GENETIC ANALYSIS OF *SERRATIA MARCESCENS*, THE CAUSAL AGENT OF CUCURBIT YELLOW VINE DISEASE

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Submitted to the Faculty of the
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in partial fulfillment of
the Degree of
DOCTOR OF PHILOSOPHY
December, 2004

GENETIC ANALYSIS OF *SERRATIA MARCESCENS*,

THE CAUSAL AGENT OF CUCURBIT

YELLOW VINE DISEASE

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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor, Dr. Jacqueline Fletcher, for her support, patience, and intelligent guidance throughout my graduate studies. Her editorial advice was essential to the completion of this dissertation. I would also like to thank Dr. Ulrich Melcher, who gave me much invaluable advice. My thanks extend to my other committee members, Dr. Carol Bender and Dr. Moses Vijayakumar, for offering me priceless assistance and suggestions.

I am very grateful to Astri Wayandande, Michael Berg, Kikin Mutaqin and Guanpingsheng, Luo, for suggestions and joys brought to me. I will also give a special thanks to Gerry Smith and other secretaries at the Entomology and Plant Pathology Department for providing me a good working atmosphere.

Last, but not least, I would like to thank my wife Kang for her understanding and love during the past few years. Her support and encouragement was in the end what made this dissertation possible.

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CHAPTER I

LITERATURE REVIEW

Introduction to Cucurbit Yellow Vine Disease

Vine declines of cucurbits have increased in number and severity in the past 20 years and are yield-limiting factors in many intensive production areas around the world (13). In 1988, a new disease called cucurbit yellow vine disease (CYVD) was observed in central Texas and Oklahoma. CYVD is characterized by rapid and general yellowing of leaves appearing over a 3-4 day period, followed by gradual or rapid decline and death of the vine in several cucurbit crops (13). Symptoms resembled those reported in diseases caused by phloem-associated bacteria in other crops. Avila et al. (6) found that the CYVD organism was detectable by polymerase chain reaction (PCR) using nonspecific primers designed from prokaryotic 16S rDNA. The deduced nucleotide sequence of the amplified 16S ribosomal DNA placed this organism within the gamma-3 proteobacteria. In 1998, pure bacterial cultures isolated from diseased zucchini and watermelon, designated Z01-A and W01-A, respectively, were considered to be candidate causal agents. Koch's postulates were completed using isolate Z01-A by mechanical inoculations and by transmission via an insect vector, the squash bug, *Anasa tristis* (14). Rascoe et al. (70), after extensive analysis of the 16S rDNA and *groE* sequences of Z01-A, showed that the cucurbit isolates Z01-A and W01-A were *Serratia marcescens*. Bacteria of this species occupy a variety of ecological niches. However, the degree of genetic homogeneity of the CYVD strains of *S. marcescens* compared to other isolates

remains unknown. In addition, the genetic factors that are responsible for the plant pathogenicity in these strains of *S. marcescens* have not been determined.

Niche Versatility of *S. marcescens*

S. marcescens, a gram-negative bacillus classified as a member of the *Enterobacteriaceae*, was the first species identified in the genus *Serratia*. Most *S. marcescens* isolates produce a red pigment, prodigiosin, which in early times was often mistaken for fresh blood (26). In the past few decades this pigmented bacterium has been identified in various ecological niches including soil, water, air, plants, insects, animals and even human beings.

When the name *S. marcescens* was assigned by Bizio (8) to this bacterium it was generally considered as a saprophytic microorganism living in soil. *S. marcescens* might play a role in the biological cycle of metals by metabolizing organic iron and dissolving gold and copper (64). On the other hand, it is one of the most frequent contaminants of laboratory cultures of bacteria. It is also found in foods, particularly in starchy variants, which provide an excellent growth environment (34).

S. marcescens is also an insect pathogen (15). Red strains of *S. marcescens* have been isolated from healthy, diseased, or dead field-collected insects, but the species is most frequently reported as a pathogen of insectary-reared insects (77), in which it causes a lethal septicemia after invasion into the hemocoel. Nonchromogenic strains are also pathogenic and exhibit no significant biochemical differences from classical red strains. Inglis et al. (36) reported that egg production from adult *Heliothis virescens* 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*.

The first description of nosocomial human infection caused by *S. marcescens* was Wheat's (85) report of 11 cases over a 6-month period at Stanford University Hospital. *S. marcescens* was isolated from preterm infants (gestational age 25-30 weeks) cared for in the intensive care unit. Later *S. marcescens* was reported to be the most common Gram-negative bacterium associated with contact lens related keratitis (65). *S. marcescens* may also be responsible for appreciable morbidity among patients with acquired immunodeficiency syndrome (AIDS), especially when the disease is accompanied by a low CD4+ cell count, neutropaenia, and hospitalization (46). Biochemical analysis showed that almost all clinical strains of *S. marcescens* secrete a cytotoxin, Sh1A, that causes hemolysis of human and animal erythrocytes and the release of inflammatory mediators from leukocytes (9).

Mention of plants as a source of *Serratia* species, predominantly *S. marcescens* and *S. liquefaciens*, can be found in several papers and culture collection catalogs. Plant-associated *S. marcescens* isolates have been investigated for a possible role in the contamination of humans (88). However, it is still unclear if the biotypes found in human patients are the same as those found in plants. *S. marcescens* may be harmful to host plants in some cases, but beneficial in other cases. For example, *S. marcescens* 90-166 was described as a plant growth promoting rhizobacterium (PGPR), which could induce systemic resistance in cucumber against different pathogenic agents including bacteria, fungi and even viruses (84). Shinde and Lukezic (76) reported that two groups of Gram-negative bacteria were consistently associated with a root and crown disease of alfalfa.

Based on biochemical characterization Lukezic et al. (44) concluded that one group was *S. marcescens*, but no further characterization of this particular strain has been reported.

Only a few bacteria have been identified to be pathogens of both plants and humans. Among them, *Burkholderia cepacia* is much better characterized than *S. marcescens.* Like *S. marcescens*, *B. cepacia* is resistant to many antibiotics and utilizes a wide variety of substrates. Genotypic and phenotypic relationships within *B. cepacia* have been studied (39, 86). The *B. cepacia* genome is comprised of multiple chromosomes and is rich in insertion sequences. These two features play a key role in the evolution of novel degradative functions and the unusual adaptability of *B. cepacia*. The same mechanism may also apply to *S. marcescens,* but whether this is true or not still needs to be verified.

The potential of *S. marcescens* to utilize a wide range of nutrients is reflected clearly by its ability to survive and grow under extreme conditions, including in disinfectants, antiseptics and double distilled water. Szewzyk et al. (81) studied the survival and growth of *S. marcescens* strain K202 at different oxygen concentrations in de-ionized water containing materials derived from blood bags. The rate of bacterial survival and growth was highest under anaerobic conditions.

S. marcescens is unique among enteric bacteria in many respects. It secretes extracellular chitinases, several proteases, a nuclease and a lipase, and produces a wetting agent or surfactant called “serrawettin”. In keeping with its varied habitats, *S. marcescens* produces alternate forms of differentially flagellated cells. These cells display different types of motility depending on whether the growth medium is solid or liquid (1).

Infections caused by *S. marcescens* may be difficult to treat because of its resistance to a variety of antibiotics, including ampicillin and cephalosporins. The resistance of *S.*

marcescens to β-lactam antibiotics may arise from two mechanisms: first, high level production of chromosomal AmpC cephalosporinases combined with decreased membrane permeability; second, synthesis of β-lactamase, which hydrolyses carbapenems (42). *S. marcescens* resistance to aminoglycosides results from its ability to prevent the drugs from reaching the target site on the ribosome in one of two ways: first, alterations in the cell envelope can prevent uptake of the drug; and second, the drug itself may be modified by an "inactivating enzyme" that adenylates, acetylates, or phosphorylates the aminoglycoside hydroxyl or amino groups.

S. marcescens Pathogenicity and Virulence

Adherence and hydrophobicity. Piliation has been shown to be a determinant of microbial adherence to host epithelial surfaces. A nosocomial isolate of *S. marcescens* possesses pili and adheres to uroepithelial cells (89). Two classes of adhesins have been suggested for *S. marcescens* (71). The pili of one class, designated mannose-resistance (MR), agglutinate chicken erythrocytes in the presence of D-mannose; those of the other class, mannose-sensitive (MS), exhibit mannose sensitive haemagglutination of guinea-pig and chicken erythrocytes. Mizunoe et al. (53) assessed the effect of bacterial piliation on *S. marcescens* interaction with human leucocytes and found that the MS-piliated strain was more susceptible to phagocytosis than was the MR- or nonpiliated strain.

S. marcescens possesses hydrophobic surfaces (55). Cell surface hydrophobicity has been linked to partitioning of *S. marcescens* at air: water and oil: water interfaces, as well as adhesion to solid surfaces including catheters and other plastics (5). When Ness-Greenstein et al. (62) tested the feasibility of increasing the cell surface hydrophobicity of

commonly used strains of *E. coli* by transformation with DNA from *S. marcescens*, the transformed *E. coli* became more hydrophobic. Mallick (45) compared the hydrophobicity of two *S. marcescens* mutant strains, of which one is non-pigmented and the other overproduces red pigment. An extra protein of the outer membrane (approximately 40 K Da) might be responsible for higher surface hydrophobicity in the non-pigmented mutant of *S. marcescens*.

Lipopolysaccharide. Lipopolysaccharide (LPS; the biologically active constituent of endotoxin) 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. Palomar et al. (63) showed that bactericidal activity of phages against *S. marcescens* depends upon the O-side chain length. Two *S. marcescens* genes involved in core LPS biosynthesis were cloned and characterized. These two genes conferred resistance to bacteriocin 28b when introduced into *E. coli* NM554 (23).

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 the pathogenesis of human sepsis (69). However, there is a poor correlation between serum LPS levels and mortality in septic patients. Luchi and Morrison (43) compared the chemical, structural, and biological differences among LPS from *S. marcescens* and other clinical bacterial isolates. The relatively minor differences in LPS activity seemed unlikely to explain the discrepancy between serum endotoxin levels and mortality in patients with Gram-negative sepsis.

Extracellular products. *S. marcescens* produces several extracellular enzymes and is one of the most efficient organisms for the biological degradation of chitin. Sundheim et al. (79) cloned two chromosomal fragments encoding chitinolytic activity from *S. marcescens* strain BJL200. Brurberg et al. (12) analyzed one of the fragments and determined the nucleotide sequence of a chitinase gene. Several different chitinase genes are present in *S. marcescens*. Suzuki et al. (80) found a novel chitinase gene, named chiC, in *S. marcescens* 2170. The protein product of this gene has a fibronectin type III-like domain.

Letoffe et al.(41) identified a *S. marcescens* extracellular protein, HasA (for heme acquisition system), which is able to bind heme and is required for iron acquisition from heme and hemoglobin by the bacterium. HasA does not have a signal peptide and has no sequence similarity to other proteins. When HasA secretion was reproduced in *E. coli*, it was shown that, like many proteins lacking a signal peptide, HasA has a C-terminal targeting sequence and is secreted by a specific ATP-binding cassette (ABC) transporter (74).

Epidemiological Typing

Typing methods for *S. marcescens* involve either phenotypic or genotypic characterization and are based on the assumption that closely related organisms will possess a unique characteristic that distinguishes them from unrelated isolates.

Phenotypic approaches based on metabolic or biological characteristics have included serotyping, biotyping, bacteriocin typing, phage typing and whole cell fingerprinting

(47). Biotyping has the advantage of using bacteriological techniques that can be employed on a routine basis. However, the method is time consuming and laborious, and biotyping cannot always distinguish between different epidemiological types (30). Serotyping is another commonly used method for typing *S. marcescens*, but has limitations associated with the tedious determination of both O and K antigens, and the presence of a few untypable mutants (40). Bacteriocin typing is a powerful method, but it also may not distinguish between different epidemiological types, while phage typing may be of value only in subdividing strains of the same O group from the same incident of infection (68).

