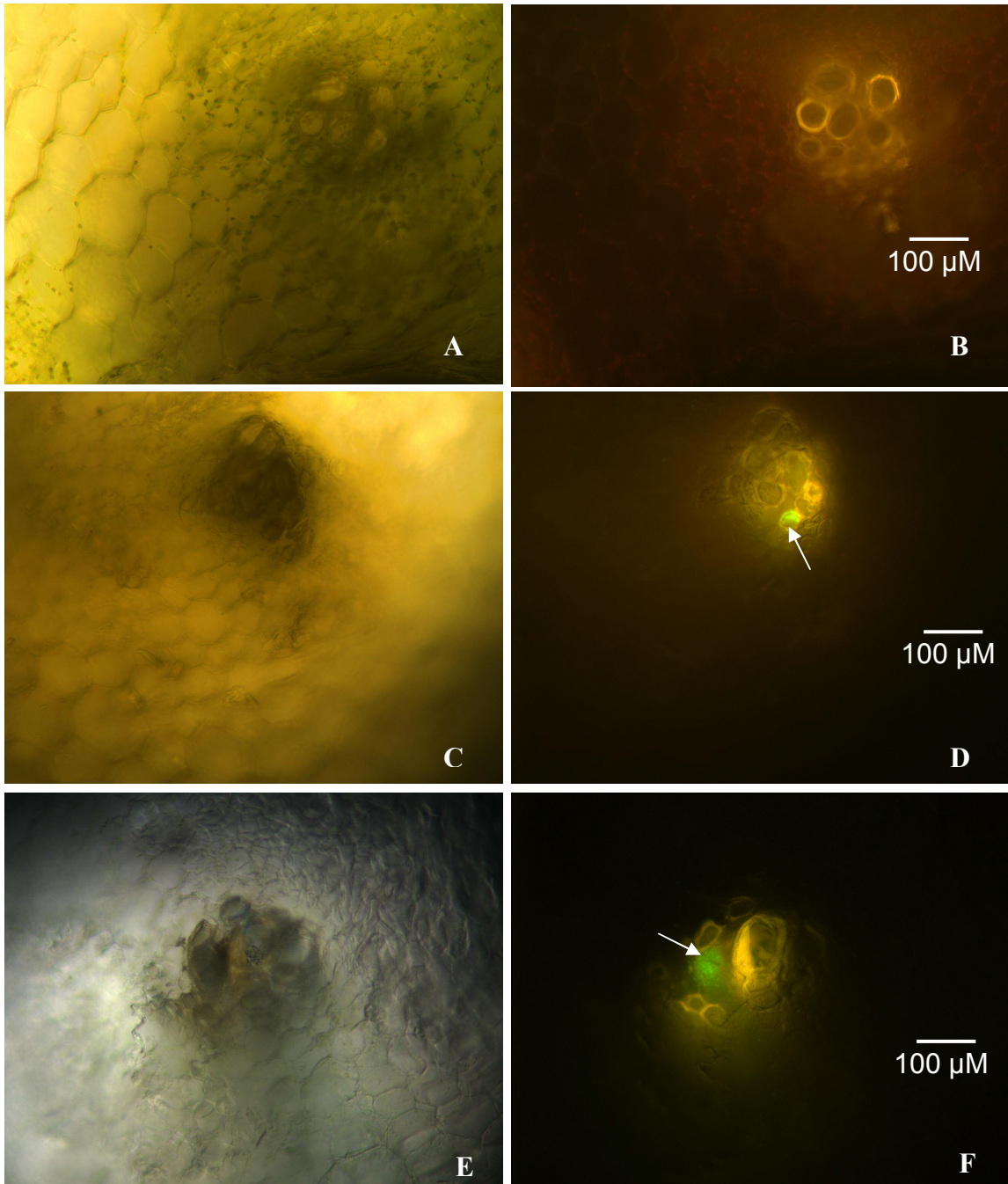


Fig. 4. Fluorescence micrographs of transverse sections of squash stems showing *Serratia marcescens* GFP-ZO1 and GFP-RO2 within xylem vessels (arrows). Plants were inoculated with: A, B, PBS; C, D, GFP-ZO1; E, F, GFP-RO2. The two micrographs of each treatment are of the same field, photographed using regular light (A, C, E), or fluorescence optics (B, D, F).

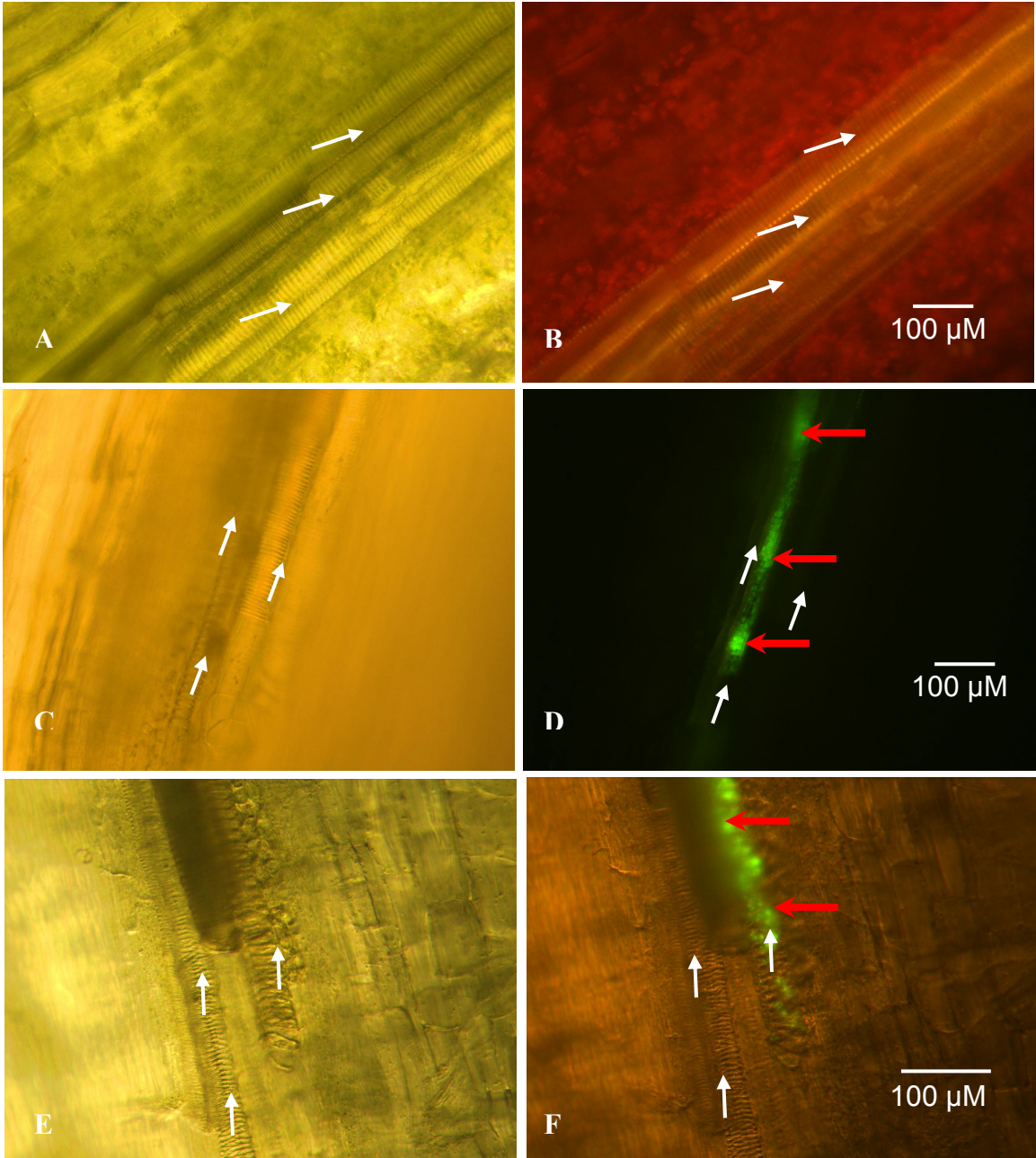


Fig. 5. Fluorescence micrographs of longitudinal sections of squash stems showing *Serratia marcescens* GFP-ZO1 and GFP-RO2 within xylem vessels. Plants were inoculated with: A, B, PBS; C, D, GFP-ZO1; E, F, GFP-RO2. The two micrographs of each treatment are of the same field, photographed using regular light (A, C, E), or fluorescence optics (B, D, F). White arrows: secondary xylem thickenings; red arrows: GFP-labeled bacteria.

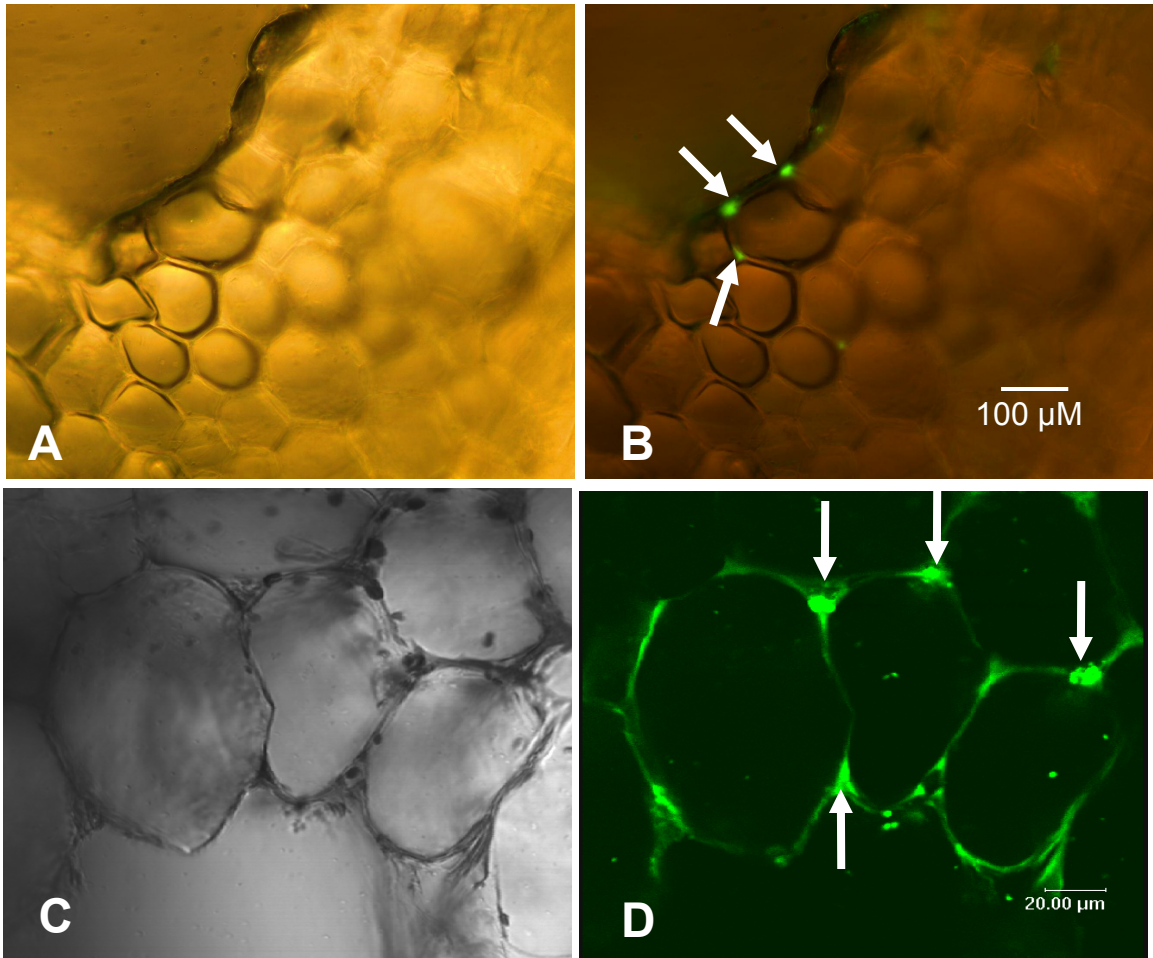


Fig. 6. *S. marcescens* GFP-ZO1 in the intercellular space of parenchyma, shown using fluorescence microscopy (40 X): A, regular light; B, fluorescence; or confocal microscopy: C, bright field; D, fluorescence. Arrows: the labeled bacteria.