Genotyping methods include pulsed field gel electrophoresis (18), plasmid profiling (60), restriction fragment length polymorphism (7), ribotyping (47) and various PCR approaches. Ribotyping was reported to be more discriminatory than biotyping, serotyping and bacteriocin typing (3). However, because of technical difficulties and the prolonged time needed for Southern blot analysis, its clinical use has been limited. Esterase electrophoretic typing of *S. marcescens* is reliable, but it is also technically difficult compared to other methods. The popularity of PCR-based typing methods is rapidly increasing due to the speed at which results are obtained. Patton et al. (66) employed different PCR-based methods to type clinical *S. marcescens* isolates. Randomly amplified polymorphic DNA (RAPD) and repetitive element (RE) based PCR were used to amplify total DNA prepared from each of 62 clinical *S. marcescens* isolates. Three different random primers designated 1060, 1254 and 1283 were used individually in RAPD-PCR. Primers representing enterobacterial repetitive intergenic consensus (ERIC) sequences, extragenic palindromic (REP) elements, and polymorphic GC-rich

repetitive sequences (PGRS) constituted the REP-PCR. The data indicated that all of these PCR-based approaches are a valid means of discriminating among isolates of *S. marcescens,* and the amount of differentiation depends on the primer used.

Genome Plasticity in *Enterobacteriaceae*

At present (August 2004) 177 bacterial genomes, including those of 18 enterobacteria, have been completely sequenced (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/Complete.html). Many other bacterial sequencing projects are underway. In particular, shotgun sequencing of *S. marcescens* db11, a spontaneous mutant of insect pathogen *S. marcescens* db10 that is resistant to streptomycin, has begun. At this writings there were 80,227 reads totaling 51.619 Mb and giving a theoretical coverage of 99.99% of the 4.6 Mb genome (http://www.sanger.ac.uk/Projects/S_marcescens/). Comparative analyses of the known bacterial genome sequences are leading us away from the view that bacterial genomes are static, monolithic structures, and towards the view that they are relatively fluid or plastic structures. Genome plasticity is manifested not only in the acquisition or loss of genetic information but also in large-scale rearrangements affecting genomic organization.

Chromosomal rearrangement. Recombination between homologous DNA regions may cause inversions, deletions, and duplications, and generate variation across large molecular distances. Chromosomal rearrangement has occurred frequently within the family *Enterobacteriaceae*. Spontaneous chromosomal duplications are common in populations of *E. coli* and *Salmonella typhimurium.* The chromosomal maps of *S. typhi,*

S. paratyphi, S. gallinarum and *S. pullorum* show rearrangements due to homologous recombination between rRNA genes (73).

Transposable elements. Transposable elements such as insertion sequences (IS) and composite transposons are another source of DNA variability. Naas et al. (59) observed a high degree of genetic diversity in bacterial subclones of a stab culture of *E. coli* K-12 that had been stored for 30 years. The authors suggested that the genetic diversity was caused mainly by transposition of IS elements. In addition, the degree of diversity was dependent upon the type of IS. Inactivation of metabolic, regulatory, or virulence genes of *E. coli* following insertion of IS has been described (11). Transposon rearrangements also exist for the aerobactin gene cluster in the pathogenicity island SHI-2 in *Shigella flexnerii* strain SA100, which is flanked by two copies of IS2 (83).

Conjugative transposons. Conjugative transposons are able to directly mediate horizontal gene transfer. Hochhut et al. (35) provided evidence that a self-transmissible element in *Salmonella* strains is in fact a conjugative transposon. This element, termed CTnscr94 and carrying genes for a sucrose metabolic pathway, integrates at two specific attachment sites into the *E. coli* chromosome, independent of RecA. R391 is another putative conjugative transposon found in *Proteus rettgeri* (56).

Plasmids. Pathogenic strains of *Enterobacteriaceae* often harbor plasmids mediating special pathogenicity traits. Examples are the EAF-plasmid of *E. coli* (82), and plasmid p0157 of enterohemorrhagic *E. coli* O157:H7 (31).

Glare (28) reported that the ability of *Serratia entomophila* and *S. proteamaculans* to cause amber disease, which causes the death of the New Zealand grass grub, *Costleytra zealandica*, is dependant on the presence of a large plasmid. The transfer of this plasmid alone to several other *Enterobacteriaceae* resulted in the ability of the transformed strains to cause the disease. Al-Harithy et al. (2) analyzed conjugative R plasmids derived from 74 clinical isolates of *S. marcescens.* They found that the phenotypes of different isolates correlated with the genetic pattern of R plasmids, and 27 percent of resistant strains transferred 32 R plasmids to *E. coli* or *Klebsiella* by mixed culture.

The integration of plasmids into the chromosome has been observed in *S. flexneri.* Insertion of a 220 kb plasmid alters the plasmid-encoded virulence factors (90). The *Y. pestis* plasmid pMT1 also integrates into the bacterial chromosome, but the virulence factors are not destroyed. All the known sequences of enterobacterial virulence plasmids point to a mosaic structure, in which virulence genes are often flanked by IS elements (11).

Bacteriophages. There are many examples of phage-mediated gene transfer between different bacterial species and even genera, e.g. between *Salmonella* and *E. coli* by phage P22 (61). The transferred genes may be virulence factors. It was observed that Shiga-toxin-producing *E. coli* is able to spread the toxin among *E. coli* strains. Figueroa-Bossi et al. (25) described two *S. typhimurium* phages, Gifsy-1 and Gifsy-2, which influence the virulence of *Salmonella* strains in mice. Another example is phage SopΦ, which encodes an effector protein translocated by a type III secretion system. SopΦ is able to infect a wide range of Gram-negative bacteria including *Shigella, S. marcescens, S.*

typhymurium, Klebsiella pneumoniae, and *Yersinia*. The horizontal transfer of the *SopE* gene into other *Enterobacteriaceae* by this phage supplies the recipient bacteria with effector proteins for interaction with host cells (52). Prophages can constitute as much as 10-20% of a bacterium's genome and are major contributors to differences between individuals within species. Many of these prophages appear to be defective and are in a state of mutational decay. Prophages, including defective ones, can contribute important biological properties to their bacterial hosts (16). Besides fully functional prophages, four additional types of prophage-related entities have been characterized: defective and satellite prophages, bacteriocins and gene transfer agents.

Pathogenicity islands (PAIs). The concept of a PAI was first established by Jorg Hacker and his colleges (32). When investigating the genetic basis of virulence of uropathogenic *E. coli* (UPEC) strains 536 and J96, this research group observed a genetic linkage of determinants encoding P fimbriae, P-related fimbriae and hemolysins. This linked group of genes was later called a PAI.

Schmidt and Hensel (75) summarized the structure of PAIs as follows: (i) PAIs carry one or more virulence genes; (ii) PAIs are present in the genomes of pathogenic bacteria but absent from the genomes of nonpathogenic representatives of the same species; (iii) PAIs occupy a large genomic region; (iv) PAIs often differ from the core genome in their base composition and may also prefer different codons for translation; (v) PAIs are often located adjacent to tRNA genes; (vi) PAIs are frequently associated with mobile genetic elements; (vii) PAIs are unstable and delete with high frequencies; (viii) PAIs often show

a mosaic structure rather than comprising homogeneous segments of horizontally acquired DNA.

Gain or loss of PAIs is thought to be a major step in the evolution of pathogenic *Enterobacteriaceae*. Incorporation of a PAI could transform a normally benign organism into a pathogen in a single step. The locus of enterocyte effacement (LEE) was initially described in enteropathogenic *E. coli* (EPEC) strains, the causal agents of infant diarrhea (49). It was demonstrated that all the genes necessary for the pathogenicity are located on LEE. Horizontal transfer of the LEE island in vitro confers the whole pathogenicity phenotype to benign laboratory *E. coli* strains (50). PAIs themselves are subject to variation. The aerobactin-encoding island of *Shigella*, SHI-2, was recently sequenced from two different *S. flexneri* strains belonging to serotypes 2a and 5 (54). Whereas the region between *selC* and the aerobactin operon was nearly identical in the two strains, the region 3' to the aerobactin operon was markedly different.

With the acquisition of an increasing amount of genomic sequence information, it became clear that the genomes of prokaryotes are highly diverse mosaic structures. A more ubiquitous occurrence of entities with properties similar to PAI was discovered. The designation PAI has been extended to include "genomic islands", which encode a wide range of functions. Hacker and Carniel (33) proposed a model for the development of specialized genomic islands. In this model, a bacterial cell first acquires blocks of genes. Selection processes may favor the maintenance and development of genomic islands that increase fitness. The "fitness islands" may then specialize as saprophytic, symbiotic or pathogenic islands.

Genome-wide Comparison Strategies

Physical and integrated maps. Pulsed-field gel electrophoresis of macro-restriction fragments, the first method enabling whole-genome comparisons, has been used as a tool for molecular epidemiological and population-genetic studies of *S. marcescens* (37). It has also been used to dissect the *Salmonella typhimurium* genome by production of physical maps and restriction fragment catalogs (87). The physical maps were useful for estimating genome size and structure, and provided a scaffold for establishing integrated maps of ordered cosmid and bacterial artificial chromosome (BAC) libraries, which are essential for the rapid completion of genome sequences.

Microarrays. DNA microarrays could be used to compare genomes between target bacteria and closely related, but sequenced, strains. Ge et al. (27) constructed the first *Rickettsia prowazekii* microarray and used it to compare genomic variation between the virulent Breinl and avirulent Madrid E strains, whose genome has been sequenced. An oligonucleotide-based Affymetrix GeneChip, in conjunction with powerful informatics, can accurately identify deletions in a region as small as 300 bp (72). But because of cross-hybridization, it is difficult to use microarrays to detect deletions in multi-gene families, insertions, inversions and duplications (10).

Subtractive hybridization. Genes that are present in certain isolates of a given bacterial species and absent from or significantly different in others can be of great interest biologically. It is impractical and expensive to sequence the entire genomes of multiple strains of a species. Subtractive hybridization allows strain specific DNAs to be

selected directly and is attractive because it eliminates the need to score any particular phenotype or to do extensive mapping or sequencing (78). A sensitive PCR-based version of this technique, suppressive subtractive hybridization (SSH), has been widely used to compare genomes of closely related bacteria (4 22). SSH allows researchers to rapidly sequence and identify unique regions in the target bacterial chromosomes or plasmids. It is especially useful to derive information on differences in life style and metabolism of related bacterial strains when one strain's genome has been sequenced.

Computational analysis. The alignment of complete genome sequences is the ultimate DNA-based comparative strategy. With the growing number of completed genome sequences, and advances in bioinformatics, highly refined comparisons of sequence variation between two strains are possible using genome alignment tools such as GLIMMER (20). This is by far the most informative approach and its power is increasing as more sequences become available. At this time, the genome-sequencing project of *S. marcescens* db11 is at the final stage of completion (http://www.sanger.ac.uk/Projects/S_marcescens/).

Chromosomal Mutation Strategies

Forward and reverse genetics are the two primary ways to link the sequence and function of a specific gene. In reverse genetics, typically scientists start with the selection of a specific sequence and try to gain insight into the phenotypic change caused by targeted gene disruption. Other approaches, such as antisense, cosuppression and RNA interference strategies also may be used (48). These approaches rely on sequence

information as retrieved from the genome and expressed sequence tag (EST) sequencing or transcription profiling projects. On the other hand, forward genetics is used to identify the sequence change that underlies a specific mutant phenotype. The starting point is an already available or a predicted phenotypic mutant of interest. In bacterial gene function analysis, both reverse and forward genetics depend heavily on mutagenesis.

Two methods are frequently used to generate bacterial genetic mutants. One involves rec-independent transposons and the other achieves gene disruption by rec-dependent recombination of cloned genomic DNA with a homologous chromosomal locus. Mutation can be random or targeted. Whether targeted or random, the successes of mutation are impacted by the general ability to deliver DNA into the specific bacterial cell.

Transposon-based gene disruption. Transposon mutagenesis is especially useful for bacterial species with poorly described genetic systems or when existing molecular tools are inadequate. Historically, there were often difficulties with this strategy. Problems include high degrees of insertion specificity, limited host range, instability after transposition. These problems were largely resolved with the development of minitransposons (38) and self-cloning transposons (51). Minitransposons are specialized transposons that arrange the cognate transposase outside of the transposon's inverted repeats**.** A self-cloning transposon has a conditional origin of replication within the basic transposon, so that the disrupted gene could be easily isolated from the genomic library of the mutant. Dennis (21) developed a modular self-cloning minitransposon, called a plasposon, for rapid genetic analysis of gram-negative bacterial genomes. This new

synthetic transposon has been successully used to engineer *Burkholderia* (17) and *Yersinia* (67).

In vitro transposition reactions have been used to generate genome-wide insertion mutations in a diverse group of bacteria (29). Typically, purified genomic DNA of the target organism is subjected to *in vitro* transposition, followed by transformation of the mutated DNA into the host with selection for a marker on the transposon. A DNA transposition complex, called a transpososome, could be constructed *in vitro* in the absence of divalent metal ions that are essential for progression of the transposition reaction to completion. The preassembled transpososomes are readily transformable and, once in the metal ion-rich intracellular environment, produce normal chromosomal transposon insertions (24). Commercial kits for performing *in vitro* mutagenesis using Tn*5* (Epicentre Technologies, Madison, WI), Tn*7* (New England Biolabs, Beverly, MA) and Mu (Invitrogen, Carlsbad, CA) transposons are now available and can be easily adapted to deliver the antibiotic resistance marker of choice. A limiting factor in transposome mutagenesis is the efficiency of transfer of the transposome particle into the cell. Mutagenesis frequencies in *E. coli* are approximately 100-fold less than electroporation frequencies of standard plasmid vectors (29).

Homologous recombination-based mutagenesis. Gene disruption by homologous recombination relies on crossover events between cloned genomic DNA and a cognate chromosomal locus. Target genes are disrupted by a single crossover or by exchange of the target locus with the plasmid-borne allele (via a double crossover). Both methods require extensive vector constructions.

A PCR mediated gene replacement method was established to simplify the bacterial genetic modification (19 57 58). This method utilizes the Red recombination system, encoded by bacteriophage lambda genes *gam*, *bet* and *exo,* that operates on linear DNA. Electroporation is used to introduce a linear DNA fragment carrying the synthesized mutation directly into the cell, where the three lambda genes are induced to assist recombination. Incorporation of the mutation into the chromosome occurs where two crossovers flank the mutant site. The PCR-mediated gene replacement is limited by the efficiency of electroporation.

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CHAPTER II

INTRODUCTION

A vine decline of melons, squashes and pumpkins known as cucurbit yellow vine disease (CYVD) has been consistently associated with rod-shaped bacteria in plant phloem elements (2). A bacterium was cultured from diseased plants, and Koch's postulates were completed by mechanical inoculations and by transmission via an insect vector, the squash bug, *Anasa tristis*, confirming that this bacterium is the causal agent of CYVD (3). Avila et al. (1) found that the CYVD organism was detectable by polymerase chain reaction (PCR) using nonspecific primers designed from prokaryotic 16S r DNA. The deduced nucleotide sequence for 16S ribosomal DNA placed this organism within the gamma-3 proteobacteria, with the nearest apparent relative being *Serratia marcescens*. Sequence analysis showed that there were limited differences between the 16S rDNA sequence of this organism and that of the *S. marcescens* type strain. Based on these differences Avila et al. (1) were able to design more specific primers, designated YV1, YV2 and YV3, for detecting CYVD. These primer sets worked in most cases for detecting the yellow vine causal agent, but these primers could not differentiate some nonphytopathogenic *S. marcescens* strains from CYVD strains. Thus one of my major objectives was to develop a more discriminatory method to detect CYVD strains. Based on a genetic marker identified by suppressive subtractive hybridization we designed a CYVD strain specific primer set. A multiplex PCR employing this primer set and a species-specific primer set were able to discriminate between the CYVD causal agent and bacteria from other ecological niches (Chapter IV).

Two CYVD strains, W01-A and Z01-A, were isolated from diseased watermelon and zucchini, respectively. To substantiate the placement of the CYVD strains within the genus *Serratia* and to ascertain its phylogenetic position, Rascoe et al. (4) sequenced two highly conserved genomic regions, 16S rDNA and *groE*, for the two CYVD isolates as well as for eight *S. marcescens* isolates from different environmental niches. The data were used to establish phylogenetic trees reflecting the relationships among these isolates as well as their relationships with other members of the *Enterobacteriacae*. All the trees confirmed that W01-A and Z01-A were almost identical and were indeed *S. marcescens* strains. Of the strains used in his study, human clinical isolates H01-A and H02-A and *S. marcescens* type strain 14880 showed the closest relationship with these two CYVD isolates.

Phenotypic typing of W01-A and Z01-A using other methods, such as Biolog, Vitek, API-20 and fatty acid analysis, also has been done (4). These two isolates were unidentifiable by Vitek. W01-A was identified as *Alcaligenes xylosoxydans* by three separate fatty acid analyses (FAME, performed by E. Dickstein, University of Florida), and as *Aeromonas veroni* using Biolog (Biolog Inc., Hayward, CA, also performed by E. Dickstein). Isolate Z01-A was identified as *Pantoea agglomerans* using API-20, as *A. xylosoxydans*, *Edwardsiella ictaluri*, and *Proteus vulgaris* in three separate fatty acid analyses, and as *V. cholerae* using Biolog. The results indicate that the metabolic capabilities and fatty acid profiles of these cucurbit bacteria are different from those of the type strain *S. marcescens*, although the microbes are clearly identified as *S. marcescens* by 16S r DNA and *groE* sequence data. Surprisingly, W01-A and Z01-A were unable to metabolize some substrates usually considered diagnostic for *S.*

marcescens. For example, neither CYVD isolate tested positive for the presence of DNase and oxidase.

There are at least two possible explanations for this apparent disparity: i) the preservation of 16S rDNA and *gro*E sequences may not represent the overall genome similarity and the phylogenetic status; ii) CYVD strains are descendants of *S. marcescens* but their genomes may have changed significantly overall, and the gain of virulence to cucurbit plants was due to a genomic insertion. In this study we used both rep-PCR and DNA-DNA hybridization (The latter experiment was done by Dr. Robert Weyant's research group at the Centers for Disease Control and Prevention, Atlanta, GA, as a part of a collaboration) to confirm the identity of CYVD strains and to examine the taxonomic position of the CYVD bacteria. In addition, genetic relationships among CYVD strains were also evaluated by these two methods to seek for the clues as to their site of origin, pattern of spread, and disease epidemiology. Our results demonstrated that CYVD associated strains clustered together as a *S. marcescens* subgroup significantly different from other strains of the species (Chapter III, already published in Phytopathology (6)).

The ultimate goal is to identify the pathogenicity determinants of CYVD strains. *S. marcescens* DNA variability may arise from plasmids, bacteriophages, transposable elements and pathogenecity islands (PAIs), through which the trait of pathogenicity was obtained. Suppressive subtractive hybridization is a powerful method for identifying genetic differences between closely related organisms. In this study, we subtracted the genome of a *S. marcescens* rice endophytic strain, IRBG502, from that of a CYVD strain, Z01-A. A library of DNA sequences specific to CYVD pathogenic strains was obtained. Sequence analysis showed that the majority of the sequences resembled genes involved in

the synthesis of bacterial surface molecules. By fosmid library construction and dot hybridization we identified two fosmid clones, FOSU1 and FOSU2, which contain multiple Z01-A specific sequences. Both fosmid clones were sequenced by Bruce Roe's laboratory at the University of Oklahoma. Interestingly, a phage gene cluster and a genome island containing a type 1 fimbrial (pilus) gene cluster were identified on FOSU2 and FOSU1, respectively. These gene clusters and other CYVD strain specific genes are good candidates for further functional analysis aimed at deciphering the CYVD phytopathogenicity (Chapter IV).

A different strategy of analyzing *S. marcescens* pathogenicity is to randomly knock out bacterial genes and screen for virulence defective or attenuated mutants. In this work, insertion mutants of Z01-A were obtained by conjugational transposon mutagenesis (Chapter V). Bacterial mutant phytopathogenicity screening, however, is extremely difficult because of the low percentage of successful plant inoculations and the inability to obtain full symptom expression following mechanical inoculation. For that reason we explored an alternative screening method. For certain pathogens capable of infecting a broad range of organisms, there exist universal virulence factors necessary for full pathogenicity regardless of the host (5). We thus evaluated the utility of *Caenorhabditis elegans,* an economic free-living worm, as an *in vivo* model for the study of *S. marcescens* virulence factors (Chapter V). Both killing assays and feeding inhibition assays showed that CYVD strains were only moderately toxic to *C. elegans*. In the future, experiments with more replications and optimized assay conditions may provide further information for evaluating this screening model.

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Phytopathology 93:1240-6

CHAPTER III

GENOTYPING OF *SERRATIA MARCESCENS* STRAINS ASSOCIATED WITH CUCURBIT YELLOW VINE DISEASE

Abstract

The bacterium that causes cucurbit yellow vine disease (CYVD) has been placed in the species *Serratia marcescens* based on 16S rDNA and *groE* sequence analysis. However, phenotypic comparison of the organism with *S. marcescens* strains isolated from a variety of ecological niches showed significant differences. In this study, we compared the genomic DNA of *S. marcescens* strains from different niches as well as type strains of other *Serratia* species through rep-PCR. CYVD strains showed identical banding patterns despite the fact that they were from different cucurbit hosts, geographic locations, and years of isolation. In the phylogenetic trees generated from rep-PCR banding patterns, CYVD strains were clearly differentiated from other strains but formed a loosely related group with *S. marcescens* strains from other niches. The homogeneity of CYVD strains was further supported by a DNA relatedness study (done by Dr. R. Weyant's research group as a part of the collaboration) in that labeled DNA from the cantaloupe isolate, C01-A, showed an average relative binding ratio (RBR) of 99% and 0.33 percent divergence to other CYVD strains. Used as a representative strain of CYVD, the labeled C01-A had a RBR of 76% and a 4.5 percent divergence to the *S. marcescens* type strain. These data confirm the previous placement of CYVD strains in species *Serratia marcescens*. Our investigations, including rep-PCR, DNA-DNA hybridization

and previous phenotyping experiments, have demonstrated that CYVD associated strains of *S. marcescens* cluster together in a group significantly different from other strains of the species.

Introduction

Cucurbit yellow vine disease (CYVD) was first observed in squash (*Cucurbita maxima*) and pumpkin (*C. pepo*) in 1988 in Oklahoma and Texas (7), but is now known to affect other cucurbits including watermelon and cantaloupe. The disease has been confirmed in Arkansas (J. C. Correll, personal communication), Tennessee (4), Massachusetts (26), Kansas, Colorado and Nebraska (diagnosis of CYVD in the latter three states is unpublished). Affected plants exhibit characteristic symptoms of yellowing, stunting, gradual decline and phloem discoloration. Losses can range from less than 5% to 100% in affected fields.

Disease symptoms are consistently associated with the presence in the phloem of a rod-shaped, Gram-negative bacterium, detected using transmission electron microscopy (7). The bacterium was cultured, and Koch's postulates were completed by mechanical inoculations and by transmission via an insect vector, the squash bug, *Anasa tristis*, confirming that this bacterium is the causal agent of CYVD (6).

Two CYVD pathogenic strains, W01-A and Z01-A, were originally isolated from diseased watermelon and zucchini, respectively. Sequence analysis of 16S rDNA and *groE* gene fragments of these two strains indicated that they shared more than 97% sequence similarity with the type strain of *Serratia marcescens* (19). The possible identity of the CYVD bacterium as *S. marcescens* was unexpected. Different strains of

this species can assume roles as soil saprophytes (17), plant endophytes (24), insect pathogens (8), and even opportunistic human pathogens (25), but the only previous reports of plant pathogenicity for this species, in sainfoin (21) and alfalfa (13), were made when the tools of bacterial identification were less definitive than those of today.

Biological characterization of Z01-A and W01-A using BIOLOG and fatty acid profiling showed that, despite the strong similarity of their 16S rDNA and *groE* genes to those of other *S. marcescens*, CYVD strains were quite unique in some other respects, lacking a number of metabolic functions and possessing different lipid complements than those present in *S. marcescens* strains from other niches (19).

This apparent disparity led us to seek a more definitive confirmation of the identity of CYVD strains and to examine the taxonomic position of the CYVD bacteria based on techniques that take into consideration the sequence and organization of the entire chromosome. In addition, the broad geographic distribution and potential devastation of this disease make it important to better understand the genetic relationships among CYVD strains, as these may provide clues as to their site of origin, pattern of spread, and disease epidemiology.