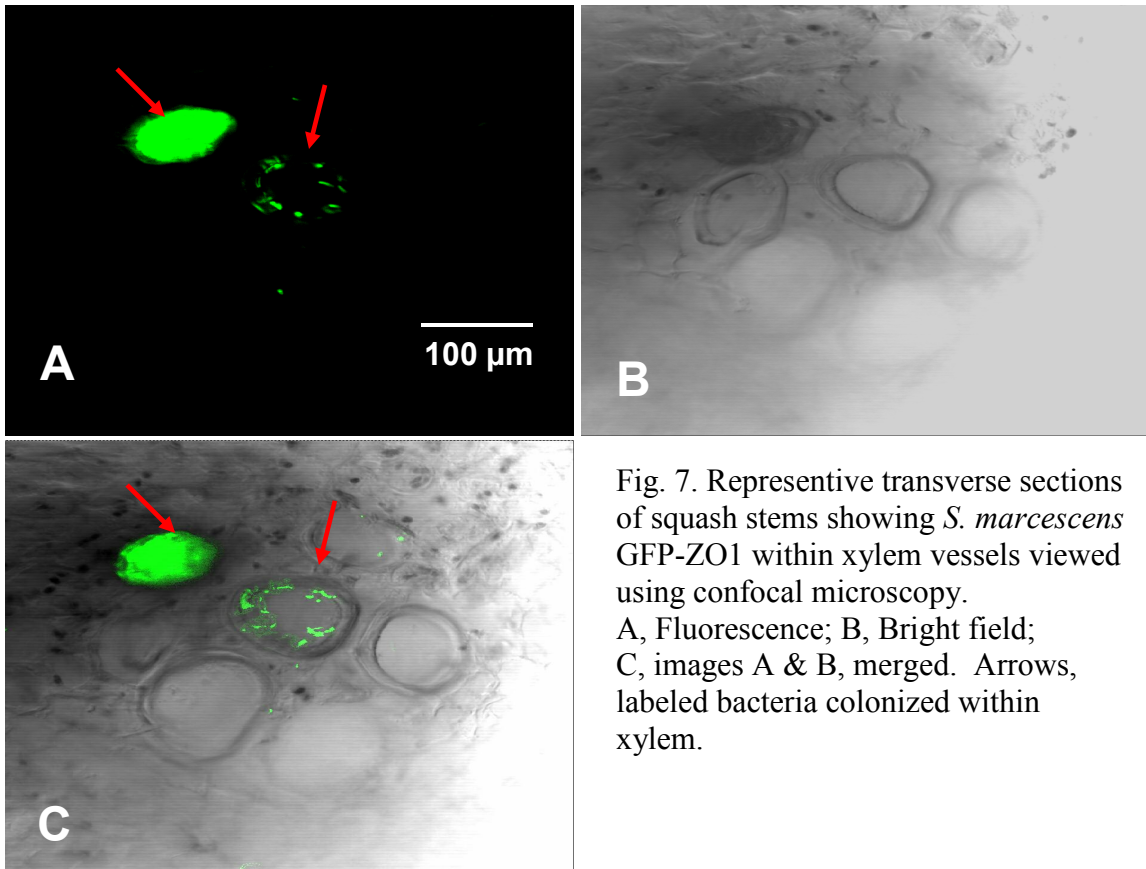


Fig. 7. Representative transverse sections of squash stems showing *S. marcescens* GFP-ZO1 within xylem vessels viewed using confocal microscopy. A, Fluorescence; B, Bright field; C, images A & B, merged. Arrows, labeled bacteria colonized within xylem.

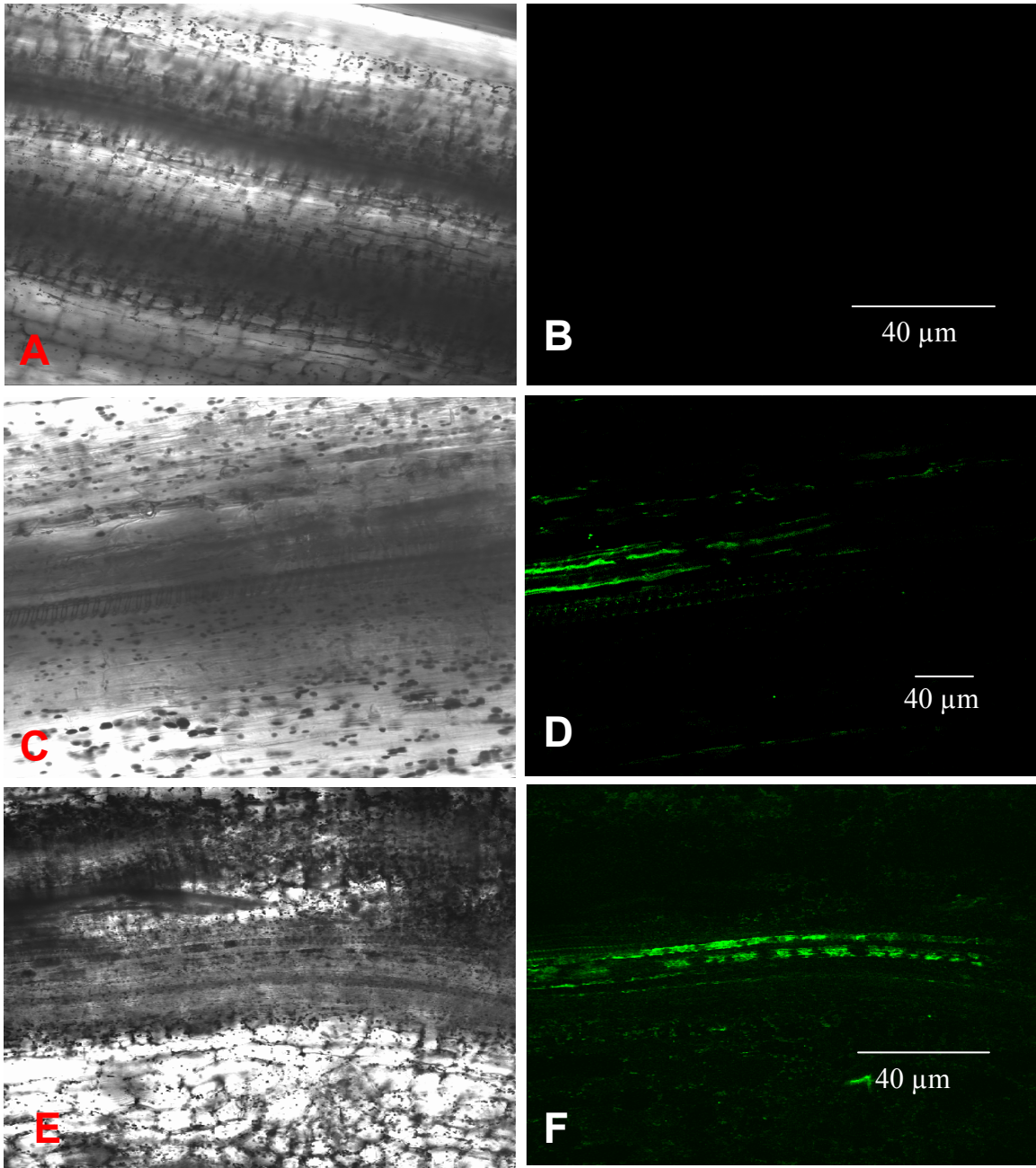


Fig. 8. Confocal micrographs of longitudinal sections of squash stems showing *Serratia marcescens* GFP-ZO1 and GFP-RO2 within xylem vessels. Plants were inoculated with: A, B, PBS; C, D, GFP-ZO1; E, F, GFP-RO2 inoculated plant. The two micrographs of each treatment are of the same field, obtained using regular light (A, C, E), or fluorescence optics (B, D, F).

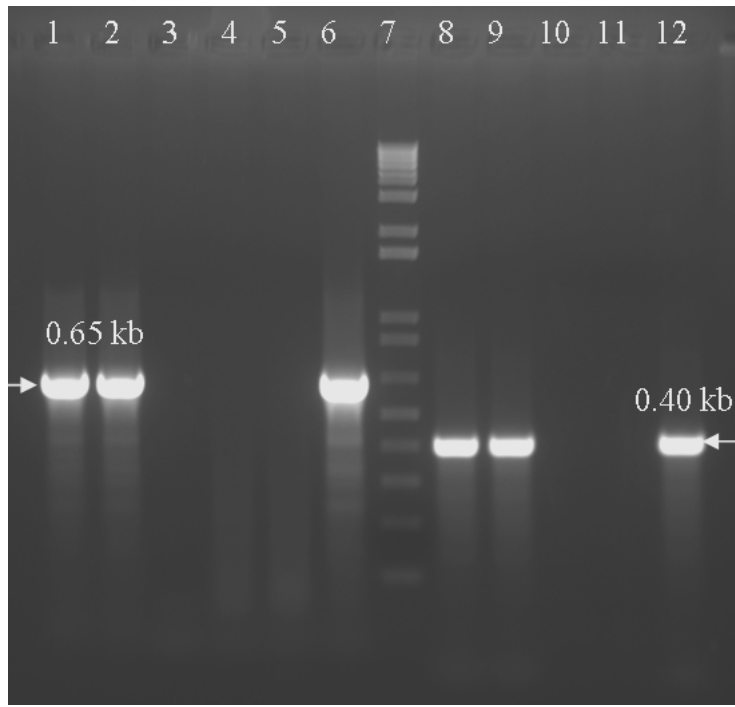


Fig. 9. PCR amplification of DNA from bacteria recovered from inoculated squash plants. Primers used GFPF and GFPR (Lanes 1-6); *Serratia marcescens* genomic target YV1 and YV4 (Lanes 8-12). Lanes 1 & 8, GFP-ZO1; lanes 2 & 9, GFP-RO2; lanes 3 & 10, PBS; lanes 4, ZO1-A; lane 5, RO2-A; lane 6, pMCS21; lane 7, 1 kb plus DNA marker; lane 11, blank; lane 12, ZO1-A.

CHAPTER IV

REDUCED VIRULENCE OF *SERRATIA MARCESCENS* ON SQUASH FOLLOWING FIMBRIAL GENE DISRUPTION

Abstract

Serratia marcescens, a cosmopolitan bacterium known primarily as an environmental microbe and a nosocomial human pathogen, causes cucurbit yellow vine disease (CYVD) in many cucurbit production areas in the U.S.A. The bacterial features responsible for pathogenicity in plants are not known. A fimbrial gene cluster identified in the chromosome of CYVD strains of *S. marcescens* and absent in non-CYVD strains was cloned in the fosmid FOSU1. Because fimbriae have been implicated in virulence of other pathogenic bacteria, we hypothesized that these appendages play a role in the interaction of *S. marcescens* with its cucurbit hosts. When a non-fimbriated bacterium, *E. coli* HB101, was transformed with FOSU1, fimbriae appeared on the transformants indicating that the fimbrial gene cluster in FOSU1 was functional. Two genes of the cluster, *fimA* and *fimH*, respectively encoding the major subunit of the fimbrial rod and the fimbrial adhesin, were chosen for disruption using the TargeTron® Gene Knockout System. Polymerase chain reaction and sequence analysis indicated that the two genes were disrupted at the desired target sites. *In vitro* growth rates in LB medium of the two mutants (designated ZO1-FimA⁻ and ZO1-FimH⁻) and of the wild type strain ZO1-A were indistinguishable. Transmission electron microscopy revealed few or no fimbriae on ZO1-FimA⁻. Although fimbrial numbers on the surfaces of the parent strain and ZO1-FimH⁻ were

similar, ZO1-FimH⁻ fimbriae were shorter than those of ZO1-A. Although symptoms caused by the mutants were similar to those caused by the parent strain, both ZO1-FimA⁻ and ZO1-FimH⁻ had significantly reduced virulence on ‘Lemon Drop’ yellow summer squash. Furthermore, these two mutants maintained their resistance to kanamycin *in planta* for at least 6 weeks in the greenhouse.

Introduction

Serratia marcescens is a motile, non-sporulating, gram-negative microbe that can colonize various niches such as water, soil, plants, insects, and vertebrates, including humans. In 1988, a new disease, cucurbit yellow vine (CYVD), was described in central Texas and Oklahoma (Bruton et al., 1995). This disease, which causes heavy losses to watermelon, pumpkin, cantaloupe and squash, is now known to occur in many U.S. cucurbit-production areas from Texas (Bruton et al., 1995) and Colorado (Bruton et al., 2003) to Illinois (Bruton et al., 1998) and Tennessee (Bost et al., 1999), and as far as north as Massachusetts (Wick et al., 2001). It is characterized by phloem discoloration, foliar chlorosis, wilting, and plant decline (Bruton et al., 2001). Previously, Zhang et al (Zhang et al., 2005) identified a cluster of fimbrial genes in the chromosome of CYVD-causing *S. marcescens* strains that was absent from the non-CYVD strains examined.

Fimbriae, also called pili, hair-like appendages that extend from the bacterial surface (Soto and Hultgren, 1999), are important in bacterial colonization and pathogenicity, mediating bacterial adherence to host tissues (Hultgren et al., 1996). For example, the fimbriae of *S. marcescens* facilitate the colonization of strain US5 in human urinary tract infections (Yamamoto et al., 1985). Fimbriae also act as a virulence factor for some phytopathogens; for example, they are involved in the infection of bean leaves by *Pseudomonas syringae* pv. *phaseolicola* (Romantschuk and Bamford, 1986), the adhesion of *Xanthomonas campestris* pv. *hyacinthi* to bean plants and enhanced entry of the pathogen into leaves (Van Doorn et al., 1994) and the polar attachment of *Xylella fastidiosa* to host xylem cells (Feil, et al., 2003). The role of fimbriae in the virulence or

pathogenicity of CYVD strains of *S. marcescens* to cucurbit plants is unclear. Since these bacteria colonize the vascular system (Bruton et al., 2001), fimbriae might help bacteria attach to plant cell walls, aggregate and form biofilms. Strong adhesion might inhibit bacterial movement through the plant.

A portion of the fimbrial gene cluster of *S. marcescens* CYVD strains is homologous to a region of fimbrial genes of the type I system of *Klebsiella pneumoniae* and *Escherichia coli* (Zhang et al., 2005). In the type I system, the fimbrial genes are clustered together (Hull et al., 1981) and have the same gene organization regardless of the genus. Among the genes within this cluster, *fimA* and *fimI* encode fimbrins, *fimG* and *fimF* encode adaptor proteins, *fimD* and *fimC* encode usher proteins, *fimH* encodes an adhesin, and *fimB* and *fimE* encode regulatory proteins (Schilling et al., 2001). Since the fimbrins make up the core of the fimbrial rod and its fibrillar tip, fimbrial construction might be prevented if the *fimA* gene were disrupted. In human infecting strains of *S. marcescens*, the adhesin FimH, located at the distal tip of the fimbriae, mediates both bacterial adherence and the invasion of epithelial cells (Martinez et al., 2000). The present study was designed to assess whether fimbriae play a role in *S. marcescens* infection and colonization of the cucurbit vascular system, or in the ability of the bacteria to cause disease in the plants. If our hypothesis, that *fimA* and/or *fimH* are important in the virulence of *S. marcescens* to cucurbit plants, is correct, then disruption of either or both of these genes should reduce the ability of the bacteria to induce yellow vine disease symptoms.