Repetitive elements-based polymerase chain reaction (rep-PCR) generates DNA fingerprints by amplifying different-sized DNA fragments lying between the repetitive elements in a genome. The amount of differentiation among the tested strains depends on the primer used, but overall the rep-PCR banding patterns of strains from the same bacterial species can be quite diverse and often reveal relationships not seen by other methods. This method has been used successfully to fingerprint clinical isolates of *S. marcescens* (18). DNA-DNA hybridization, on the other hand, has the advantage of

comparing organisms at the whole chromosome level. The goals of this work were to type CYVD strains, based on repetitive elements, and to determine the DNA relatedness among CYVD strains as well as between plant pathogenic strains and those from other niches.

Materials and Methods

Bacterial isolates and growth conditions. Tested bacteria included type strains of several *Serratia* species, several *S. marcescens* strains from different ecological niches, and several CYVD isolates from different cucurbit hosts, geographical locations and years of isolation (Table 1). Bacteria were stored, -80ºC, in aliquots in 1.5 ml LB broth containing 15% glycerol. For use in experiments, bacteria were streaked onto Luria-Bertani (LB) agar (20) and incubated at 28ºC for 24 h.

DNA isolation. Single colonies were transferred to 5 ml of LB broth and incubated at 28ºC, shaking at 220 rpm, for 18 hr. DNA was extracted using a modified version of the hexadecyl trimethyl ammonium bromide (CTAB) method (2). Briefly, 5 ml of bacterial culture were centrifuged 5 min at 10,000 g, 4ºC, the supernatant was removed and the pellet was resuspended in 200 µl TE buffer (10mM Tris-HCl, 1 mM EDTA, pH 8.0). The mixture was incubated 30 min at 60ºC, then 100 µl of 5 M NaCl and 80 µl CTAB solution (10% CTAB in 0.7 M NaCl) were added and mixed, and the suspension was incubated at 60ºC for 10 min. The solution was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The aqueous phase was mixed with 0.6 vol isopropanol, and DNA precipitated at -20ºC for at least 15 min. DNA was collected by

centrifugation (20,000 x g, 15 min, 4ºC) and washed with 70% ethanol. The DNA pellet was air dried at room temperature for 15 min and was then dissolved in 100 ul TE buffer (pH 8.0). The DNA solution was stored at -20ºC until use.

Rep-PCR. The BOX primer (14), PGRS primer (18) and ERIC primers (11) were synthesized by the Oklahoma State University Recombinant DNA/ Protein Resource Facility. Amplification was performed with each of three primer sets: BOX/BOX, PGRS/PGRS, and ERIC1R/ERIC2. Each reaction consisted of 5 µl 1x Gitshier buffer (16.6 mM $[NH_4]_2SO_4$; 67 mM Tris-HCl, pH 8.8; 6.7 mM $MgCl_2$; 6.7 mM EDTA; 30 mM β-mercaptoethanol), 10% bovine serum albumin, 10% dimethyl sulfoxide (v/v) (Fluka Chemical Corp., Ronkonkoma, NY), 50 pmoles of primer, 125mM of each dNTP, 2 units of Taq polymerase (Promega, Madison, WI), and 1 µl of the bacterial DNA solution. The total volume per reaction was 25 µl. Amplification was carried out in a DNA thermal cycler 480 (Perkin-Elmer Cetus, Norwalk, CT). Reaction conditions were slightly modified from previous studies (18). With all three primer sets, each round of amplification began with initial incubation at 94ºC (3 sec) and 92ºC (30 sec). This was followed by 52ºC (1 min) and 65ºC (8 min) for the BOX primer, 50ºC (1 min) and 65ºC (8 min) for the ERIC primers, or 54ºC (1 min) and 72ºC (8 min) for the PGRS primers. A total of 35 cycles was used. An 8 µl aliquot of the PCR product was analyzed on a 1.5% agarose gel, electrophoresed at 70 V (4 V/cm) for 5 h in 1x TAE buffer (pH 8.3). Gels were stained with 0.5 µg/ml ethidium bromide and differences in DNA fingerprint patterns were assessed visually.

Comparisons of DNA fingerprint patterns were performed by measurement of band positions of PCR products. The presence or absence of each band in each bacterial strain

was converted into binary data (1 for presence and 0 for absence). These data were then used as an input file for the MIX parsimony program of PHYLIP (10) to generate a phylogenetic tree. In cases of multiple data sets the sets were combined to generate a consensus tree using CONSENSE (9). Finally all the trees were visualized using the TREEVIEW program (16). The reliability of the dendrogram was assessed by a bootstrap analysis with the SEQBOOT program (9). One thousand repeated samplings with replacement were made in each analysis. The frequencies with which each group was formed in repeated cycles of dendrogram construction were used as a measure of the relative reliability of the clusters of the strains.

DNA-DNA hybridization. Purification of genomic DNA was performed using the method of Brenner et al. (5). Briefly, the bacterial cells were lysed with 0.05 M TE buffer containing 0.5% SDS and 50 µg/ml pronase, followed by phenol extraction. DNA from the aqueous phase was then precipitated with 95% ethanol and washed with 70% ethanol. The resulting crude DNA solution was then incubated overnight at 37ºC with 50 µg/ml RNase containing SDS (0.5% final concentration) and pronase (50 µg/ml final concentration). The DNA was extracted once with phenol and twice with chloroform, followed by three precipitations with ethoxyethanol. DNAs were sheared by sonication at 4ºC (9).

The procedures used for the determination of DNA relatedness by hybridization in free solution by the hydroxyapatite method have been described previously (5). The DNAs were labeled enzymatically in vitro with [^{32}P]dCTP by nick translation. DNA-

DNA hybridization experiments were performed at 60ºC for optimal DNA reassociation and also at the stringent temperature of 75ºC. The percent divergence of hybrids was calculated as decrease of 1ºC in thermal stability of a heterologous DNA duplex, which correlates to approximately 1% unpaired bases within related DNA. The guidelines of Wayne et al. (23) were used in defining hybridization groups. These guidelines indicate that strains within the same species should have a DNA relatedness, as measured by relative binding ratio (RBR), of greater than or equal to 70% and a divergence less than or equal to 5%. The experiments of DNA-DNA hybridization were performed twice.

Results

Rep-PCR. A total of 29 CYVD associated *S. marcescens* strains, 16 CYVD non-associated *S. marcescens* strains and five other *Serratia* type strains were tested by Rep-PCR. Each reaction was repeated at least three times and only the repeatable banding patterns were used for phylogenetic analysis. The PCR products of all 29 CYVD associated *S. marcescens* strains, regardless of the geographic location, plant host species or collection date, showed a homogeneous pattern (data not shown). Five of these strains were finally selected to compare with other strains (Figure 1.A, 1.B, 1.C, lanes 1-5) and to generate phylogenetic trees (Figure 2). *S. marcescens* isolates from other ecological niches, as well as the type strains of different *Serratia* species, showed a variety of patterns, all of which were different from those of CYVD isolates (Figure 1.A, 1.B, 1.C, lanes 6-24).

Distinguishable banding patterns were generated in all PCR primer combinations. For reactions using PGRS and BOX primers, the size of the amplicons ranged from 0.5 kb to

6 kb. Two rice endophyte strains, R02-A (IRBG 502) and R03-A (IRBG 505), showed identical patterns using both PGRS and BOX primers. Two insect pathogenic strains, I06-A and I09-A, were also identical with each other. Two bands generated from PGRS PCR, with sizes of 1.2 kb and 1.4 kb, respectively, were shared by all the *S. marcescens* strains. In BOX-primed PCR one band of 0.6 kb was shared by all the *S. marcescens* strains. Twenty-seven bands were amplified using ERIC primers, and the size of amplified DNA ranged from 0.3 kb to 5 kb. Although fewer bands were generated from ERIC primers, the banding patterns of *S. marcescens* appeared to be as diverse as those from BOX and PGRS reactions.

The banding patterns of BOX, PGRS and ERIC PCR products were each scored separately as binary data. Phylogenetic trees were generated for each of the three rep-PCR reactions. The three trees were similar in that the CYVD strains grouped together within a large branch containing the *S. marcescens* type strain (data not shown). When the data from all three PCR reactions were combined, the consensus phylogenetic tree was similar to the trees generated from individual reactions except that branches with low bootstrap value were collapsed. All the *S. marcescens* strains tested fell into two groups.

DNA-DNA hybridization. DNA-relatedness studies were conducted using labeled reference DNA from C01-A, the *S. marcescens* type strain, G03-A (JM-983), the *S. proteomaculans* type strain and G02-A (JM-965), respectively (Table 2). As a representative CYVD strain, C01-A, from cantaloupe, was hybridized to all the strains listed in Table 2 at both 60ºC and 75ºC, while the other labeled reference strains were hybridized only at 60ºC to a limited number of strains as shown. At the optimal

reassociation temperature (60ºC), labeled C01-A DNA showed an average RBR of 99% (range from 94 to 100%) and 0.33 percent divergence (from 0.0 to 1.0) to other CYVD strains. At the same temperature, C01-A hybridized with the *S. marcescens* type strain with a 76% RBR and 4.5 percent divergence. In addition, C01-A DNA had an RBR of 73 to 90% relative to all the other *S. marcescens* reference strains, with an average percent divergence of 2.1 (range from 1.0 to 3.5). In contrast, the relatedness values between C01-A and type strains of other *Serratia* species were well below 70%, and the percent divergences were 10.5 to 13.5. Labeled DNA from the *S. marcescens* type strain hybridized to unlabeled C01-A DNA with a 69% RBR, slightly lower than the 76% RBR obtained when labeled C01-A DNA reacted with the type strain. However, the percent divergences obtained from both reactions were lower than 5.0. According to the criteria of species definition (23), our data clearly illustrate that C01-A and other CYVD strains group together and belong to species *S. marcescens*. In more stringent hybridization reactions carried out at 75ºC, labeled C01-A DNA showed an average RBR of 72% (range from 62 to 88%) to *S. marcescens* DNA from different niches, which reflects the reliability of results obtained at 60ºC.

Discussion

As a PCR based fingerprinting method, rep-PCR has the advantages of convenience and sensitivity. In our study 29 CYVD strains showed 100% similarity by rep-PCR despite the fact that they were collected from different plant species, in different years and at different geographic locations. This method clearly discriminated CYVD-associated strains from strains of *S. marcescens* not associated with CYVD.

Rep-PCR, based on repetitive elements such as ERIC, BOX and PGRS, has been used to study the epidemiology of a wide array of microorganisms. These three families of unrelated repetitive DNA sequences vary in location throughout the chromosome and show different discriminatory ability depending on the species examined. In this study ERIC PCR generated fewer bands than did BOX and PGRS, similar to the observation of Patton et al. (18) for *S. marcescens* but in contrast to a previous study by Liu et al.(12) using the same species. Nevertheless, the phylogenetic trees generated in our study from PCR patterns amplified by all three primer sets were very similar. The final consensus phylogenetic tree generated from rep-PCR banding patterns was also similar to the ones generated from 16S rDNA and *gro*E sequence analysis (19). All the CYVD strains were identical to each other and fell into a group containing CYVD non-associated *S. marcescens* strains.

Results from the DNA-DNA hybridization study also illustrate that CYVD strains form a closely related group. As a reference strain of this group, C01-A was more than 70% related to the *S. marcescens* type strain, with a percent divergence lower than 5.0. This result supported the placement of CYVD strains in the species *S. marcescens*. In a previous study (19) the biological tests BIOLOG, Vitek, API-20E and FAME indicated that the metabolic capabilities and fatty acid composition of the CYVD strains were quite different from those of CYVD non-associated strains, including the *S. marcescens* type strain. The approximately 25-30% difference at the genome level might be responsible for these phenotypic differences.

The DNA relatedness between C01-A and other CYVD strains ranged from 94% to 100%, as shown by DNA-DNA hybridization. This result is in contrast to the unanimous

identity revealed in rep-PCR analysis. Considering the facts that in rep-PCR the amplicons may only represent a small portion of the genome, and bands with the same electrophoretic mobility were not compared for DNA sequence similarity, we believe that DNA-DNA hybridization is likely a more accurate measure than is rep-PCR of the genetic complementarities. Even the lowest relatedness measured among CYVD strains (94% RBR between C01-A and P01-A) reflects the overall similarity among pathogenic strains, and the percent divergence of 0.0 suggests that the C01-A and P01-A similarity is actually even higher than suggested by the 94% RBR. This is in contrast to the results for *S. marcescens* clinical isolates, in which the rep-PCR banding patterns of different clinical isolates were quite diverse. This result suggests to us that CYVD strains of *S. marcescens* may have diverged from an ancestor much more recently than the human isolates have.

Rascoe et al. (19) found that strain G02-A (JM965), which had been tentatively identified as *S. marcescens,* is significantly different from the *S. marcescens* type strain by 16S rDNA and *groE* analysis. In this study, the 15% RBR and 13.0% divergence clearly demonstrated that G02-A belongs to a species other than *S. marcescens*. Strain G03-A (JM983), which had been tentatively designated as a strain of *S. plymuthica*, has a RBR greater than 70% with *S. proteomaculans*, suggesting that it belongs to this species.

Both rep-PCR and DNA-DNA hybridization results showed that, among the CYVD non-associated bacteria tested, the rice endophytic strains R02-A and R01-A were those most closely related to CYVD strains. Rice strain R03-A, which was not tested by DNA-DNA hybridization, was indistinguishable from R02-A based on BOX and PGRS PCR, a result in good agreement with data of Tan et al. (22), who characterized the rice

endophytic strains more fully. Like the CYVD strains, all rice strains were non-pigmented and differed from the *S. marcescens* type strain in several key nutrient utilization characteristics. Preliminary results (Bruton et al., unpublished) showed that none of the three rice strains were pathogenic to cucurbit plants. Comparing the rice endophytes with the phytopathogenic CYVD strains on the genetic level may be a starting point to the identification of virulence factors of CYVD strains.

In a separate study, Bextine et al. (3) demonstrated that the squash bug, *Anasa tristis*, is a vector of CYVD strains of *S. marcescens*. Insects of two other taxa (leafhoppers and mirids) also were able to transmit the CYVD pathogen under artificial conditions (1). However, in the work reported here, all strains isolated from field collected insects were much more distantly related to CYVD strains than were the rice endophytes. These findings suggest the possibility that *S. marcescens* strains associated with plants may have followed two routes of evolutionary adaptation, one as a plant pathogen and the other as a harmless and possibly even beneficial endophyte. They also indicate that different strains of *S. marcescens* have different types of relationships with insects that harbor them, some as entomo-pathogens and others in a vector-host relationship.

Compared to the *S. marcescens* endophyte 90-166 and clinical isolate H01-A, CYVD strains lack 30 of the 95 metabolic capabilities in the BIOLOG test (19). Whether CYVD strains fail to produce those enzymes as a result of regulatory control of gene expression or by loss of genes due to genomic rearrangements during evolution, or both, is still unclear. Ochman (15) recently suggested that changes in genome repertoire, occurring through gene acquisition and deletion, are the major events underlying the emergence and evolution of bacterial pathogens and symbionts. In the future, analysis of *S. marcescens*

from other niches that are more closely related to CYVD strains of *S. marcescens* might provide clues as to the origin and the evolutionary path of plant pathogenic strains.

Acknowledgments

We thank J. K. Ladha, J. Kloepper, A. H. Purcell, D. Adamson, S. P. Deng and American Type Culture Collection (ATCC) for providing bacterial cultures and M. Berg and P. V. Alejandro for thoughtful review of the manuscript. The work was funded by the Oklahoma State University Targeted Research Initiative Program, the Oklahoma Agricultural Experiment Station, Project #2092, and the USDA Southern Regional IPM Program.

The DNA-DNA hybridization experiment was done by Dr. Robert Weyant's research group at the Centers for Disease Control and Prevention, Atlanta, GA, as a part of a collaboration. This paper was originally published in Phytopathology (27). The complete paper is presented here for integrity.

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Table 1. Strains of cucurbit yellow vine-associated and non-associated *Serratia marcescens*, and other *Serratia* species, used in this study.

Strain		Host	Location	Collection date	Rep-PCR [h]	DNA-DNA hybridization [h]
Strain designation used in this study	Alternative designation, if any					
CYVD strains						
W01-A--W01-J [a]		Watermelon	Texas	1999	Y	Y
W04-A		Watermelon	Arkansas	2000	Y	ND
W05-A		Watermelon	Arkansas	2001	Y	Y
W08-A		Watermelon	Oklahoma	1997	Y	Y
W09-A		Watermelon	Oklahoma	1997	Y	Y
Z01-A		Zucchini	Oklahoma	1999	Y	Y
Z01-B		Zucchini	Oklahoma	1999	Y	ND
Z02-A		Zucchini	Oklahoma	1999	Y	ND
Z02-B		Zucchini	Oklahoma	1999	Y	Y
P01-A		Pumpkin	Oklahoma	1999	Y	Y
P01-B		Pumpkin	Oklahoma	1999	Y	ND
P02-A		Pumpkin	Oklahoma	1999	Y	Y
P02-B		Pumpkin	Oklahoma	1999	Y	ND
P03-A	99-2515	Pumpkin	Oklahoma	2000	Y	ND
P07-A	00B058	Pumpkin	Massachusetts	2001	Y	ND
P08-A	00B060	Pumpkin	Massachusetts	2001	Y	Y
C01-A		Cantaloupe	Oklahoma	1999	Y	Y
C01-B		Cantaloupe	Oklahoma	1999	Y	ND
C02-A		Cantaloupe	Oklahoma	1999	Y	Y
C02-B		Cantaloupe	Oklahoma	1999	Y	ND
Endophytic strains						
R01-A [b]	IRBG 501	Rice	Philippines	Unknown	Y	Y
R02-A [b]	IRBG 502	Rice	Philippines	Unknown	Y	Y

R03-A [b]	IRBG 505	Rice	Philippines	Unknown	Y	ND
G01 [c]	90-166	Cotton	Alabama	Unknown	Y	Y
G02 [c]	JM-965	Cotton	Unknown	Unknown	Y	Y
G03 [c]	JM-983	Cotton	Unknown	Unknown	Y	Y
Animal pathogenic strains						
I02-A [d]	0-8-12	Insect	California	1961	Y	ND
I06-A [d]	56-1-1	Insect	California	1960	Y	ND
I09-A [d]	73-1-1	Insect	California	1961	Y	ND
H01-A [e]		Human	Oklahoma	1999	Y	Y
H02-A [e]		Human	Oklahoma	1999	Y	Y
V01-A [e]	94100675	Feline	Oklahoma	Unknown	Y	ND
Saprophytic						
S01-A [c]	CP01(4)CU	Rhizosphere	Unknown	Unknown	Y	Y
S02-A [c]	98A-742	Soil	Florida	Unknown	Y	Y
S03-A [f]	ROX6	Soil	Oklahoma	2001	Y	ND
Serratia species						
S. fonticola [g]		spring water	ATCC #29844	Unknown	Y	Y
S. grimesii [g]		unknown	ATCC #14460	Unknown	Y	Y
S. liquefaciens [g]		milk	ATCC #27592	Unknown	Y	Y
S. marcescens [g]		water	ATCC #13880	Unknown	Y	Y
S. plymuthica [g]		water	ATCC #183	Unknown	Y	Y
S. rubidaea [g]		unknown	ATCC #27593	Unknown	Y	Y
S. ficaria [g]		unknown	ATCC #33105	Unknown	ND	Y

S. proteamaculans [g]	unknown	ATCC #19323	Unknown	ND	Y
S. odorifera [g]	unknown	ATCC #33077	Unknown	ND	Y

[a] For the cucurbit isolates, the strains were named as follows: W01-A, W=type of host plant; 01=number assigned to the plant from which the strain was isolated; A=number assigned to one particular strain isolated from that plant. W01-A-W01-J represents 10 isolates, and all other lines list only single isolates. Strains isolated from the same host, state and year were from the same field.

[b] Provided by J. K. Ladha, International Rice Reasearch Institute, Los Banos, Philippines.

[c] Provided by J. Kloepper, University of Alabama, Auburn, AL.

[d] Provided by A. H. Purcell, University of California, Berkeley, CA.

[e] Provided by D. Adamson, Medical Arts Laboratory, Oklahoma City, OK.

[f] Provided by S. P. Deng, Oklahoma State University, Stillwater, OK

[g] Provided by the American Type Culture Collection, Manassas, VA. All the strains listed are the type strain of the respective species.

[h] Y=test performed; ND=not done.

Table 2. DNA relatedness disclosed by DNA-DNA hybridization, with respect to strains of CYVD-associated and non-associated strains of *Serratia marcescens*, and other *Serratia* species.

	Source of labeled DNA										
	C01-A			*S. marcescens*		G03-A		*S. proteomaculans*		G02-A	
Source of unlabeled DNA	RBR[a] 60°C	%D[b]	RBR 75°C	RBR 60°C	%D	RBR 60°C	%D	RBR 60°C	%D	RBR 60°C	%D
CYVD strains											
C01-A	100	0.0	100	69	3.5	50	11			20	
C02-A	100	0.0	95	71	3.0						
P01-A	94	0.0	95								
P02-A	100	0.5	100								
P04-A	100	0.5									
P08-A	99	1.0	100								
W01-A	97	0.5									
W05-A	99	0.5									
W08-A	100	0.0	100								
W09-A	100	0.0	100								
Z01-A	100	0.5	99								
Z02-B	100	0.5	100								
S. marcescens											
R01-A	82	1.5	79								
R02-A	90	1.5	88								
H01-A	73	3.0	63	87	0.5						
H02-A	82	1.0	77								
G01-A	76	1.0	75								
S01-A	75	3.5	65	93	2.0						

S02-A	73	3.5	62	91	1.0						
Strains to be identified											
G02-A	15	13.0				22				100	0.0
G03-A	46	9.5				100		74	6.1	22	
Serratia type strain											
S. marscescens	76	4.5	66	100	0.0	44				18	
S. ficaria	66	10.5				52				29	
S. plymuthica	51	11				61		64	11.2	22	
S. liquefaciens	49	12.5				74	8.5	65	9.1	21	
S. proteomaculans	48	13.5				79	6.5	100	0.0	21	
S. odorifera	42	13.0				41				22	
S. grimsii	41	12.0				58		65	9.7	16	
S. rubidaea	40	12.0				42					
S. fonticola	39	12.5				40					

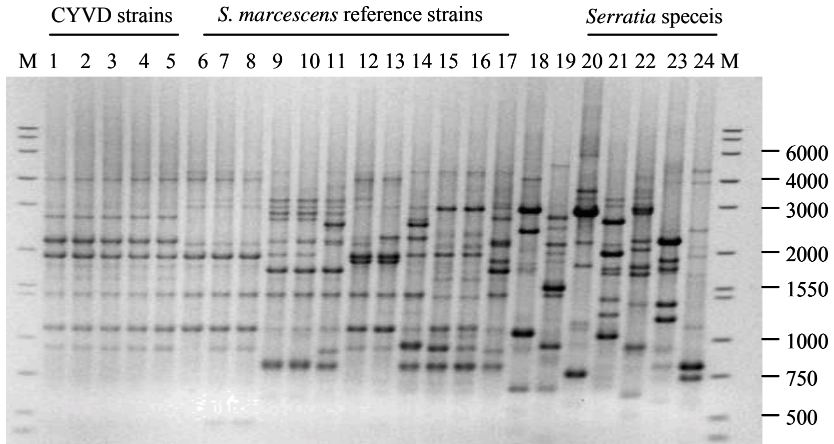
[a] RBR, or relative binding ratio, is determined by the equation: (percent bound to hydroxylapatite in heterologous reactions) / (percent DNA bound in homologous reactions) x 1000.

[b] %D, percent divergence, assumed that a 1ºC decrease in thermal stability of a heterologous DNA duplex compared with that of the homologous duplex was caused by 1% unpaired bases.