Efficient bacterial gene disruption in several bacteria has been reported using the TargeTron® Gene Knockout System (Karberg et al., 2001). In this system, mobile group II intron uses a mobility mechanism termed retrohoming, mediated by a ribonucleoprotein complex containing the intron-encoded reverse transcriptase and excised intron lariat RNA (Karberg et al., 2001). Compared to conventional methods, this system is reported to be more rapid and to have excellent site specificity and high efficiency (Frazier et al., 2003).

Materials and Methods

Bacterial strains and growth conditions. Tested bacteria, plasmids and primers are listed in Table 1. *E. coli* BL21 (DE3), obtained from Carol L. Bender (Oklahoma State University), was used to maintain plasmid pAR1219 (Matsuura et al., 1997). *E. coli* HB101, obtained from Cold Spring Harbor (New York), was used as a host for plasmid pACD4K-C (Greg Davis, personal communication). *S. marcescens* ZO1-A was isolated from CYVD-affected zucchini (*Cucurbita pepo*) as previously described (Bruton et al., 1998). *S. marcescens* RO2-A, also known as a rice endophyte IRBG502, obtained from P. Gyaneshwar (International Rice Research Institute, The Philippines) was used as a fimbriae-negative control because it lacks the chromosomal fimbrial gene cluster (Zhang et al., 2005). Bacteria were stored at -80°C in aliquots of 1.5 ml Luria-Bertani (LB) broth (Sambrook et al., 1989) containing 15% glycerol. For use in the experiments, bacteria were streaked onto LB or nutrient agar (Sambrook et al., 1989) and incubated overnight at 30°C for *S. marcescens* or at 37°C for *E. coli*.

Confirmation of the functional fimbriae gene cluster. Using suppressive subtractive hybridization, Zhang et al. (2005) identified a fimbrial gene cluster in the chromosome of *S. marcescens* CYVD strains that was absent in non-CYVD strains. To test whether this fimbrial gene cluster is functional, a fosmid containing the cluster (Zhang et al., 2005) was extracted from *E. coli* DH5 α (FOSU1) by alkaline lysis mini-prep (Sambrook et al., 1989) and transformed into *E. coli* HB101, a strain lacking fimbriae (Miller, 1972), using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, CA) at 1.8 Kv, 200 ohms and 25 μ F. Transformants were selected on LB agar plates containing chloramphenicol (Cm, 25 μ g/ml). A chloramphenicol-resistant colony, designated *E. coli* HB101 (FOSU1), was chosen for further tests. Overnight colonies of ZO1-A, RO2-A, *E. coli* HB101 and *E. coli* HB101(FOSU1) cells were placed into individual tubes of LB broth (containing 25 μ g/ml Cm for the transformant only) and grown without shaking at 30°C for *S. marcescens* or at 37°C for *E. coli*. About 1 μ l of bacterial culture from each sample was tested by colony PCR using primers FimAF and

FimAR, and FimHF and FimHR (Table 1). Electron microscopy was conducted as in a previous study (Mizunoe et al., 1988). Briefly, overnight bacterial cultures were pelleted (900 X g) and washed twice in 0.5 ml 2% ammonium acetate. The cells were fixed 1 min on a formvar grid and negatively stained 20 s with 2% phosphotungstate (pH 7.0). Samples were viewed using a JEOL 100CX ASID transmission electron microscope (JEOL Company, Japan) at 80 KV.

Mutation of the *fimA* and *fimH* genes. The plasmids and primers used in new plasmid constructs are shown in Table 1. Gene disruption was accomplished using the TargeTron® Gene Knockout System (Sigma-Aldrich, Inc., St. Louis, MO), following the manufacturer's protocol. Briefly, target sites within the known *fimA* (551bp) and *fimH* (381bp) sequences (Zhang et al., 2005) were chosen using the TargeTron algorithm (sigma-aldrich.com/targetronaccess). From the output design options, the target site with the lowest E-value within the region of the desired genes was selected. Polymerase chain reaction (PCR) was done using a GeneAmp PCR System 9600 (Perkin-Elmer Cetus, Norwalk, CT) thermal cycler under the following reaction conditions: 50 µl reaction mixtures containing approximately 20 ng intron PCR template (TargeTron® Gene Knockout System, Sigma-Aldrich), 25 µl GoTaq Master Green Mix (Promega, Madison, WI), 10 mM each of primers IBS and EBS1dA, and 4 mM each of EBS2A and EBS universal primers (Table 1). The reaction was subjected to a 30 s denaturation step at 95°C followed by 30 cycles at 94°C for 15 s, 55°C for 30 s and 72°C for 30 s. The reaction mixture was maintained at 72°C for 2 min and then placed at 4°C until analyzed on a 1.5% agarose gel. The expected 350 bp band was recovered using a Wizard® SV Gel and PCR Clean-Up System (Promega). The recovered product was double digested with *Hind*III and *Bsr*GI as follows: 15 µl purified PCR product (~3000 ng), 3 µl 10 X restriction enzyme buffer (Promega), 1 µl *Hind*III (10 U/µl), 1 µl *Bsr*GI (10 U/µl), and 10 µl water (molecular biology reagent, Sigma) to a total volume of 30 µl. The mixture was incubated at 37°C for 3 h and 60°C for 1 h and inactivated at 80°C for 20 min. The digested product was analyzed on a 1.5% agarose gel and the appropriate band recovered as above. Then 15 µl of the mixture, which included 2 µl pACD4K-C linear vector (66 ng),

0.2 μ l *Hind*III/*Bsr*GI digested intron PCR product (350 bp, ~12ng), 3 μ l 5X DNA ligation buffer (Invitrogen, Carlsbad, CA), 9.7 μ l water (molecular biology reagent grade, Sigma) and 0.1 μ l T4 DNA ligase (1U/ μ l), was incubated at 25°C for 1 h. Competent ZO1-A cells were prepared as in Sambrook et al (1989). A volume of 1 μ l of the ligated product was added to 50 μ l of competent ZO1-A cells and the mixture was incubated on ice for 10 min and pulsed at 2.5 Kv, 200 ohms and 25 μ F using a Bio-Rad Gene Pulser (BioRad Laboratories). A volume of 450 μ l of room temperature SOB Medium (0.5% yeast extract, 2.0% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, pH 7.0) was added immediately and the suspension incubated at 30 °C with shaking at 250 rpm for 2 h. ZO1-A transformants were selected on LB agar plates containing Cm (25 μ g/ml), 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal, 30 μ l of 20 mg/ml), and isopropyl- β -D-thiogalactopyranoside (IPTG, 30 μ l of 100 mM IPTG spread on plates). White colonies (successfully re-targeted) were chosen for further study. Helper plasmid pAR1219, encoding T7 polymerase, was extracted from *E. coli* by an alkaline lysis mini-prep (Sambrook et al., 1989). It was transformed into the ZO1-A transformants as described above. Since ZO1-A has resistance to ampicillin (Luo, unpublished data), carbenicillin was used to select the helper plasmid for the transformants. After 2 h incubation at 30°C in SOB, 100 ml of this transformation reaction mixture was transferred to 3 ml LB (containing 1% glucose, 25 μ g/ml Cm and 75 μ g/ml carbenicillin) at 30°C overnight. A volume of 40 μ l of the overnight culture was added to 2 ml LB (containing 1% glucose, 25 μ g/ml Cm and 75 μ g/ml carbenicillin) at 30°C to an OD₆₀₀ of approximately 0.2. IPTG was added to a final molarity of 500 μ M and the mixture was incubated at 30°C for 30 min with shaking as above. The culture was immediately centrifuged at 16000g for 1 min and the pellet was resuspended in 1.0 ml of LB containing 1% glucose (no Cm or carbenicillin) and incubated at 30°C for 1 h with shaking. Transformants were selected on LB containing 25 μ g/ml kanamycin (Kan). They were designated ZO1-FimA⁻ and ZO1-FimH⁻ for *fimA*⁻ and *fimH*⁻ mutants, respectively.

Confirmation of gene disruption and sequence analysis. About 10 colonies were selected randomly from the LB-Kan plates for colony PCR. Each amplification was performed in a 25 μ l reaction mixture containing one colony as template, 1x Gitshier buffer (Gibco BRL, Carlsbad, CA), 100 μ M of each dNTP, 5 mM of each primer, 2 mM MgCl₂ (Gibco BRL), and 5 U *Taq* DNA polymerase (Gibco BRL) per reaction. This mixture was placed in a GeneAmp PCR System 9600 thermal cycler and subjected to a 5 min denaturation step at 95 °C followed by 35 cycles at 95°C for 45 s, 56°C for 30 s and 72°C for 3 min. The reaction mixture was next held at 72°C for 10 min and then placed at 4°C until analyzed on a 1.5% agarose gel. The PCR products were purified using a Wizard® SV Gel and PCR Clean-Up System (Promega) and sequenced by the Oklahoma State University Recombinant DNA/Protein Resource Facility. The sequences were aligned using SDSC Biology Workbench 3.2 (<http://workbench.sdsc.edu/>).

Growth of the transformants and wild type ZO1-A. Overnight colonies of wild type ZO1-A, ZO1-FimA⁻, and ZO1-FimH⁻ cells were grown individually in 5 ml LB broth (containing 25 μ g/ml Kan for the mutants only) with shaking (250 rpm) at 30°C. Samples (0.1 ml) were taken every 3 h, and serially diluted in sodium phosphate buffer (0.01 M, containing 0.15 M NaCl, pH 7.0, PBS) and spread onto LB agar plates; colony counts were made after 24 h incubation at 30°C.

Electron microscopy. Overnight colonies of ZO1-A, ZO1-FimA⁻, and ZO1-FimH⁻ cells were placed into individual tubes of LB broth (containing 25 μ g/ml Kan for the mutants only) and grown without shaking at 30°C overnight. Samples were prepared as above but viewed with a JEM 2100 (JEOL Company, Japan) transmission electron microscope. Fimbrial dimensions were averaged from measurements of at least 10 fimbriae from at least 10 cells.

Pathogenicity tests. Summer squash var ‘Lemon Drop’ seedlings (*Cucurbita pepo*) were grown 4-7 d in a growth chamber at 30°C or in the greenhouse at 22 to 35°C of night-day variation, to the closed cotyledon stage. At the same time, *S. marcescens* ZO1-A, ZO1-FimA⁻ and ZO1-FimH⁻ were grown on nutrient agar with appropriate antibiotics (25 μ g/ml) at room temperature for 4 d. Bacterial

lawns were suspended in PBS (pH 7.0) to achieve a cell number of about 1×10^{11} cells/ml (determined using a UV/vis spectrophotometer at OD₄₂₀) (Bruton et al., 2003). For plant inoculations, a multi-pronged “inoculating fork” (Bruton et al., 2001) was dipped into the bacterial suspension, picking up a drop of about 10 μ l. The abaxial sides of squash cotyledons were jabbed about 15-20 times each with the fork such that bacteria were introduced into the interior tissues of the growing point. Plants inoculated with PBS served as controls. For each treatment, 100 squash plants were inoculated and then placed in the greenhouse for disease development. The test was performed three times and the symptoms and percentage of diseased plants were recorded weekly. The squash crowns were examined using light microscopy and Dienes’ stain (Bextine et al., 2001) for vascular tissue necrosis, and bacterial presence was confirmed by PCR as previously described (Bextine et al., 2001).

Bacterial titer and recovery from inoculated plants. Five squash plants from each treatment were randomly selected for bacterial isolation. Each week after inoculation, two stem slices, each about 0.5 g, were excised; one at the inoculation site and a second from the stem about 3 cm below the inoculation site. These samples were surface-sterilized in 70% ethanol for 30 s and 10% bleach for 1 min and then rinsed three times in sterile distilled water. The tissue was macerated using a plastic pestle in 1.5 ml microtubes, serially diluted in PBS and plated on LB agar (for wild type ZO1-A and the control) or on LB containing 25 μ g/ml Kan (for the two transformants). To confirm the identity of the recovered bacteria, primers a79F and a79R (Zhang et al., 2005), which are specific for *S. marcescens* CYVD strains, and primers specific for the mutants, FimAF and FimAR (for ZO1-FimA⁻), and FimHF and FimHR (for ZO1-FimH⁻), were used for colony PCR.

Results

The functional gene cluster of FOSU1. When the fosmid FOSU1, containing the fimbrial gene cluster, was transformed into *E. coli* HB101, a non-fimbriated strain (Fig. 1 A), chloramphenicol-resistant colonies were obtained on the selective medium. Colony PCR (using primers FimAF and FimAR, FimHF and FimHR) indicated that *fimA* (encoding the major fimbrial rod) and *fimH* (encoding an adhesin at the fimbrial tip) were present in *E. coli* HB101 (FOSU1). Transmission electron microscopy revealed fimbriae on the surface of *E. coli* HB101 (FOSU1) (Fig. 1 B), indicating that FOSU1 contained a functional fimbrial gene cluster. *S. marcescens* ZO1-A (a CYVD strain) had numerous hair-like fimbriae on the surface (Fig. 1 C), while RO2-A, which lacks the fimbrial gene cluster (31), lacked such appendages (Fig. 1 D).