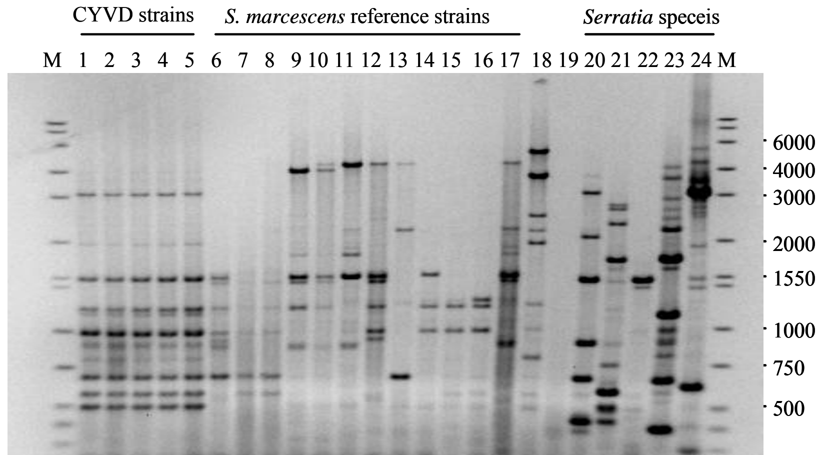
Figure 1. Agarose electrophoresis gels of rep-PCR products generated from CYVD strains, *Serratia marcescens* from various niches, and other *Serratia* type strains. A=PGRS primer, B=ERIC primers, and C=BOX primer. Lanes labeled M were run with the DNA ladder (DirectLoad™, Sigma, St. Louis, MO), sizes of fragments are listed on the right. Lane 1, C01-A; Lane 2, P01-A; Lane 3, W01-A; Lane 4, Z01-A; Lane 5, P03-A; Lane 6, R01-A; Lane 7, R02-A; Lane 8, R03-A; Lane 9, S01-A; Lane10, S02-A; Lane 11, H01-A; Lane 12, H02-A; Lane 13, G01-A; Lane 14, V01-A, Lane 15, I06-A; Lane 16, I09-A; Lane 17, *S. marcescens*; Lane 18, G02-A; Lane 19, G03-A; Lane 20, *S. grimesii*; Lane 21, *S. plymuthica*; Lane 22, *S. rubideae*; Lane 23, *S. liquefaciens*; Lane 24, *S. fonticola*.

Figure 2. Consensus phylogenetic tree of the relationships among *Serratia* strains and species, compiled from binary data based on the banding patterns of three different rep-PCR reaction sets. Branches with bootstrap values less than 500 were collapsed.

A



B



C

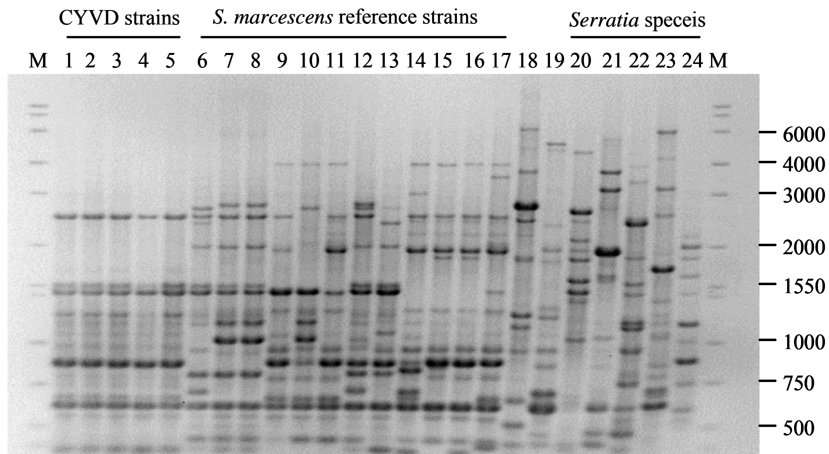


Figure 1.

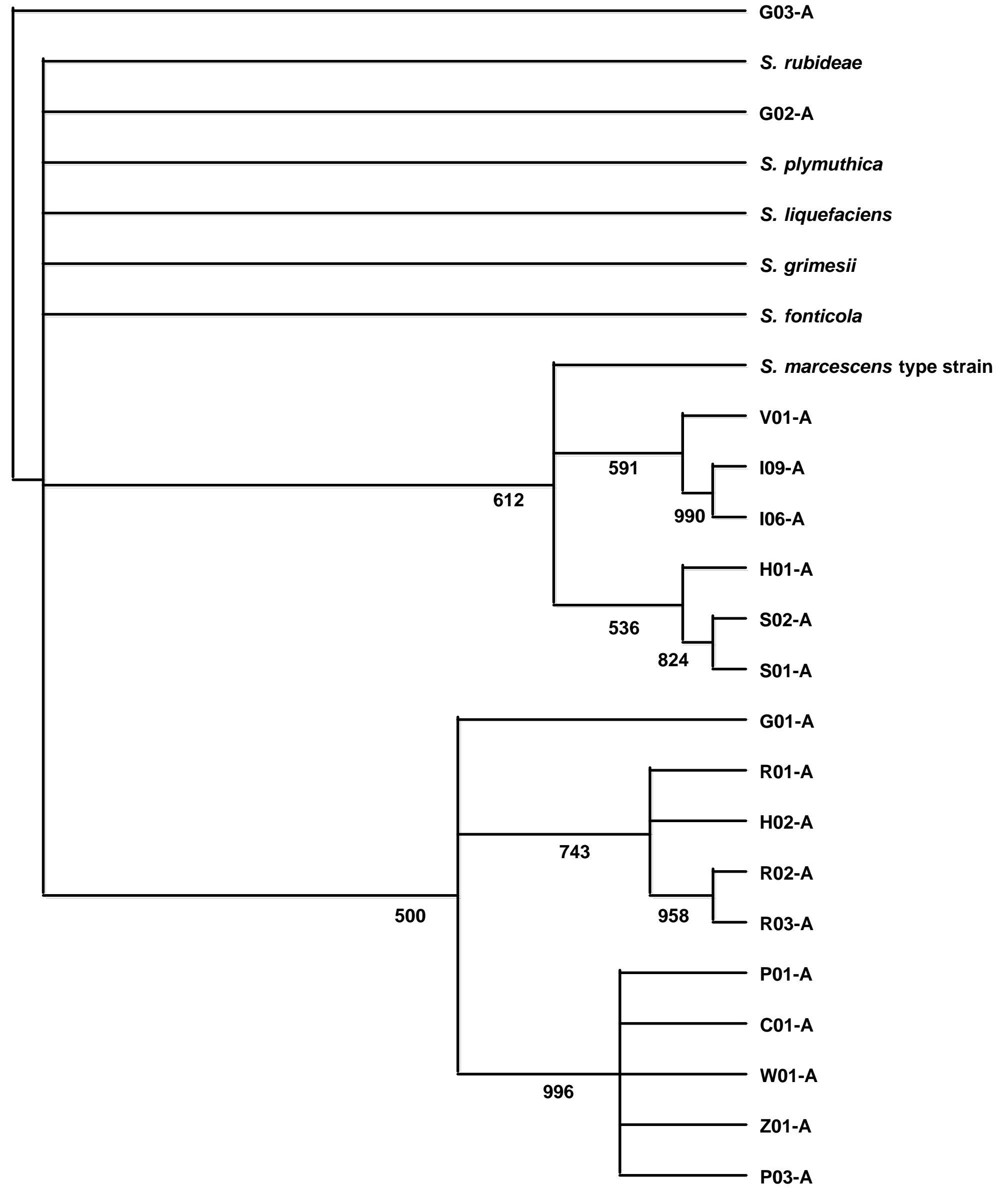


Figure 2.

CHAPTER IV

GENOMIC COMPARISON OF PLANT PATHOGENIC AND NON-PATHOGENIC *SERRATIA MARCESCENS* STRAINS USING SUPPRESSIVE SUBTRACTIVE HYBRIDIZATION

Abstract

The cucurbit yellow vine disease (CYVD) is caused by a cosmopolitan bacterium *Serratia marcescens.* CYVD-associated *S. marcescens* strains, however, are significantly different from non-phytopathogenic strains as shown by the chemical utilization phenotypes. To identify the genetic differences responsible for pathogenicity related phenotypes, we used a suppressive subtractive hybridization strategy (SSH). *S. marcescens* strain Z01-A, isolated from CYVD-affected zucchini, was used as the tester, whereas *S. marcescens* rice endophytic strain R02-A (IRBG 502) was used as the driver. SSH revealed 48 sequences that were present in Z01-A, but absent in R02-A. Sequence analysis showed that a large proportion of these sequences resembled genes involved in synthesis of surface structures. By construction of a fosmid library and colony hybridization, a phage gene cluster and a genome island containing a fimbrial gene cluster were identified. A multiplex PCR employing primers designed from a strain specific marker and a species specific marker was able to discriminate between *S. marcescens* strains causing CYVD and those from other ecological niches.

Introduction

Cucurbit yellow vine disease (CYVD) was first observed in squash (*Cucurbita maxima*) and pumpkin (*C. pepo*) in 1988 in Oklahoma and Texas (6). Since then the disease has been confirmed in nine other states (4) (29) and Fletcher, J., personal communication). CYVD is characterized by rapid and general yellowing of leaves appearing over a 3-4 day period, followed by gradual or rapid decline and death of the vine in several cucurbit crops (5).

The causal agent of CYVD was identified as the cosmopolitan bacterium *S. marcescens* (18). Non-CYVD strains of this species can assume roles as soil or water resident saprophytes, plant endophytes, insect pathogens, and even opportunistic human pathogens. None of these other strains is able to cause CYVD and phenotypic differences between them and CYVD-pathogenic strains include substrate utilization and fatty acid profiles (18). Rep-PCR fingerprinting and DNA-DNA hybridization also revealed significant differentiation between CYVD pathogenic strains and *S. marcescens* strains from other niches (31). Much remains to be learned, however, about the nature of the genetic materials that are responsible for the differences.

Suppressive subtractive hybridization is a powerful method for identifying DNA fragments that are present in one organism (tester) but absent from the other (driver), especially if these two organisms are closely related (1). It has been widely used for bacterial genome analysis to discover new epidemiological markers, virulence factors or host specificity determinants (30). In this work, my goal was to identify the genetic differences responsible for pathogenicity in cucurbits. We compared the genomes of a CYVD strain, Z01-A, and a rice endophytic strain, R02-A, in an effort to identify genes

or genetic markers present in the phytopathogenic strain but absent from a closely related nonpathogenic strain. We report the identification of a pool of DNA sequences specific to CYVD pathogenic strains. In addition, we identified a phage gene cluster and a genome island containing a type 1 fimbrial (pilus) gene cluster. Furthermore, we designed a primer set specific for CYVD strains of *S. marcescens* based on an identified genetic marker.

Materials and Methods

Strains. Test bacteria included several *S. marcescens* strains from different ecological niches, and several CYVD strains from different cucurbit hosts (Table 1). Bacteria were stored, -80ºC, in aliquots of 1.5 ml Luria-Bertani (LB) broth (22) containing 15% glycerol. For use in experiments, bacteria were streaked onto LB agar and incubated at 28ºC for 24 hr. *E. coli* was grown at 37ºC on LB agar or in LB broth.

DNA manipulation. Genomic DNA of CYVD and non-phytopathogenic *S. marcescens* strains was isolated using a bacterial genomic DNA miniprep protocol (2). Plasmid DNA of subtracted library colonies was isolated by the alkaline lysis mini-prep protocol (22).

Suppressive subtractive hybridization and differential screening. CYVD strain Z01-A was subtracted from a rice endophytic *S. marcescens* strain, R02-A, using CLONTECH PCR-select^{TM} Bacterial Genome Subtraction Kit (Clontech, Palo Alto, CA, USA). The hybridization temperature was set at 68ºC to accommodate the high G+C

content of the *S. marcescens* species. The numbers of cycles for primary and secondary PCR were 26 and 12, respectively. Subtracted sequences were inserted into the TA vector using the TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA, USA) and electroporated into One Shot® TOP10 Electrocomp™ *E. coli* according to the manufacturer's protocol. Subtracted library clones were randomly selected as template for PCR amplification using nested primers 1 and 2R provided by the genome subtraction kit. PCR products were then transferred to duplicate nylon membranes with a dot blot method (13). Each membrane was hybridized (2) to an individual probe made from a CYVD or a non-phytopathogenic strain. The digoxigenin-labeled probe was detected with CSPD (Roche, Switzerland), and exposures were from 30 min to 3 h.

DNA sequencing and analysis. Insert sequences in the subtracted library were sequenced by the Oklahoma State University (OSU) Recombinant DNA/ Protein Resource Facility. Sequencing reactions were performed as recommended by the supplier (Applied Biosystems, Inc.) and analyzed on an ABI 3100 automated DNA sequencer. Probable biological functions of the products encoded by genes in the raw sequences were identified using MyPipeOnline 2.00b, a program designed by the OSU bioinformatics group (3). Based on the analysis, redundant sequences were identified and contigs were formed. Individual sequences were also analyzed by BLASTX and/or BLASTN searches against the GenBank database.