***fimA* and *fimH* gene disruption.** We chose L1.1trB, an *E. coli*-based selection system having a group II intron, derived from *Lactococcus lactis*, to disrupt the two fimbrial genes because *S. marcescens*, like *E. coli*, belongs to the family *Enterobacteriaceae*. For each gene three unique PCR primers, (IBSA, EBS2A, and EBS1dA for *fimA*; IBSH, EBS2H, and EBS1dH for *fimH*, as shown in Table 1), were used to retarget the intron to insert at the specific site. The intron introduced by the primer-mediated mutation is about 350 bp. After PCR modification of the intron target site, pACD4K-C was transformed into ZO1-A competent cells and transformants were selected on LB plates containing Cm. In this system, because the T7 RNA promoter is inserted upstream of the group II intron, it is critical that the recipient strain express T7 RNA polymerase to induce the intron-mediated gene disruption. Since *S. marcescens* ZO1-A has no T7 RNA polymerase gene, the helper plasmid pAR1219, which contains the polymerase gene (Davanloo et al., 1984), was required to disrupt ZO1-A genes. Compared to co-transformation of the donor plasmid pACD4K-C and the helper plasmid pAR1219, it was more efficient to first disrupt the target gene by transformation of the donor plasmid pACD4K-C into ZO1-A, selecting for Cm resistant colonies, and to then transform the helper plasmid into those lines. After induction by IPTG,

about 20 colonies (insertion frequency of the intron into ZO1-A about 2×10^{-7}) grew on each Kan LB plate. To confirm fimbrial gene disruption, primers specific for the sequences flanking the insertion site were used in colony PCR. Using *fimA*-specific primers FimAF and FimAR, the amplicon from the parent strain was 0.5 Kb, while that from ZO1-FimA⁻ was about 2.5 Kb (Fig. 2 B). Similarly, using *fimH*-specific primers FimHF and FimHR, the amplicon from the parent strain was 0.35 Kb, while that from ZO1-FimH⁻ was about 2.35 Kb (Fig. 2 C). Based on colony PCR (20 colonies per mutant), 80% of ZO1-A transformants (Kan^R) underwent disruption of *fimA* and 85% of *fimH*, and a fragment of about 2 kb inserted into each gene. To confirm that the disruption was due to intron insertion, the mutants' PCR products were sequenced using the fimbrial primers FimAF/FimAR and FimHF/FimHR. Alignment results confirmed that the 2 kb fragment was from the intron and that it had inserted into the desired target site (data not shown).

Comparison of the mutants and wild type ZO1-A. Overnight colonies of ZO1-FimA⁻ and ZO1-FimH⁻ were similar in appearance to those of the parent strain. All were off-white in color and circular on LB agar. ZO1-A and ZO1-FimA⁻ colonies averaged ~0.8mm (0.8 ± 0.18 mm) in diameter while those of ZO1-FimH⁻ averaged ~0.5mm (0.5 ± 0.16 mm) in diameter. Grown in LB broth, ZO1-A reached its highest titer (about 5.3×10^9 CFU/ml) at 24 h while ZO1-FimA⁻ and ZO1-FimH⁻ did so (reaching maximal titers of 4.2×10^9 and 3.9×10^9 CFU/ml, respectively) only after 30 h (Fig. 3). However, a *t* test ($p=0.05$) found these differences in cell numbers and growth rates to be insignificant, indicating that fimbrial gene disruption did not significantly affect bacterial growth under these conditions.

Electron microscopy. To determine if *fimA* and *fimH* disruption affected fimbrial structure, bacterial cells were examined by transmission electron microscopy. Numerous fimbriae were present on the surface of most (94.5% or 86/91) of the wild type ZO1-A and most (95.2% or 59/62) of the ZO1-FimH⁻ observed (Fig. 4 A, C). In contrast, very few (12.5% or 7/56) of ZO1-FimA⁻ cells had fimbriae. Though the number of fimbriae on the surface of ZO1-FimH⁻ cells

was similar to that on the wild type cells, the appendages of this mutant were shorter (177.5 ± 13.8 nm; Fig. 4 C) than those of the parent strain (239.0 ± 20.2 nm; Fig. 4 A). No difference in fimbrial width was observed between the FimH⁻ mutants and the parent strain (fimbrial width on both strains ranged from 2.7-5.1 nm).

Pathogenicity to squash plants. Foliar symptoms first appeared 2 wk after inoculation of ZO1-FimA⁻, ZO1-FimH⁻ and ZO1-A into squash seedlings, and became more severe with time. Regardless of the bacterial strain inoculated, symptoms were indistinguishable and included stunting and leaf cupping. Control plants had no disease symptoms. However, the severity of disease differed depending on the bacterial strain, with ZO1-A causing significantly more stunting than either mutant (Figs. 5, 6). Though the heights of all the inoculated plants were similar within the first three wk, there was a significant difference in plant height beginning at wk 4 among the plants inoculated with the parent strain, ZO1-FimA⁻, ZO1-FimH⁻, or PBS (Fig. 6). Disease incidence also was affected by the bacterial strain; 42.8 ± 6.3 % of squash plants inoculated with ZO1-A showed foliar symptoms while only $22.3 \pm 1.8\%$ of those inoculated with ZO1-FimA⁻ and $21.7 \pm 2.3\%$ of those inoculated with ZO1-FimH⁻ exhibited disease symptoms. Dienes' stain results were positive in about 48.9 ± 1.1 %, 27.6 ± 1.8 %, and $25.1 \pm 0.9\%$ of squash seedlings inoculated with ZO1-A, ZO1-FimA⁻ and ZO1-FimH⁻, respectively. All symptomatic plants were Dienes' stain positive. When all inoculated plants were assessed for presence of *S. marcescens* by PCR, about $54.6 \pm 2.7\%$, $30.9 \pm 1.7\%$, and $29.4 \pm 2.5\%$ were positive for ZO1-A, ZO1-FimA⁻ and ZO1-FimH⁻, respectively.

Bacterial population and recovery from inoculated plants. As shown in Fig. 7, the bacterial titer increased in the first five wk in the PBS-inoculated control plants. In the plants inoculated with *S. marcescens*, regardless of whether they were wild type or mutants, the bacterial titer decreased in the first wk, then increased in the following two wk. The bacterial population reached the highest level ($\sim 4 \times 10^6$ cells/g tissue) after 3-4 wk, which was significantly different that of control plants. There was no significant difference in cell numbers between the

two mutants (ZO1-FimA⁻ and ZO1-FimH⁻) or between the mutants and the wild type strain. ZO1-FimA⁻ and ZO1-FimH⁻ were re-isolated from the inoculated plants on LB-kan plates, indicating that the cells retained the kanamycin resistance gene for at least 6 wk *in planta* without selection. The results of PCR with fimbrial primers (FimAF and FimAR or FimHF and FimHR) revealed that the group II intron was still present in these genes (data not shown), demonstrating that the disruption by the group II intron was stable at least six wk without selection.

Discussion

This is the first report that the TargeTron® Gene Knockout System, an *E. coli*-based selection system (Zhong et al., 2003), can be used to disrupt the genes of *S. marcescens*. Because ZO1-A has no T7 RNA polymerase gene, a helper plasmid, pAR1219, is necessary for successful gene disruption using this system. This finding extends the range of bacteria in which the TargeTron® Gene Knockout System has been shown to work. Compared to conventional transposon mutagenesis, this system is more rapid and has excellent site specificity and high efficiency, consistent with a previous report (Frazier et al., 2003).

In this study, *fimA* and *fimH* were chosen as knockout targets because of their location in a gene cluster that is present in cucurbit-infecting strains of *S. marcescens*, but absent in a closely related, non-pathogenic, plant endophytic strain (Zhang et al., 2005). In other bacteria, these genes play important roles in the type I fimbrial gene cluster (Hull et al., 1981) and in bacterial pathogenicity, with *fimA* encoding fimbrins, the major fimbrial structural elements, and *fimH* encoding an adhesin at the fimbrial tip that mediates bacterial adherence and cell invasion (Martinez et al., 2000). *fimH* is highly conserved within the *Enterobacteriaceae* (Levine et al., 1983).

Transmission electron microscopy (TEM) demonstrated that fosmid FOSU1, carrying *S. marcescens* ZO1-A fimbrial genes, contains a functional fimbrial gene cluster because fimbriae were observed on *E. coli* HB101 (FOSU1) (Fig. 1 B) but

not on wild type *E. coli* HB101 cells (Fig. 1 A). TEM also showed an extensive array of fimbriae on the surface of the wild type strain ZO1-A (Fig. 1C and Fig. 4A), consistent with a previous report (Adegbola and Olds, 1982) that most tested *S. marcescens* strains have multiple fimbriae. Furthermore, the length and width of ZO1-A's fimbriae are similar to those reported for other strains (Jingushi et al., 1987). The fact that no, or very few, fimbriae were visible on ZO1-FimA⁻ may reflect that FimA is the major structural subunit of the fimbrial rod (Schilling et al., 2001). Our result is similar to the finding by Feil et al (2003) that disruption in *X. fastidiosa* of *fimA* or *fimF* reduced the number of fimbriae. In the absence of a functional *fimA* gene, the process of fimbrial assembly may be interrupted, limiting the number of fimbriae formed (Fig. 4 B). In our study, the finding that a few (7/56) ZO1- FimA⁻ cells had fimbriae may be due to a small rate of reversion to wild type. Because the TargeTron group II intron is inserted into the sense DNA strand, there is a greater chance of reversion than if its insertion had been in the other strand (Greg Davis, Sigma-Aldrich, Inc., personal communication).

The number and width of fimbriae on ZO1-FimH⁻ were similar to those in wild type ZO1-A, consistent with previous results (Minion et al., 1986). The fact that ZO1- FimH⁻ fimbriae, when present, are significantly shorter than those of the parent strain is consistent with the finding of Jones et al (1995) that mutation of *fimH* of *E. coli* reduced the average fimbrial length from 16 nm to 3 nm. *fimH* encodes the adhesin FimH, which locates at the distal tip of the fimbriae, and it is possible that the fimbriae of ZO1- FimH⁻ are shorter because they lack the tip. There may be several copies of *fimH* in ZO1-A, as in *E. coli* (Jones et al., 1995). Why a small number of cells of wild type ZO1-A and of ZO1-FimH⁻ had no fimbriae, resembling ZO1-FimA⁻, is not clear. It is possible that the fimbriae were removed by washing or were broken during the preparation because broken fimbrial fragments could be seen unattached and scattered on the grids (Fig. 4). These results suggest that disruption of *fimA* has a significant effect ($p < 0.01$) on fimbrial formation and that mutation of *fimH* had a significant impact ($p < 0.01$) on fimbrial length.

Growth rates of *S. marcescens* were affected by mutations in *fimA* and *fimH*. Under our experimental conditions, the highest bacterial titers were reached later for ZO1-FimA⁻ and ZO1-FimH⁻ (30 h *in vitro* and 4 wk *in planta*) than for the wild type (24 h *in vitro* and 3 wk *in planta*).

The maximum *in planta* bacterial titers recorded were similar in the wild type and mutant treatments, when plant extracts were spread on agar plates and bacterial colonies enumerated. However, a significantly ultimate bacterial titer was recorded in control plants inoculated with PBS. It is possible that vascular puncture by the inoculating fork results in colonization of the plant interior by whatever bacteria were present on the surface of the plant at the time of inoculation, and that such bacteria (which may be either natural epiphytes or the prepared *S. marcescens* inoculum or both). It is logical to suspect that the bacteria enumerated in the wild type and mutant treatments are, at least predominantly, ZO1-A, ZO1-FimA⁻ and ZO1-FimH⁻ but confirmation would require quantitative PCR or ELISA on each of the bacterial populations.

fim gene disruption reduced the virulence of *S. marcescens* on squash. ZO1-A, ZO1-FimA⁻ and ZO1-FimH⁻ caused similar symptoms (stunting, leaf cupping, and petiole shortening) but symptoms on plants infected with either mutant were less severe than those on ZO1-A infected plants (Fig. 5). The incidence of disease in inoculated test plants depended on the bacterial strain as well. Compared to squash plants inoculated with the wild type ZO1-A, of which 42.8 ± 6.3 % exhibited macroscopic symptoms, only $22.3 \pm 1.8\%$ of ZO1-FimA⁻ and $21.7 \pm 2.3\%$ of ZO1-FimH⁻ inoculated plants were symptomatic. Dienes' stain, developed as a specific stain for animal mycoplasma colonies on agar (Dienes et al., 1948), was used for detection of mollicute infection of plant phloem sieve elements by Deeley et al (1979). Although Dienes' staining appeared to be slightly more sensitive than visual assessment, showing about 48.9 ± 1.1 %, 27.6 ± 1.8 %, and $25.1 \pm 0.9\%$ infection in plants inoculated with ZO1-A, ZO1-FimA⁻ and ZO1-FimH⁻, respectively, this sensitivity difference was not statistically significant. These results are consistent with those of a previous study in which the phloem of all CYVD symptomatic squash plants was Dienes' stain positive

(Bextine et al., 2001). PCR was more sensitive than either symptom assessment or Dienes' staining, showing infection in about $54.6 \pm 2.7\%$, $30.9 \pm 1.7\%$, and $29.4 \pm 2.5\%$ of plants inoculated with ZO1-A, ZO1-FimA⁻, and ZO1-FimH⁻, respectively.

Why *fim* gene disruption reduced the virulence of *S. marcescens* to squash is not known. It is possible that fully functional fimbriae are necessary for the bacteria to adhere to host plant cells and/or to form biofilms, activities known to be important in pathogenicity in some other systems (Walker et al., 2004). Future work will be needed to assess the role of *fim* genes in these activities in *S. marcescens*' invasion and disease development in cucurbit plants.