Fosmid cloning and hybridization. A Z01-A fosmid library was constructed using a CopyControl™ Fosmid Library Production Kit (Epicentre, Madison, WI, USA). Library construction employed recommended protocols, with slight modifications. Briefly, gel

slices containing fractionated DNA were washed twice with 1X GELase Digestion Buffer before being incubated with GELase for digestion. To induce fosmid clones to high copy number, the induction solution was added just before the bacterial culture reached log phase. The incubation period was held to three hours or less to avoid toxicity.

E. coli colonies containing fosmid clones were transferred and fixed to Hybond N+ nylon membranes (Amersham Biosciences, Piscataway, NJ, USA) following the manufacturer's instructions. PCR amplicons of subtracted Z01-A unique sequences, amplified using nested primers 1 and 2R provided by the genome subtraction kit, were equally pooled and labeled using a DIG DNA Labeling Kit (Roche Applied Science, Mannheim, Germany). The probe was then hybridized to the blot as described for colony hybridization.

Once a few fosmid clones were identified to contain phytopathogenic-strain-unique sequences, reverse hybridizations were conducted to confirm the actual number of unique sequences located in each fosmid clone. PCR amplicons of subtracted Z01-A unique sequences were arrayed on a nylon membrane (as described above) and hybridized to a Dig probe made from one fosmid clone. Similar arrays were made and hybridized to other individual fosmid clones as well.

Plasmid or chromosome origin of subtracted sequences determination.

Subtracted clone a43, which is clustered with a putative plasmid partition gene in FOSU1 locus, was selected as a target for quantitative PCR to determine whether the subtracted sequences are located on the chromosome or on a plasmid. A pair of primers, designated a43F (5'- CGCAGAACATCAACATATCTTAGCC -3') and a43R (5'-

TACCGTAGTAGTGCTGCATGAG -3’), were designed based on a43 using Primer3 software (20). Amplification of a segment of 16S rDNA, the genetic marker on the chromosome, was carried out as a control. Genomic DNA and plasmid DNA of Z01-A were prepared from 1.5 ml and 150 ml of log-phase bacterial culture, respectively, and suspended in 50µl distilled sterile water. Each DNA preparation was diluted 50x, 100x, 200x, 400x and 800x and 1 µl of each solution was used in each PCR reaction. PCR conditions for amplification of 16S rDNA were as follows: one initial denaturation cycle at 95°C for 5 min, followed by 26 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min 30 s and one final extension cycle of 72°C for 10 min. The amplification of a43 was carried out under the same conditions except that the annealing temperature was 60°C.

Multiplex polymerase chain reaction (PCR) development. Primers were designed based on CYVD pathogenic strain specific sequences using Primer3 software (20). Primers YV1 and YV4, which were designed from the 16S rDNA region of the *S. marcescens* genome and shown to be specific to this species (15), were used together with the new primers in a multiplex PCR to obtain both species and strain specificity. Bacteria grown in broth culture were washed once with 0.5M sodium chloride and resuspended in distilled water, and 1 µl suspension was used as template. Multiplex PCR, performed with the GeneAmp PCR System 9600 (Perkin–Elmer Cetus, Überlingen, Germany), was carried out in 25-µl volumes including 5µl of 5X Git buffer, 0.5 mM of dNTP, 0.1 µM of each primer and 2U of *Taq* DNA polymerase. PCR conditions were as follows: one initial denaturation cycle at 95°C for 5 min, followed by 34 cycles of 94°C

for 40 s, 60°C for 1min, and 72°C for 1 min 30 s and one final extension cycle of 72°C for 7 min.

Results

Suppressive subtractive hybridization of R02-A from Z01-A. A Z01-A subtracted library of approximately 400 clones was produced by SSH. Clones from which no amplicon or multiple amplicons were amplified using nested primers 1 and 2R were discarded. Selection of clones for further characterization was based on their sizes to avoid repeated sampling of clones containing the same insert sequences. A total of 183 clones were end sequenced, among which 94 different insert sequences were identified. Forty nine percent of the 94 inserts were represented at least twice. PCR products of the clones representing the 94 were then arrayed as described (Materials and Methods) on duplicate nylon membranes and hybridized to Z01-A and R02-A genomic DNA probes, respectively. As expected, all the sequences showed hybridization signals with strain Z01-A (data not shown). Forty-six sequences were shown to be false positives for SSH, as they hybridized to tester strain R02-A. The remaining 48 DNA fragments were present in Z01-A but not in R02-A.

A large proportion of the tester specific sequences resembling genes involved in synthesis of surface molecules. Analysis of the 48 subtracted sequences by BLASTX search showed that eight of them resembled hypothetical bacterial genes of unknown function, while nine other sequences had no significant matches with known sequences (Table 2). Among the clones that had significant similarity to sequences with known

function, a large proportion was judged to be involved in the synthesis of bacterial surface molecules. Clones A40, C19 and C34 resembled genes *rmlC, wbbL* and *wbbA*, respectively, which encode proteins of the rhamnose synthesis pathway and have been previously identified in the *S. marcescens* N28 wb O antigen gene cluster (21). The O antigen is the most external component of lipopolysaccharides (LPS), and its structure consists of a polymer of oligosaccharide repeating units. The sequence of the clone B1 insert resembled that of a surA isomerase gene, whose product participates in the assembly of outer membrane proteins (10). A defined *surA* deletion mutant of *Salmonella enterica* serovar Typhimurium C5 was defective in the ability to adhere to and invade eukaryotic cells (26). Phosphatase amsI, the predicted product of clone A3, may participate in changes of phosphorylation required for the biosynthesis of extracellular polysaccharide (EPS) (7).

In addition to the clones mentioned above, nine clones contained sequences resembling fimbrial genes and six others had ORFs resembling phage proteins. Although only one gene for a transposase, namely *IstA* of insertion element IS21 (19), was identified, it was represented in 38 subtracted clones, indicating its high copy numbers in the genome.

Identification of two gene clusters. To study the linkage of multiple fimbrial genes as well as other subtracted sequences, a fosmid library of strain Z01-A containing 410 clones was constructed and maintained on a LB plate. The average size of each clone insert was 40kb. Using pooled Z01-A specific sequences as the probe (Materials and Methods), 19 fosmid clones were positive in colony hybridization. The ten clones having

the strongest hybridization signals were chosen to study the linkage of individual Z01-A specific sequences in the genome. Clones were individually labeled and hybridized to 34 Z01-A specific sequences arrayed as described (Materials and Methods) on duplicate nylon membranes. Hybridization results suggested three categories of clones: 1) two clones, one of which was designated FOSU1, overlapped each other as shown by hybridization to 13 sequences, including genes that may be involved in type 1 fimbriae (pili) synthesis; 2) four other clones, one of which was designated FOSU2, were from the another locus as they all hybridized to seven Z01-A specific sequences, including five encoding putative phage proteins; 3) four clones duplicated each other in hybridizing to two Z01-A specific sequences. Because of their content of multiple Z01-A sequences, FOSU1 and FOSU2 were selected for shot-gun sequencing. The partial sequence of FOSU1 (accession number: AC148074) and the full sequence of FOSU2 (accession number: AC148075) were annotated and one gene cluster was identified in each clone.

Sequence analysis showed that a total of 13 insertion sequences occurred in multiple loci of the FOSU1 clone (Appendix I) (Figure 1). The average G+C content for FOSU1 is 51.67%, a value much lower than the approximately 59% G+C content calculated for *S. marcescens* db11, for which shot-gun sequences are available in the Sanger Institute database (http://www.sanger.ac.uk/Projects/*S_marcescens*/). These two features together indicate that the FOSU1 locus is likely a genome island obtained by lateral gene transfer. A fimbrial gene cluster, which may be involved in synthesis of filamentous surface adhesive organelles called type1 pili, was identified at the end of the genome island (Figure 1). Putative proteins encoded by genes within this cluster include: the fimbrins fimA and fimI, the adaptor proteins, fimG and fimF, the usher proteins, fimD and fimC,

the adhesin, fimH, and the regulatory proteins, fimB and fimE (23). The gene cluster has the same gene organization as those of other fimbrial gene clusters from related organisms, except that the regulatory gene *fimE* is truncated and *fimB* is located 21kb upstream. Interestingly, the G+C content of this gene cluster is only 49.94%, even lower than that of the FOSU1 genome island.

FOSU2 also contained a gene cluster, in which each ORF had a corresponding homolog in the genome of Fels-1, a phage of *Salmonella typhimurium* LT2 (Appendix II) (14). The highest match for each ORF, however, was from various phages, including Fels-1, Gifsy-2 (14), N15 (ref|NP_046907.1|), CP933U (17) , CP933K (8) and HK97 (9). Interestingly, all these phages have similar genomic architecture at the gene cluster region. The Z01-A prophage, however, lacks a virulence factor, superoxide dismutase SodCIII, that is present in Fels-1. The G+C content of the Z01-A prophage gene cluster is 57.26%, which is close to the 56.46% G+C content for Fels-1 in *Samonella typhimurium* LT2 (14).

The location of FOSU1 island on the chromosome. The identification of an ORF putatively encoding a plasmid partitioning protein within the FOSU1 locus raises the question of whether flanking sequences are from a plasmid or from the chromosome. Theoretically, it is possible that a trace amount of plasmid DNA could have remained in the genomic DNA preparations, and vice versa. However, plasmid DNA would be enriched in the plasmid preparation and chromosomal DNA would be enriched in the genomic DNA preparation. In this study, the intensity of a43 sequence amplicon amplified from the genomic DNA preparation was much greater than amplified from the

plasmid preparation, clearly showing that a43, together with the flanking sequences in FOSU1, are located on the bacterial chromosome (Figure 2).

The conservation of subtracted sequences among *S. marcescens* strains. The extent of polymorphism among *S. marcescens* strains for the subtracted sequences was assessed by dot hybridization. Sequences were arrayed on duplicate nylon membranes and hybridized to different CYVD strains or non-phytopathogenic strains. All the 94 sequences hybridized to probes made from genomic DNA of CYVD strains P01-A, C01-A, W01-A and W09-A, respectively. However, the intensity of hybridization signal varied among different sequences (data not shown). Forty nine, 39 and 41 of the total 94 sequences hybridized to nonpathogenic strains R01-A, 90-166 and db11, respectively. The conservation of sequences among *S. marcescens* strains varied (Table 3). Thirty-four sequences were present only in strain Z01-A. To confirm the reliability of the hybridization result, all the sequences were subjected to BLASTN search against the genome sequences of *S. marcescens* db11 (Table 3). A homolog with identity greater than 87% was found in the *S. marcescens* db11 genome database for every Z01-A subtracted sequence that hybridized to the db11 genomic DNA probe. On the other hand, no homolog was found for any of the sequences that were negative for hybridization to db11. Although clone c98 had a match in the db11 database with an expect value of e-19, the identity was only 62%.

Multiplex PCR diagnosis. Neither BLASTN nor BLASTX searches of GenBank database revealed sequence similarity to subtracted clone a79. In addition, the DNA sequence of a79 was negative for hybridization to all tested CYVD non-phytopathogenic

strains (Table 3). A new pair of primers, designated a79F (5'-CCAGGATACATCCCATGATGAC-3') and a79R (5'-CATATTACCTGCTGATGCTCCTC-3'), were designed based on a79. The multiplex PCR was carried out as described (Materials and Methods). A PCR amplicon of 338 bp was amplified from CYVD strains, but not from non-phytopathogenic *S. marcescens* strains. As a comparison, a 452bp amplicon was amplified from all *S. marcescens* strains using primers YV1 and YV4. No fragments were amplified from non- *S. marcescens* strains (Figure 3).

Discussion

DNA-DNA hybridization results from a previous study (31) showed that *S. marcescens* endophytic strain R02-A, of all the strains tested, was most closely related to CYVD strains. With a relative binding ratio of 90% and a 1.5 percent divergence from CYVD representative strain C01-A, and confirmed to be non-pathogenic to cucurbits, R02-A is an ideal driver strain to subtract from the well-studied CYVD pathogenic strain Z01-A.