Interestingly, there was no significant difference in disease incidence or severity in plants inoculated with ZO1-FimA⁻ and ZO1-FimH⁻. Disruption of *fimA*, the basal rod of fimbriae, prevented the assembly of fimbriae, leading to no fimbriae on the mutant cell surface (Fig. 4 B). It is possible in both *fimA* and *fimH* mutants fail to produce a functional adhesin, FimH. FimH may be necessary for *S. marcescens* to attach to host plant cells, leading to an indistinguishable disease incidence or severity in plants inoculated with these two mutants. These results indicate that fimbriae are virulence factors rather than pathogenicity determinants, a conclusion consistent with that of a previous study (Feil., 2003) in which *fimA* and *fimF* (as *fimH* of this test) gene disruption in *X. fastidiosa* had no effect on pathogenicity to grapes.

In the varied interactions between bacterial pathogens and their host plants, fimbriae may have evolved to play different roles. Further study is needed to clarify the ability of FimA⁻ and FimH⁻ mutants of *S. marcescens* to adhere to other bacterial cells as well as to those of their plant hosts and insect vectors, and to assess their movement and colonization in plants and insects. Ultimately, such activities are likely to be relevant to the occurrence of a fimbrial gene cluster in CYVD strains, but not in non-CYVD strains, of the versatile microbe, *S. marcescens*.

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Table 1. Bacteria, plasmids and primers used in this study

Strains	Relevant characteristics or primer sequence	Source or reference
ZO1-A	<i>Serratia marcescens</i> wild type CYVD strain	Bruton et al., 1998
ZO1-FimA ⁻	ZO1-A <i>fimA</i> mutant, kanamycin resistance	This study
ZO1-FimH ⁻	ZO1-A <i>fimH</i> mutant, kanamycin resistance	This study
RO2-A	<i>Serratia marcescens</i> rice endophyte	Gyaneshwar et al., 2001
<i>E. coli</i> DH5 α (FOSU1)	F- ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17</i> (rk-, mk+) <i>phoA supE44</i> <i>thi-1 gyrA96 relA1 tonA</i> , FOSU1	Zhang et al., 2005
<i>E. coli</i> BL21(DE3)	<i>hsdS gal</i> (λ <i>clts857 ind1 Sam7</i> <i>nin5 lacUV5 T7 gene 1</i>)	Sambrook et al., 1989
<i>E. coli</i> HB101	<i>supE44 supE58 hsdS3</i> (<i>r_B⁻m_B⁻</i>) <i>recA13</i> <i>ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</i>	Sambrook et al., 1989
<i>E. coli</i> HB101 (FOSU1)	<i>supE44 supE58 hsdS3</i> (<i>r_B⁻m_B⁻</i>) <i>recA13</i> <i>ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</i> , FOSU1	This study
Plasmids		
FOSU1	Type I fimbrial gene cluster	Zhang et al., 2005
pACD4K-C	T7 promoter, kanamycin RAM marker, td group I intron, LtrA ORF, T1/T2 transcriptional terminator, chloramphenicol resistance	TargeTron Gene Knockout System (Sigma-Aldrich)
pAR1219	lac UV5 promoter T7 polymerase ampicillin and carbicillin resistance	TargeTron Gene Knockout System (Sigma-Aldrich)
Primers		
YV1	5'- GGGAGCTTGCTCCCCGG	Melcher et al., 1999
YV4	5'- AACGTC AATTGATGAACGTATTAAGT	Melcher et al., 1999
a79F	5'-CCAGGATACATCCCATGATGAC	Zhang et al., 2005
a79R	5'-CATATTACCTGCTGATGCTCCTC	Zhang et al., 2005
FimAF	5'-ACTGCTAACCTGTCCCATGC	This study
FimAR	5'-TGCTGTCAATGCTGAGAACC	This study
FimHF	5'-TATCTCGGAAAGGCTGGAGA	This study
FimHR	5'-GCTTCCTATGCACGAACCAC	This study
EBS universal	5'-CGAAATTAGAACTTGC GTTCAGTAAAC	This study
EBS1dA	5'-AGATTGTACAAATGTGGTGATAACAGATA AGTCGCGCTGGCTAACTTACCTTTCTTTGT	This study
IBSA	5'-AAAAAAGCTTATAATTATCCTTAGGTAAC GCGCTGGTGCGCCAGATAGGGTG	This study
EBS2A	5'-TGAACGCAAGTTTCTAATTTTCGATTTTACC TCGATAGAGGAAAGTGTCT	This study
EBS1dH	5'-CAGATTGTACAAATGTGGTGATAACAGAT AAGTCGATCCATCTAACTTACCTTTCTTTGT	This study
IBSH	5'-AAAAAAGCTTATAATTATCCTTATCACCCG ATCCAGTGCGCCAGATAGGGTG	This study
EBS2H	5'-TGAACGCAAGTTTCTAATTTTCG GTTGGTGA TCGATAGAGGAAAGTGTCT	This study

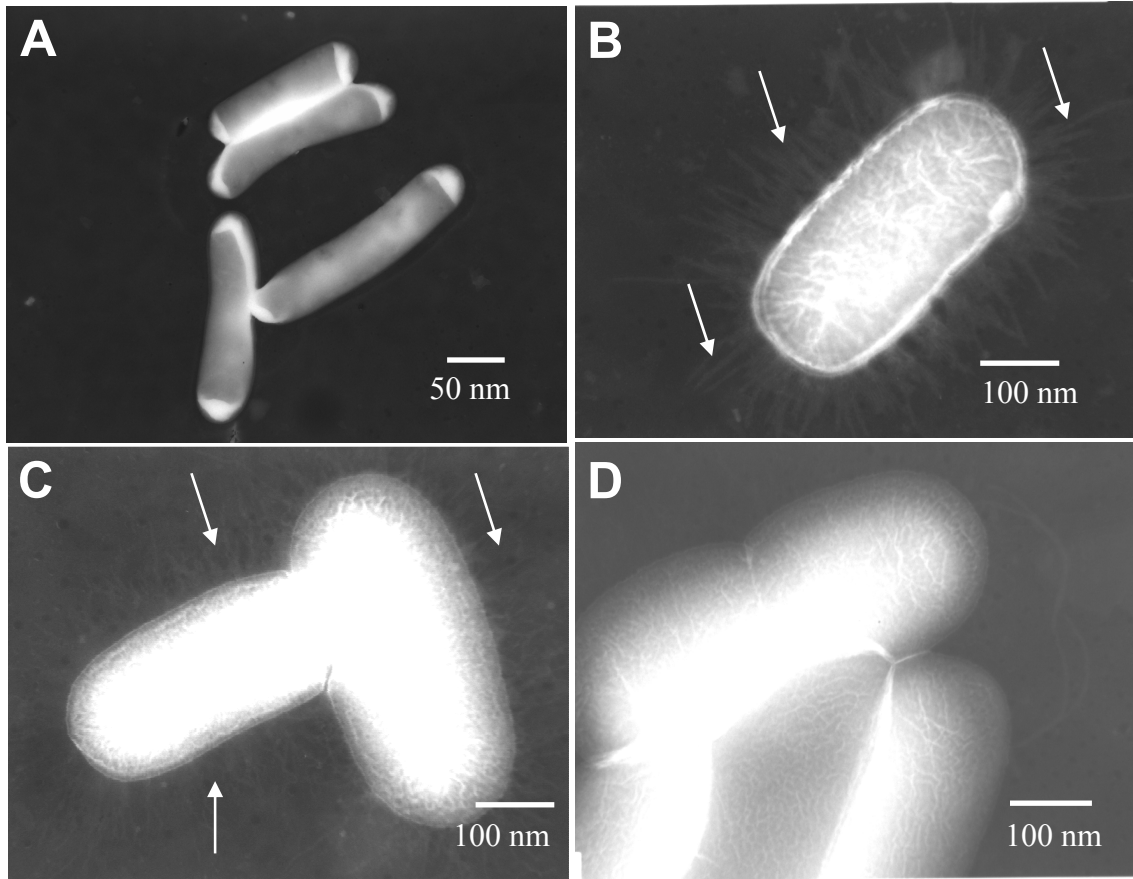


Fig. 1. Transmission electron micrographs showing fimbriae on bacterial cell surfaces. A, *Eschechichia coli* HB101; B, *E. coli* HB101 (FOSU1); C, *Serratia marcescens* ZO1-A; D, *S. marcescens* RO2-A. Arrows indicate fimbriae.

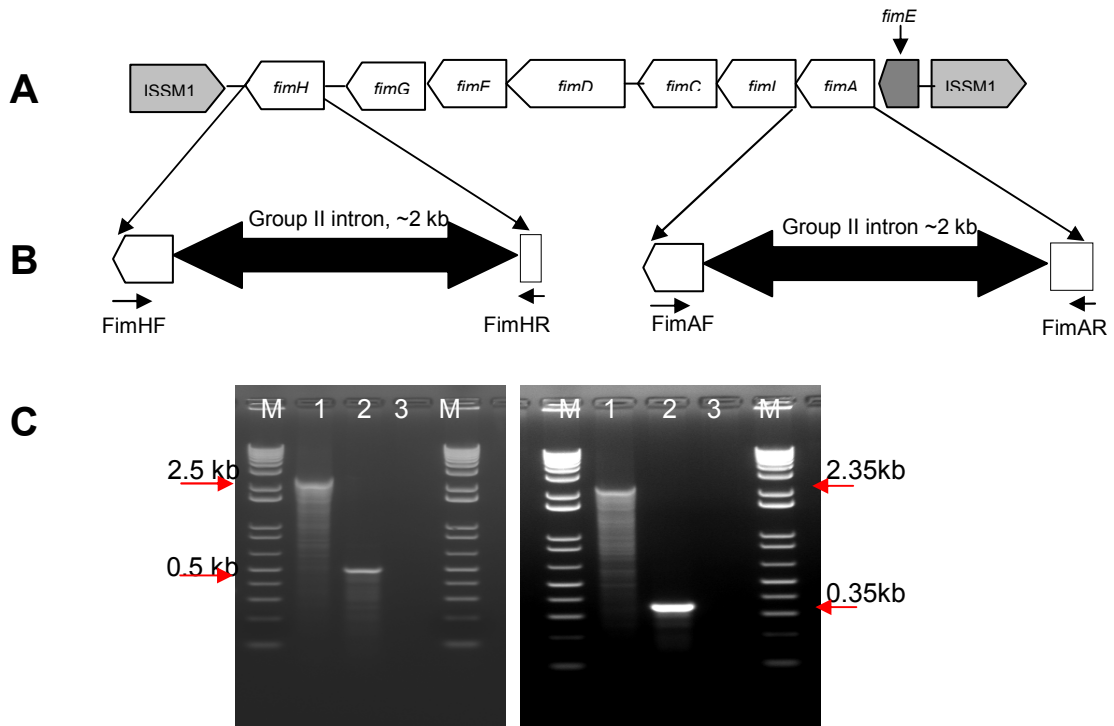


Fig. 2. (A) *Serratia marcescens* ZO1-A fimbrial gene cluster, diagram modified from Zhang et al. (34). (B) Illustration of group II intron insertion into *fimH* of mutant ZO1-FimH⁻ and *fimA* of mutant ZO1-fimA. (C) Left panel: confirmation of *fimA* disruption by PCR with primers FimAF and FimAR. Lane M: 1Kb plus DNA ladder; Lane 1: ZO1- FimA⁻; Lane 2: wild type ZO1-A; Lane 3: RO2-A. Right panel: confirmation of *fimH* disruption by PCR with primers FimAH and FimAR. Lane M: 1Kb plus DNA ladder; Lane 1: ZO1- FimH⁻; Lane 2: wild type ZO1-A; Lane 3: RO2-A.

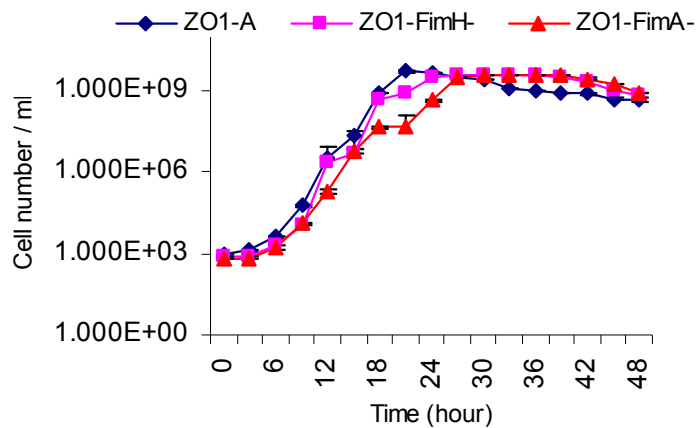


Fig. 3. Growth of *Serratia marcescens* ZO1-A, ZO1- FimA⁻ and ZO1-FimH⁻ in LB medium, Error bars are \pm S.D.

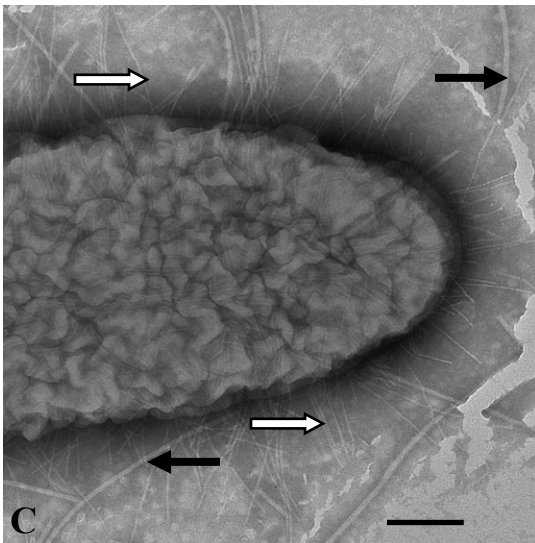
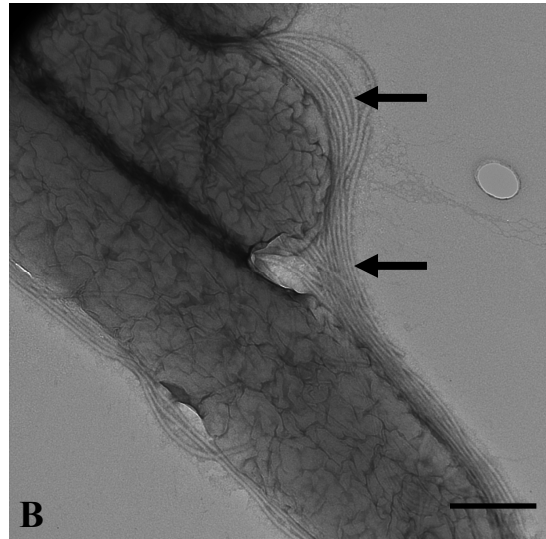
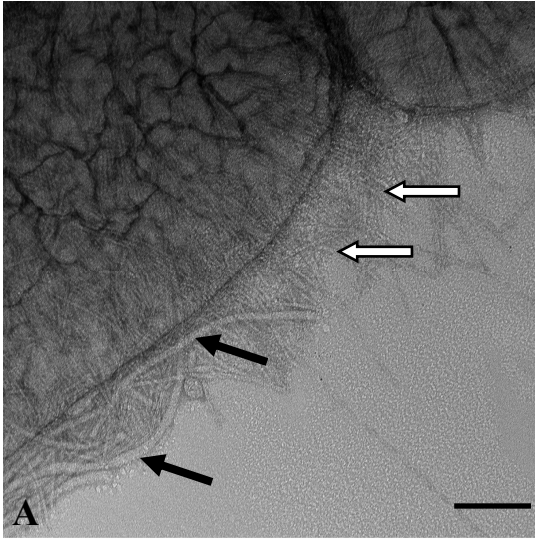


Fig. 4 Transmission electron micrographs of fimbriae on *Serratia marcescens*. Cells grown in LB broth without shaking at 30°C. A, ZO1-A; B, ZO1-FimA⁻; C, ZO1-FimH.
 ⇨ Fimbriae → Flagellae
 Bars, 100nm.

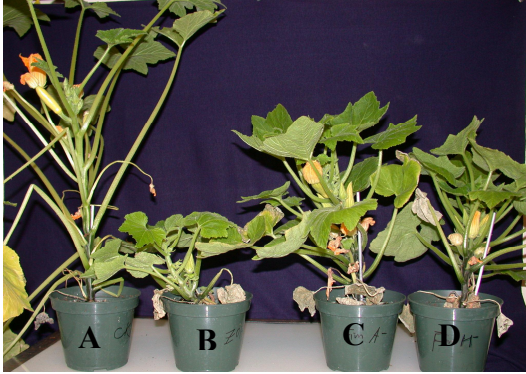


Fig. 5 Squash plants inoculated with two *Serratia marcescens* fimbrial mutants or with the parent strain, ZO1-A, 4 wk after inoculation. Inoculum: (A), PBS; (B), ZO1-A; (C), ZO1-FimA⁻; (D), ZO1-FimH⁻.

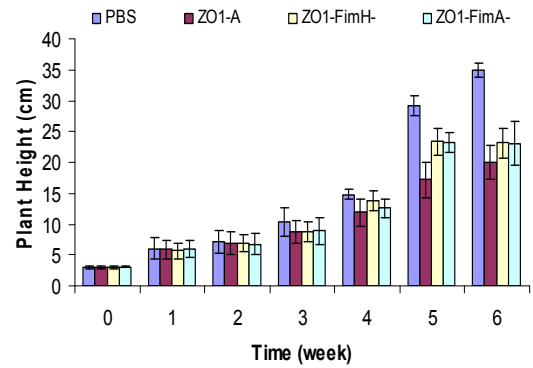


Fig. 6 Squash plant height at different times after inoculation with *Serratia marcescens* ZO1-A, ZO1-FimA⁻, ZO1-FimH⁻, or PBS. Five plants from each treatment were selected randomly for evaluation every week. Error bars are \pm S.D.

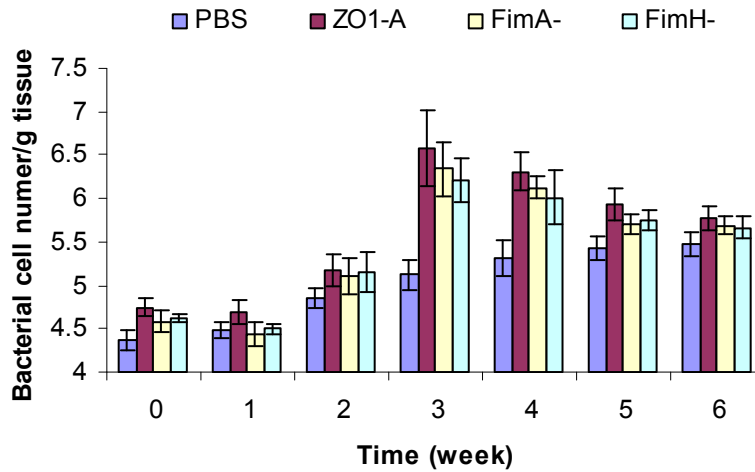


Fig. 7. Bacterial titer in squash plants inoculated with *Serratia marcescens* ZO1-A, ZO1-FimA⁻, ZO1-FimH⁻ or PBS. Bacteria were recovered from stems, three cm below the inoculation site, of five randomly selected plants from each treatment every week. Error bars are \pm S.D.

CHAPTER V

HEMOLYSIN AND CHITINASE: PUTATIVE VIRULENCE FACTORS OF CYVD STRAINS OF *SERRATIA MARCESCENS*

Abstract

Serratia marcescens, the causal agent of cucurbit yellow vine disease (CYVD), can colonize various ecological niches and produce many pathogenicity or virulence factors, including adhesins, proteases, cytotoxins, hemolysin, chitinases, siderophores and lipopolysaccharides. Some types of bacterial virulence mechanisms are conserved during evolution. Known virulence factors, including hemolysin and chitinase, were analyzed by BLASTN or TBLASTN in GenBank and the *S. marcescens* Db11 database. Primers were designed from hemolysin and chitin-related conserved sequences. Hemolysin and chitinase activities were tested on blood agar and chitin plates, respectively. Southern, northern or reverse-transcription PCR were performed to identify the genes. CYVD-strains ZO1-A, WO1-A, PO1-A and CO1-A of *S. marcescens* had hemolysin gene B (*shlB*) but lacked hemolysin gene A (*shlA*), and did not hemolyse red blood cells. The same strains had chitinase genes *chiA*, *chiB* and *chiC*, but demonstrated no chitinase activity on chitin agar. All of the tested non-CYVD strains (90-166, RO1-A, RO2-A, RO3-A, HO1-A, HO2-A, Db11, and ATCC13880) had hemolysin genes *shlA* and *shlB*, and chitinase genes *chiA*, *chiB* and *chiC*. Strains RO1-A, RO2-A, RO3-A, HO1-A, and HO2-A had both hemolysin and chitinase activities on test plates, while the remainder of the non-CYVD strains had chitinase function but lacked hemolysin activity.

Introduction

The causal agent of cucurbit yellow vine disease (CYVD), *Serratia marcescens*, is a cosmopolitan, gram-negative microbe that can be isolated from diverse niches, including soil (Pares M., 1964), water (Ajithkumar et al., 2003), insects (Bucher G. E. 1960), plants (Lukezic et al., 1982; Wei et al., 1996; Gyaneshwar et al., 2001), invertebrates, and humans (Wheat, 1951).

CYVD strains of *S. marcescens*, artificially introduced into different plant species including cucurbits, tobacco, onion and carrot, cause adverse symptoms that varied with the hosts (Chapter II). *S. marcescens* strains from non-plant niches also caused symptoms in these plants after experimental inoculation, but their interactions with the plant hosts differed from those of CYVD strains (Chapter II). In nature, phytopathogenic strains must effectively invade the plant and survive plant defense reactions as well as competition from other microbes. Pathogenicity or virulence factors, such as adhesins, proteases, cytotoxins, hemolysin, chitinases, siderophores and lipopolysaccharides have already been identified in *S. marcescens* (Chapter I). For instance, fimbriae, also called pili, hair-like appendages that extend from the bacterial surface (Sambrook et al., 1989), are important in bacterial colonization and pathogenicity, mediating bacterial adherence to host tissues (Hull et al., 1981).

Almost all strains of *S. marcescens* secrete a hemolysin, responsible for lysis of human and animal erythrocytes and the release of inflammatory mediators from leukocytes (Braun et al., 1993). Hemolytic activity is mediated by two proteins: ShlA and ShlB. *ShlA* encodes the hemolysin and *shlB* encodes an outer membrane protein required for the secretion of hemolytic ShlA across the outer membrane (Poole et al., 1988). In the absence of ShlB, inactive ShlA (ShIA*) accumulates in the periplasm (Schiebel, et al., 1989). ShIA* can be activated *in vitro*, after sonic disruption of the cells, by incubation with a lysate of cells expressing the ShlB protein. ShIA* is activated by ShlB through a covalent modification. ShlA and ShlB are synthesized as precursor proteins with typical amino-terminal signal sequences that are removed to yield the mature forms

(Poole et al., 1988). ShlA is secreted into the culture medium while ShlB is found in the outer membrane (Schiebel, et al., 1989). Export of both proteins through the cytoplasmic membrane probably occurs via the Sec system, since inhibition of SecA ATPase activity by sodium azide causes accumulation of unprocessed ShlA and ShlB precursors.

S. marcescens produces various chitinases (Monreal et al., 1969). *S. marcescens* strain 2170 produces at least three (ChiA, ChiB and ChiC), as well as a chitobiase and a chitin-binding protein (CBP21) (Suzuki, et al., 2002). These five proteins may represent the complete chitinolytic machinery of the bacterium. The primary sequences of ChiA, ChiB and ChiC1, as well as comparisons of available three-dimensional structures, show that these enzymes have a modular structure, which is very common for enzymes degrading insoluble biopolymers such as chitin and cellulose. All three *Serratia* chitinases contain a catalytic domain with the characteristics of family 18 glycosyl hydrolases (Herissat et al., 1997). ChiA has at least six well-defined subsites, one for each of the glucopyranose units of the polysaccharide substrate (Petraatos et al., 1998). The role of chitinase B is to digest the shorter GlcNAc (N-acetyl-D-glucosamine) oligomers, and render them capable of entering the periplasm (Brurberg et al., 1994). The most prominent difference between ChiC and the other two *S. marcescens* chitinases is the lack of the so-called $\alpha+\beta$ domain in the former. This domain makes up one of the walls of the substrate binding groove in ChiA and ChiB. CBP21, secreted by *S. marcescens* when induced by chitin, is a 18.8-kDa protein belonging to a family 33 carbohydrate-binding module and is known to bind to β -chitin (Suzuki et al., 1998). CBP21-like proteins play a role in microbial attachment to chitin or enhance substrate availability by disruption of crystalline chitin (Schnellmann et al., 1994). Vaaje-Kolstad et al. (2005) showed that CBP21 facilitates efficient chitin degradation by binding to the insoluble crystalline substrate, leading to structural changes and increasing substrate accessibility.

S. marcescens chitinase genes have been investigated as possible tools for the biocontrol of plant pathogens. Two chromosomal fragments encoding chitinolytic (and antifungal) activity from *S. marcescens* strain B JL200 were cloned

(Sundheim et al., 1988). The *chiA* and *chiB* genes from *S. marcescens* have been transformed into other bacterial species such as *Pseudomonas fluorescens* and *E. coli* in an attempt to improve their ability to control fungal plant pathogens (Downing et al., 2000) or to create new biocontrol agents.

Some types of virulence mechanisms have been conserved during evolution (Rahme et al., 2000). For instance, some virulence factors of *S. marcescens* Db11 were responsible for the microbe's pathogenic effects on *Drosophila* flies, *Caenorhabditis elegans* and *Arabidopsis thaliana*. An extracellular and heat-labile cytotoxin, an important virulence factor, was detected in phytopathogenic, entomopathogenic and clinical *S. marcescens* strains (Escobar et al., 2001). Similarly, nearly all clinical isolates of *S. marcescens* have hemolysin activity (Ruan et al., 1990), and 99% of 147 *S. marcescens* isolates from clinical and environmental sources have siderophore activity (Franczek et al., 1986).

It is unknown whether any of these factors also play a role in the phytopathogenicity of CYVD strains of *S. marcescens*. The purpose of the present study was to identify whether CYVD strains of *S. marcescens* have gene encoding the putative virulence factors hemolysin and chitinases and what function these genes play for disease development. We hypothesize that those genes are important virulence factors for *S. marcescens* on squash plants.

Materials and Methods

Bacterial strains and growth conditions. Strains of *S. marcescens* used in this study are shown in Table 1. Bacteria were stored at -80°C in aliquots of 1.5 ml Luria-Bertani (LB) broth containing 15% glycerol. For use in the experiments, bacteria were streaked onto LB or nutrient agar (Sambrook et al., 1989) and incubated overnight at 30 °C.

Primer design. Known hemolysin and chitinase gene sequences were searched from GenBank and aligned using SDSC Biology Workbench 3.2 (<http://workbench.sdsc.edu/>). The conserved sequences were analyzed by BLASTN and TBLASTN searches against the *Serratia marcescens* Db11

database (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/s_marcescens). Primers, designed using Primer 3 (Workbench 3.2) based on conserved sequences after BLASTN and TBLASTN searches, were designated *chiAF* and *chiAR* for chitinase gene A (*chiA*), *chiBF* and *chiBR* for chitinase gene B (*chiB*), *chiCF* and *chiCR* for chitinase C, *hemoAF* and *hemoAR* for hemolysin gene A (*shlA*), and *hemoBF* and *hemoBR* for hemolysin gene B (*shlB*) (Table 1). Colony PCRs were performed in a 25 μ l volume using these primers and the following conditions: 1 μ l of each primer, 12.5 μ l GoTaq Master Green Mix (Promega, Madison, WI), 1 μ l of bacterial suspension ($\sim 10^8$ cells/ml) and 9.5 μ l double-distilled (dd) H₂O. The reaction was subjected to a 5 min denaturation step at 95 °C followed by 34 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 2 min. The reaction mixture was held at 72°C for 10 min and then placed at 4°C until analyzed on a 1.5% agarose gel.

Hemolysis assay. From each bacterial strain (Table 1), an overnight colony was selected, grown in LB broth with shaking at 200 rpm overnight at 30 °C and diluted to about 1×10^9 CFU/ml with 0.85% saline. Blood agar plates were purchased (BUG W/BLOOD, BIOLOG, Hayward, CA), or prepared [TY (Sambrook et al., 1989) + 5 % goat blood]. Overnight bacterial cultures were centrifuged at 5000 X g for 3 min and resuspended in phosphate buffered saline (PBS, 0.01M, pH 7.0, containing 0.15 M NaCl) to approximately 1×10^8 cells per ml. Three drops (10 μ l) bacterial suspension were dropped onto a blood agar plate, two or three strains per plate and at least three plates for each strain. These plates were incubated overnight at 30 °C then maintained at 4 °C. This test was performed a total of three times.

Chitinase assays. Bacteria were prepared as described above. Petri dishes (90 mm) were layered with 12~13 ml YEM agar (Vincent, 1970) on the bottom, covered with 5 ml of a mixture of 0.5 % agar and 2% chitin (from crab shells, Sigma). To test the effect of pH on chitinase activity, chitin plates (YEM + 0.2 % chitin) were prepared at pH 7.5 and pH 8. An overnight colony from each bacterial strain was stabbed into the chitin plates using sterile toothpicks (three stabs per plate and 3-4 plates/strains). These plates were incubated 60 h at 30 °C

and the diameter of the clear zone was recorded as a measure of chitinase activity. This test was performed a total of three times.

Southern hybridization. Genomic DNAs were extracted from *S. marcescens* ZO1-A and RO2-A using a DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA). Two μg of extracted DNAs were digested with *Sma*I (Invitrogen, Carlsbad, CA) for 3 h at 30 °C. The PCR product of *shlA* was labeled with digoxigenin-11-dUTP (DIG) (Roche, Indianapolis, IN) by the random primed DNA labeling method according to the manufacturer's instructions. Southern hybridization was carried out according to Sambrook et al. (1989).

Northern blot and reverse transcription-PCR (RT-PCR). To assess chitinase gene expression, seven strains of *S. marcescens*, three CYVD (ZO1-A, WO1-A, and CO1-A), two endophytes (RO2-A and 90-166), one human isolate (HO1-A), and the type strain (ATCC13880), were grown 36 h at 30 °C in YEM containing 0.2% chitin. Total RNA from each test strain was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA concentration was determined by Nanodrop ND 1000 Spectrophotometer (Wilmington, DE). PCR products of *chiA* and *chiB* (amplified using primers in Table 1) were recovered using a Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI) and labeled with digoxigenin-11-dUTP (DIG) by the random primed DNA labeling method according to the manufacturer's instructions. Northern blot analysis was carried out as in Sambrook et al. (1989). SuperScript® II Reverse Transcriptase (Invitrogen) was used for reverse transcription PCR (RT-PCR) as described previously (Uppalapati et al., 2005), with slight modifications. Briefly, 4-5 μg of bacterial total RNA, 1 μl of DNase I reaction buffer and 1 μl of DNase I (Invitrogen) were suspended in a final volume of 10 μl with RNase-free double-distilled (dd)H₂O. The mixture was incubated 15 min at room temperature, and then the DNase I was inactivated by adding 1 μl 25 mM EDTA. The RNA was denatured by heating at 65°C for 10 min, cooled on ice and then incubated with 1 μl of RNaseOUT™ (Invitrogen). One μl of random hexamer primer was added and the mixture was incubated 2 min at room temperature. A master mix (8 μl) containing 5X Superscript RT buffer

(Invitrogen), dNTPs (1 μ l containing 10 mmol each dNTP), 0.1 M DTT (2 μ l), and RNase-free ddH₂O (1 μ l) was prepared and gently added to the RNA-RT mix. Each sample was heated for 3 min at 42°C, and 2 μ l (400 U) Superscript IITM reverse transcriptase (Invitrogen) was added. The reaction mixture was incubated at 42°C for 1.5 h, and then at 70°C for 15 min to stop the reaction and hydrolyze template RNA. The reaction was neutralized by adding 1 M Tris-HCl (5 μ l, pH 7.5). Two μ l of these cDNAs were used for PCR as described above.

Results and Discussion

Chitinase and hemolysin genes were highly conserved in *Serratia marcescens*. Many strains of *S. marcescens*, including QMB1466, BJL200 (Brurberg et al., 1995), KCTC 2216 (Gal et al., 1997), 27117 (Jones et al., 1986) and 2170 (Watanabe et al., 1997), contain chitinase genes A, B and C. BLAST analysis using the GenBank and *S. marcescens* Db11 databases showed that all three *S. marcescens* chitinases contain a highly conserved catalytic domain with the characteristics of family 18 glycosyl hydrolases. Highly conserved sequences also were observed in hemolysin genes A (*shlA*) and B (*shlB*), as shown in Fig. 1.

PCR to assess gene presence. PCR, using primers based on conserved gene sequences, showed that all the tested strains of *S. marcescens*, regardless of their source (water, cucurbits, rice, cotton, *Drosophila* flies or humans), had chitinase genes *chiA*, *chiB* and *chiC* (Table 2). These results were consistent with previous reports (Brurberg et al., 1995; Gal et al., 1997; Jones et al., 1986; and Watanabe et al., 1997), indicating conservation of these genes over time among *S. marcescens* strains, rather than the acquisition of *chiA*, *chiB* and *chiC* by lateral gene transfer. However, hemolysin gene B (*shlB*), a regulatory gene, also was present in all the tested strains, but only non-CYVD strains had hemolysin gene A (*shlA*), encoding the hemolytic protein.

Hemolysin assay and Southern hybridization. On blood agar, CYVD strains of *S. marcescens* (ZO1-A, WO1-A and CO1-A) and three of the non-

CYVD strains, 90-166 (a cotton endophyte), ATCC13880 (a pond water isolate) and Db11 (a *Drosophila melanogaster* isolate) failed to hemolyze the red blood cells and no clear zones were observed (Fig. 2). However, three rice endophytes (RO1-A, RO2-A and RO3-A) and two human isolates (HO1-A and HO2-A) were hemolytic and formed a clear zone on the blood agar plates (Fig. 2). This variation may result in the different niches of these test strains. For instance, hemolysin may be a key pathogenicity or virulence factor in the two human isolates HO1-A and HO2-A. However, why three rice endophytes expressed hemolysin activity but the cotton endophyte did not is still unclear.

PCR results showed that CYVD strains possessed hemolysin gene B (*shlB*) but not hemolysin gene A (*shlA*) (Table 2). To confirm that finding, Southern hybridization was conducted using the PCR product of *shlA*, labeled by DIG, as probe. Hybridization results confirmed that CYVD strains of *S. marcescens* lacked *shlA* but the rice endophytic strains possessed it (Fig. 4).

Why do CYVD strains of *S. marcescens* have *shlB*, a regulatory gene, but lack *shlA*, encoding the hemolytic protein? It is possible that CYVD strains lost *shlA* during coevolution with its plant host or with the vector squash bug. Hemolysin also lyses many other types of cells, including lipid membranes (Schönherr et al., 1994) and epithelial cells (Hertle et al., 1999), perhaps because these environments provided no substrate for it, or possibly because hemolysin destroys the host tissue, thus acting as a negative selection factor. *shlB*, on the other hand, may function in a regulatory cascade, involving genes other than *shlA* that are critical to the plant or insect host. RO2-A, which expressed hemolysin activity in the present study, caused greater damage than did ZO1-A to squash bugs (Heppler, *unpublished data*).

Chitinase assay. All non-CYVD strains of *S. marcescens* digested chitin and formed a clear zone on chitin plates. HO1-A and HO2-A formed the largest zones, $\sim 18 \pm 4$ mm and 17 ± 3.5 mm in diameter, respectively, at pH 7.5, followed by the three rice endophytes, RO1-A, RO2-A and RO3-A, which formed zones of 8 ± 2 mm, 7 ± 3.5 mm, and 8 ± 3 mm respectively. The clear zones produced at pH 7.5 were larger than those produced at pH 8.0. None of the CYVD

strains had chitinase activity, although they had chitinase genes (Table 2 and Fig. 3).

Northern blot and reverse transcription-PCR (RT-PCR). Since CYVD-strains of *S. marcescens* did not digest chitin and form clear zones despite the fact that the chitinase genes were present, it may be that gene expression is repressed. To test chitinase (*chiA* and *chiB*) gene expression, total RNA from each strain of *S. marcescens* was extracted from chitin-induced cultures. PCR products of *chiA* and *chiB* were DIG-labeled and used as probes. Northern blots showed no *chiA* and *chiB* expression in any of the test strains of *S. marcescens*. Since the detection limit of this method, the concentration of mRNA, is relatively high, it is possible that *chiA* and *chiB* expression occurred at a level too low to be detected. A more sensitive method detecting as little as 1 pg RNA, two-step reverse transcription-PCR (RT-PCR), revealed that non-CYVD strains expressed at least chitinase genes *chiA* and *chiB*, but CYVD strains did not. Work currently underway will reveal in which strains, if any, the third chitin-related gene, *chiC*, is expressed.

The lack of CYVD strain chitinase activity could be due to several factors. Partial deletion of *chiA* or/and *chiB* could impede gene function, but the primers used in our study, designed from highly conserved sequences, showed that CYVD strains had *chiA*, *chiB* and *chiC* genes. It is also possible that there is no promoter for *chiA*, *chiB* and *chiC* expression in CYVD strains, or that a negative regulator is present. In the field, the squash bug transmits from plant to plant (Bruton et al., 2003). Because chitin is a major component of insect's cuticle (Cohen, 1991), chitinase activity in such bacteria may damage the vector and delay or prevent transmission. What causes these variations, and what functions hemolysin and chitinase play in non-CYVD strains of *S. marcescens* need further study.

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Table 1. Strains of *Serratia marcescens* and primers used in this study

<i>Serratia marcescens</i>	Bacterial host or primer sequence	Source or reference
CYVD strains		
ZO1-A	Zucchini	Bruton et al., 1998
WO1-A	Watermelon	Bruton et al., 1998
PO1-A	Pumpkin	Fletcher
CO1-A	Cantaloupe	Fletcher
Non-CYVD strains		
RO1-A	Rice (endophyte)	Gyaneshwar et al., 2001
RO2-A	Rice (endophyte)	Gyaneshwar et al., 2001
HO1-A	Human	Rascoe et al., 2003
HO2-A	Human	Rascoe et al., 2003
90-166	Cotton (endophyte)	J. W. Kloepper
ATCC 13880	Pond water	ATCC
Db11	<i>Drosophila melanogaster</i>	Flyg et al., 1980
Primers		
<i>chiAF</i>	5'-GCGCAAATTTAATAAACCGC	This study
<i>chiAR</i>	5'-GCTGTTGAGAATATCGCCGT	This study
<i>chiBF</i>	5'-GCAAAGCCGTTATTGGGTA	This study
<i>chiBR</i>	5'-GCCAGACGTAGCCTTGGTAG	This study
<i>chiCF</i>	5'-GCCTGGATATCGATCTGGAA	This study
<i>chiCR</i>	5'-GAGGATGCAAGCGTTTTAGC	This study
<i>shlAF</i>	5'-ACAGCGAGAAAACCAC	This study
<i>shlAR</i>	5'-GCTGCTGCTGCGTTT	This study
<i>shlBF</i>	5'-AACAGTGCATCAGCAGCAAC	This study
<i>shlBF</i>	5'-GCAGCGAATAGAGCAGGGTA	This study

Table 2. Study of chitinase and hemolysin genes in *Serratia marcescens*

<i>Serratia marcescens</i>	PCR					Function assay	
CYVD strains	<i>chiA</i>	<i>chiB</i>	<i>chiC</i>	<i>shlA</i>	<i>shlB</i>	Chitin plates	Blood agar
ZO1-A	+	+	+	-	+	-	-
WO1-A	+	+	+	-	+	-	-
CO1-A	+	+	+	-	+	-	-
Non-CYVD strains							
RO1-A	+	+	ND	+	+	+	+
RO2-A	+	+	+	+	+	+	+
RO3-A	+	+	ND	+	+	+	+
90-166	+	+	ND	+	+	+	-
HO1-A	+	+	+	+	+	+	+
HO2-A	+	+	ND	+	+	+	+
ATCC13880	+	+	ND	+	+	+	-
Db11	+	+	+	+	+	ND	-

For function assay, ND-not done; -no activity; + clear zone;

For PCR test, - negative; + positive

Db11_SHLA	MKNNNFRLSAAGKLAALAIILAASAGAYAGEIVAANGANGPGVSTAATGAQVVDIVAPN
MG1-SHLA	MKNNNFRLSAAGKLAALAIILAASAGAYAAEIVAANGANGPGVSTAATGAQVVDIVAPN
W1436-SHLA	MKNNNFRLSAAGKLAALAIILAASAGAYAAEIVAANGANGPGVSTAATGAQVVDIVAPN
	*
Db11_SHLA	GSGLSHNQYQDFNVNQPAGVLLNSREAGLSQLAGQLGANPNLGGREASVILNEVIGRNP
MG1-SHLA	GNSLSHNQYQDFNVNQPAGVLLNSREAGLSQLAGQLGANPNLGGREASVILNEVIGRNP
W1436-SHLA	GNSLSHNQYQDFNVNQPAGVLLNSREAGLSQLAGQLGANPNLGGREASVILNEVIGRNP
Db11_SHLA	LLHGQQEIFGMAADYVLANPNGISCSQCGFINTSRSSLVVGNPLVENGLLQGYSTLGNRN
MG1-SHLA	LLHGQQEIFGMAADYVLANPNGISCSQCGFINTSRSSLVVGNPLVENGLLQGYSTLDNRN
W1436-SHLA	LLHGQQEIFGMAADYVLANPNGISCSQCGFINTSRSSLVVGNPLVENGLLQGYSTFGNRN
	* * *
Db11_SHLA	TLSLSGTLNAGVLDLIAPKIDSRGDVIVQDFTHRNGKVTAAAINAISGLNRVARDGKVL
MG1-SHLA	TLSLNGTLNAGVLDLIAPKIDSRGDVIVQDLTHRNGSVTPAAAINAISGLNRVARDGKVL
W1436-SHLA	TLSLNGTLNAGVLDLIAPKIDSRGEVIVQDFKQSNKVTSAAINAISGLNRVARDGTVQ
	* * *# #
Db11_SHLA	ASEQPTTALDSYYLGSMQAGRINIINTAQGSGVKLAGTLAAGDELKVKAYDIRSESRIED
MG1-SHLA	ASVQPTTALDSYYLGSMQAGRINIINTAQGSGVKLAGTLAAGDELKVKAYDIRSESRVDD
W1436-SHLA	ASQQMPTALDSYYLGSMQAGRINIINTAQGSGVKLAGSLNAGDELKVKAYDIRSESRVDD
	# # * # **
Db11_SHLA	ASSNKGGDNYQNYRGGIYVNDRSSSQKLRTELKGNISLVADNHAHLTATDIRG----
MG1-SHLA	ASSNRNGGENYQNYRGGIYVNDRSSSQKLRTELKGNISLVADNHAHLTATDIRG----
W1436-SHLA	ASSNKGGDNYQNYRGGIYVNDRSSSQTLRTELKGNISLVADNHAHLTATDIRGEDIT
	* * #####

Fig. 1. Alignment of hemolysin A protein (SHLA) of three strains of *Serratia marcescens*. Letters in colors indicate fully conserved residues, *conservation of strong groups, # no consensus. Db11, an insect pathogenic strain of *S. marcescens*; MG1 and W1436, human isolates of *S. marcescens*, sequence obtained from GenBank.

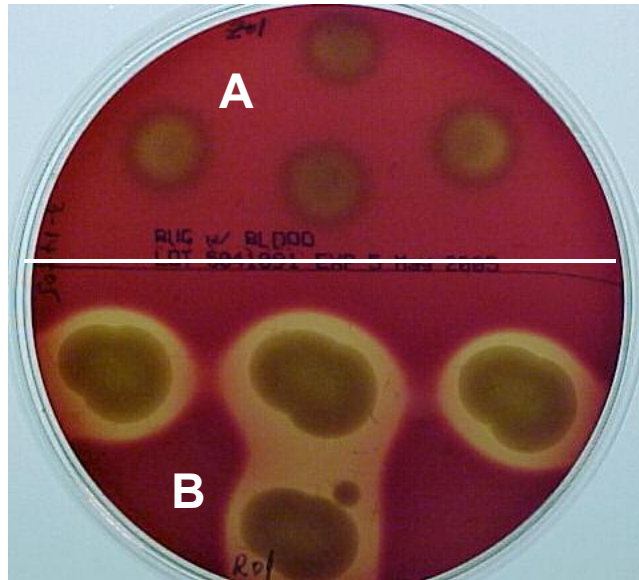


Fig. 2 Clear zones produced by dropping suspensions of *Serratia marcescens* onto a 5% blood agar plate. Strains: A, ZO1-A; B, RO1-A.

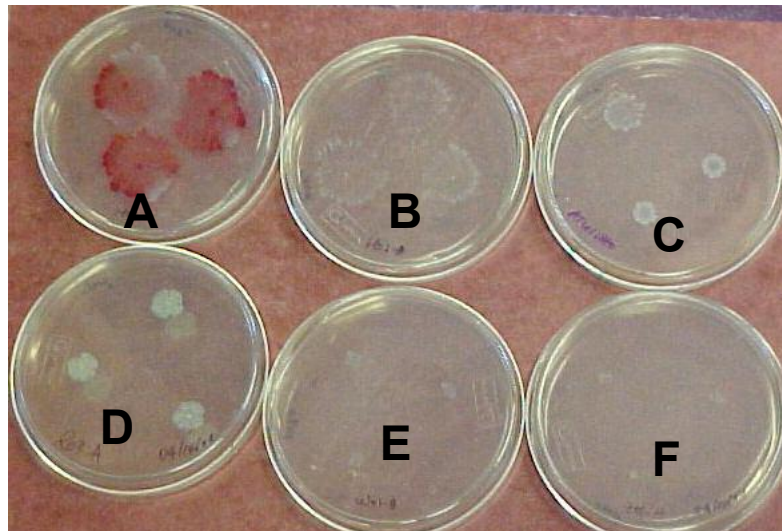


Fig. 3 Growth zones of *Serratia marcescens* on YEM chitin plates. Strains are as follows: A, HO1-A; B, HO2-A; C, ATCC13880; D, RO2-A; E, WO1-A; F, ZO1-A.

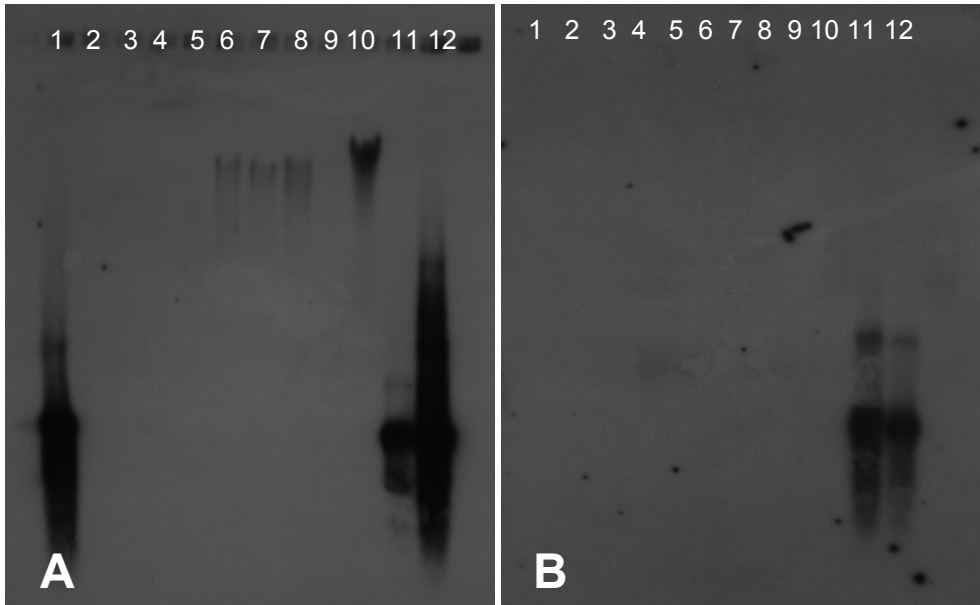


Fig. 4. Southern hybridization analysis of *Sma*I-digested strains of *Serratia marcescens* probed with DIG-labeled PCR product of the hemolysin structural gene *shlA* (A) or hemolysin regulatory gene *shlB* (B). Lane1, Db11; Lane 2, ZO1-A; Lane 3, WO1-A; Lane 4, CO1-A; Lane 5, PO1-A; Lane 6, ATCC13880; Lane 7, 90-166; Lane 8, HO1-A; Lane 9, HO2-A; Lane 10, RO1-A; Lane 11, RO2-A; Lane 12, RO3-A.

Chapter VI

Suggestions for further research

The work reported in this thesis demonstrated that strains of *Serratia marcescens* had different pathogenicity on various plant species, including squash, tobacco, carrot and onion. These differences may be due to gene presence or absence, or to differential gene expression of the tested strains. For instance, a type I fimbrial gene cluster was found in cucurbit yellow vine disease (CYVD) strains of *S. marcescens* but was absent in all of the non-CYVD strains used in our work. Although all the tested strains of *S. marcescens* had chitinase gene A (*chiA*) and B (*chiB*), CYVD-strains did not digest chitin and no growth was observed on chitin plates. Interestingly, all the tested strains of *S. marcescens* had hemolysin regulatory gene B (*shlB*), while CYVD strains lacked the hemolysin gene A (*shlA*), encoding hemolytic protein ShlA. Because CYVD strains are transmitted by an insect vector, the squash bug (*Anasa tristis*), chitinase and hemolysin of these bacteria may be virulence factors in the bacteria-vector relationship. The fact that *fimA* and *fimH* disruption significantly reduced the virulence of *S. marcescens* ZO1-A on squash plants demonstrated that fimbriae are a virulence factor. It is unclear whether the disruption of these genes affects the mutants' behaviors. The tracking of GFP-labeled *S. marcescens* in squash plants revealed that the bacteria colonized and moved in the xylem, but not in the phloem, after mechanical inoculation. This finding was surprising, since previous work had demonstrated the presence of *S. marcescens* in sieve tubes of CYVD-affected cucurbits.

It is possible that the vector squash bug (*A. tristis*) plays an important role for CYVD strains of *S. marcescens* to enter and colonize the vascular system of squash plants. There are still many questions about the interactions between *S. marcescens* and their host plants, and their insect vector. Answers to the following issues and questions may help us elucidate the nature of these interactions:

- 1) Further studies on the pathogenicity of *fimA* and *fimH* mutants on carrot and onion. As shown in chapter II, CYVD strains and non-CYVD strains of *S. marcescens* had different incidence of disease on these two plants. Fimbriae, present in CYVD strains but absent in all non-CYVD-strains tested in our study, may be a factor that contributes the difference in incidence of disease.
- 2) Analysis of the behaviors of *fimA* and *fimH* mutants, including adhere to the bacterial cells as well as to those of their plant hosts and insect vectors, to assess their movement and colonization in plants and insects
- 3) Why do CYVD strains of *S. marcescens* have the hemolysin regulatory gene B (*shlB*) and what function does this gene have in this species?
- 4) Why is it that CYVD strains of *S. marcescens* have chitinase gene A (*chiA*) and B (*chiB*), yet they do not express chitinase activity? Is it due to chitinase gene deletion or to the absence of a promoter?
- 5) In what cells and tissues of squash do CYVD strains of *S. marcescens* colonize and move after being introduced by squash bugs in greenhouse?
- 6) If the fimbrial gene cluster from CYVD strains of *S. marcescens* is transformed into the non-CYVD strain, RO2-A, will the transformed strain become pathogenic to squash?
- 7) Can full virulence of *fimA*- and *fimH*- mutants be restored by translocation of FOSU1, containing the fimbrial gene cluster into these lines?

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Scope and Method of Study:

Serratia marcescens, the causal agent of cucurbit yellow vine disease (CYVD), can colonize in many niches, including soil, water, insects, plants and humans. It is unclear whether strains from one niche can colonize others and whether these bacteria can enter into plants through natural openings. Strains of *S. marcescens* have many pathogenicity and virulence factors including proteases, nucleases, cytotoxin, hemolysin, chitinase, siderophores, lipopolysaccharide and fimbriae. However, it is not known what virulence factor (s) phytopathogenic strains use for disease development in plants.

Findings and Conclusions:

In this research, different plant species were used to assess the pathogenicity of various strains of *S. marcescens*. Only CYVD- causing strains of *S. marcescens* caused stunting on squash. However, all the tested strains of *S. marcescens*, regardless of their natural niche, caused chlorotic lesions and necrosis on tobacco leaves, wilt on carrot seedlings and water-soaking and rotting of onion bulbs. This is the first report that *S. marcescens* strains from non-plant niches cause symptoms in plants, and that their interactions with the plant host differ from those of CYVD-strains.

The TargeTron Gene Knockout System was used to mutate the fimbrial genes *fimA* and *fimH* of *S. marcescens* ZO1-A. *fimA* mutation prevented fimbrial formation, while *fimH* mutation reduced fimbrial length. Both mutants significantly reduced virulence on squash plants.

GFP-labeled *S. marcescens* cells were introduced onto squash plants. Fluorescence and confocal microscopy showed no evidence that *S. marcescens* entered into the plant interior through the stomata after being dropped onto leaf surface. *S. marcescens* did move into the plant interior through stomata by vacuum infiltration, but bacterial cells remained in the vicinity of the stomata and did not enter the vascular system. After wound inoculation, GFP-*S. marcescens* cells were detected in intercellular spaces as well as within xylem vessels, and were present as much as 31 mm away from the inoculation site, suggesting that systemic translocation occurred. This is the first report of *S. marcescens* entry and movement within xylem. Our results indicate that the nature and location of *S. marcescens* colonization and translocation in squash plants are apparently dependent upon how and where the bacteria are inoculated.

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