This is the first report of using SSH to identify the genetic differences between *S. marcescens* strains, which generally have high G+C content. About 51% of the subtracted differential sequences were confirmed by dot hybridization to be present in Z01-A, but absent in R02-A. The reliability of dot hybridization was further validated by hybridization of subtracted Z01-A sequences with *S. marcescens* db11, in which a hybridization signal always corresponded to greater than 87% sequence identity. In addition, except for *IstA* of IS21, which had a number of copies on the Z01-A genome, all

other sequences were represented no more than 4 times, indicating that the library was not significantly biased (data not shown). The fact that about half of the differential sequences were represented only once suggests that the potential library of Z01-A specific DNAs was not exhausted in our experiments.

A large number of subtracted clones identified in this study resembled known genes involved in synthesis of O antigen and type 1 pili, both of which are important bacterial surface structures. O antigen is the outmost component of LPS, the major structural and immunodominant molecules of the outer membrane. Interestingly, there was evidence that lateral gene transfer of O antigen genes occurred among subspecies of *Salmonella enterica* (11). A large variation in genes encoding surface structures has also been revealed in previous genome sequencing of pathogenic bacteria, such as *Escherichia coli* O157:H7 (27) and *Streptococcus pneumoniae* (28). It has been suggested that the variation is obtained by lateral gene transfer as a result of diversifying selection pressure to evade host immune defenses. A similar phenomenon was reported by Nesbo et al. (16), who compared different strains of *Thermotoga* using SSH. It was reasoned, however, that variation in surface structures might be a general feature of closely related bacterial strains. In this study, clone A40 and B1 were detected by hybridization in non-phytpathogenic strains, suggesting that at least some of the genetic variation was a result of divergence instead of lateral gene transfer. The CYVD pathogen investigated in this study lives predominantly in the phloem sieve tubes, a tissue that is rich in nutrients and high in osmotic pressure. In addition, it is transmitted by an insect vector, the squash bug (*Anasa trisits*). Although it is not known whether CYVD strains of *S. marcescens* are able

to colonize other ecological niches, it is certainly possible. Adaptation to such widely diverse niches might be the driving force for the genetic variation of surface molecules.

The most striking result in this study is the identification of two gene clusters present in CYVD strains of *S. marcescens*, but absent in closely related, but nonphytopathogenic strains. Several lines of evidence suggest that the fimbrial gene cluster was likely part of a genome island acquired from other species: i) the prevalence of putative insertion sequences at the flanking region; ii) low G+C content; iii) gene organization similar to that of related bacterial species; iv) the presence of the fimbrial gene cluster only in CYVD strains, and not in any CYVD non-phytopathogenic strains, as shown by the DNA hybridization. In other bacteria the whole gene cluster is responsible for the production and control of type 1 fimbriae (25). The adhesin fimH, which is located at the distal tip of the pilus, mediates not only bacterial adherence, but also invasion of human bladder epithelial cells (12). The expression of pilins, however, could be turned on and off by site-specific DNA inversion of a 312 bp fragment containing the promoter region of *fimA* (24). The switch process is catalyzed by two recombinases, fimB and fimE. Our work showed that Z01-A had only a truncated version of fimE, and that fimB is located 21kb further upstream of the gene cluster in Z01-A than it is in other bacteria. These features would impact the production of type one pili. In the future, electron microscopic observation, fimbrial gene cluster knockout and complementation experiments would provide evidence whether this cluster of genes contributes to CYVD strain pathogenicity.

We also studied the distribution of the subtracted sequences, including the two gene clusters, among CYVD strains isolated from different cucurbit species and among non-phytopathogenic strains from various niches. All CYVD strains tested were positive by

hybridizations for the subtracted sequences with various degrees of intensity, consistent with their average 99% relatedness determined in a DNA-DNA hybridization study (31). The three CYVD non-associated *S. marcescens* strains, on the other hand, each hybridized to a distinct portion of the subtracted Z01-A sequences. Notably, R01-A and R02-A shared the greatest number of common sequences among the CYVD nonpathogenic strains (data not shown), confirming the close relatedness of these strains that was predicted earlier from DNA-DNA hybridization and rep-PCR experiments (31). Testing the hybridization of the subtracted sequences to a more extensive set of *S. marcescens* strains might reveal more genetic variations that underlie the evolutionary process.

SSH proved to be a useful method for identifying molecular markers for epidemiological studies. When choosing the markers we purposely avoided the two gene cluster regions as the occurrence of gene transfer among related bacterial species could complicate marker identification. Using a primer pair designed based on a subtracted Z01-A specific sequence, a79, we were able to differentiate CYVD strains from non-phytopathogenic strains of *S. marcescens*. The selected marker is likely to be specific as it has no sequence similarity, at either the DNA or the translated protein level, with any sequence in GenBank. The multiplex PCR technique, which includes a primer pair to detect the bacterium at the species level, added further reliability to the new diagnostic tool. In the future it can be used in vector identification, field disease diagnoses, and contamination-free examination in laboratory.

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Table 1. Bacterial strains tested in this study and their relative relatedness to CYVD representative strain C01-A.

Strain		Source	RBR to C01-A [e]
Strain designation used in this study	Alternative strain designation, if any		
CYVD-pathogenic strains			
W01-A		Watermelon	100
W09-A		Watermelon	100
Z01-A		Zucchini	100
P01-A		Pumpkin	94
P07-A	00B058	Pumpkin	ND
C01-A		Cantaloupe	100
CYVD-non-pathogenic strains			
R01-A [a]	IRBG 501	Rice	82
R02-A [a]	IRBG 502	Rice	90
R03-A [a]	IRBG 505	Rice	ND
G01 [b]	90-166	Cotton	76
H01-A [c]		Human	73
H02-A [c]		Human	82
Db11[d]		Insect	ND

[a] Provided by J. K. Ladha, International Rice Reasearch Institute, Los Banos, Philippines.

[b] Provided by J. Kloepper, University of Alabama, Auburn, AL.

[c] Provided by D. Adamson, Medical Arts Laboratory, Oklahoma City, OK.

[d] Provided by the American Type Culture Collection, Manassas, VA.

[e] RBR=relative binding ratio; ND=not done. Results were reported by Zhang et al. (31).

Table 2. Sequence analysis of Z01-A specific clones.

Group and Clone	First BLASTX match[d]	Organism	Identity	Expect value
Surface structure				
A3	AmsI, phosphatase	*Shigella flexneri*	58%	9e-12
A28 [a]	FimC, chaperon	*Escherichia coli*	68%	3e-26
A40	RmlC, epimerase	*S. marcescens*		
A48 [b]	FimD, usher protein	*Escherichia coli*	56%	3e-19
B1	SurA, isomerase	*Yersinia pestis*	77%	1e-13
C15 [c]	FimH, adhesin	*Escherichia coli*	55%	3e-43
C19	A. Rhamnosyl transferase	*Klebsiella pneumoniae*	64%	0.004
	B. Inverted repeat	*S. marcescens*	93%	8e-09
C24 [a]	FimC, chaperon	*Escherichia coli*	75%	9e-21
C25 [a]	FimC, chaperon	*Escherichia coli*	53%	1e-37
C34	Glycosyltransferase	*Campylobacter jejuni*	28%	1e-04
C63 [c]	FimH, adhesin	*Escherichia coli*	86%	3e-10
C89 [b]	FimD, usher protein	*Escherichia coli*	70%	1e-101
C98 [b]	FimD, usher protein	*Escherichia coli*	67%	6e-47
D6	FimB, recombinase	*Escherichia coli*	58%	4e-56
Phage				
A6	Clp protease	*Salmonella typhimurium*	55%	2e-52
C22	Phage tail protein	*Yersinia pestis*	31%	8e-16
C62	Bacteriophage integrase,	*Escherichia coli*	72%	9e-53
C67	Tail assembly protein	*Escherichia coli*	64%	2e-78
D1	Phage terminase large subunit	*Escherichia coli*	80%	7e-87
Unknown function (17 clones)				
Other				
A23	Chitobiase	*S. marcescens*	89%	1e-33
A27	Plasmid partitioning protein	*Yersinia enterocolitica*	86%	5e-28
A66	Retinitis pigmentosa GTPase regulator	*Bos taurus*	32%	0.29
A68	Uridylyltransferase	*Aeromonas hydrophila*		
A89	Endonuclease		56%	3e-18
B21	Dam, regulatory protein	*Salmonella typhimurium*	59%	1e-46
C26	IstA, transposase	*Escherichia coli*	78%	3e-67
C68	pVS1 resolvase	*Corynebacterium glutamicum*	72%	3e-07
C93	TaxC Nickase	*Escherichia coli*	36%	0.017
D2	A. TaxC Nickase	*Escherichia coli*	34%	7e-18
	B. Transcriptional regulator	*Escherichia coli*	44%	1e-05

D26	A. Putative exported protein	*Yersinia pestis*	67%	8e-28
	B. Inverted repeat	*S. marcescens*	95%	6e-11

[a] Clones A28, C24 and C25 matched with different parts of fimC.

[b] Clones A48, C89 and C98 matched with different parts of fimD.

[c] Clones C15 and C63 matched with different parts of fimH.

[d] If more than one gene was covered by the clone, they are given as follows: A, gene X; B, gene Y.

Table 3. Conservation of tester specific sequences among *Serratia marcescens* strains.

Clone	Note[a]	Hybridization to bacterial strains[b]				
		Z01	R02	R01	90-166	Db11 (e-value)[c]
A3		+	-	-	-	-
A6	FOSU2	+	-	-	-	-
A13		+	-	+	-	-
A23		+	-	+	-	+ (e-41)
A27	FOSU1	+	-	-	-	-
A28	FOSU1	+	-	-	-	-
A30		+	-	-	-	-
A33		+	-	+-	-	+ (e-52)
A40		+	-	-	+	-
A43	FOSU1	+	-	-	-	-
A46		+	-	-	-	-
A48	FOSU1	+	-	-	-	-
A57		+	-	-	-	-
A64	FOSU1	+	-	-	-	-
A66		+	-	-	-	-
A68		+	-	-	+	+ (e-31)
A71		+	-	-	+	-
A76		+	-	-	-	+ (e-65)
A79	Diagnostic marker	+	-	-	-	-
A89	FOSU1	+	-	-	-	-
A99		+	-	+	+	+ (e-55)
B1		+	-	+	+	+ (e-58)
B6	FOSU1	+	-	-	-	-
B17	FOSU1	+	-	-	-	-
B27		+	-	-	-	-
C14		+	-	+	-	-
C15	FOSU1	+	-	-	-	-
C19		+	-	-	-	-
C22	FOSU2	+	-	-	-	-
C24	FOSU1	+	-	-	-	-
C25	FOSU1	+	-	-	-	-
C34		+	-	-	-	-
C48		+	-	-	-	-
C63	FOSU1	+	-	-	-	-
C67	FOSU2	+	-	-	-	-
C68	FOSU1	+	-	-	-	-
C69		+	-	+	-	-
C71		+	-	-	-	+ (e-29)
C89	FOSU1	+	-	-	-	-
C93		+	-	-	-	-
C98	FOSU1	+	-	-	-	- (e-19)
C99	FOSU2	+	-	-	-	-
D1		+	-	-	-	-
D2	FOSU2	+	-	+	-	-
D6	FOSU1	+	-	-	-	-
D20	FOSU2	+	-	-	-	-

D21		+	-	-	+	-
D26		+	-	-	+	+ (e-140)
D37	FOSU1	+	-	-	-	-

[a] Whether the clone was located in FOSU1 or FOSU2 is indicated here.

[b] The designation + indicates positive hybridization, whereas – indicates negative hybridization; the designation +-: indicates ambiguous hybridization signal.

[c] Expect values were obtained by BLASTN search of individual tester specific sequences against *S. marcescens* db11 draft sequences.

Figure 1. Sequence analysis of FOSU1 and its fimbrial gene cluster

A. Diagrammatic representation of FOSU1. Spotted rectangles represent different insertion sequences. Plain grey rectangles represent ORFs of unknown function or structural proteins.

B. Putative ORFs in the fimbrial gene cluster identified in this study.

C. Putative ORFs in the fimbrial gene cluster from *E.coli* O157:H7 EDL933 (upper) and *Shigella flexneri* 2a str 2457T (lower).

D. G+C content of the fimbrial cluster.

Figure 2. Agarose gel electrophoresis of 16S rDNA (Lanes 1-5) and sequence a43 (Lanes 6-10) amplified from a dilution series of genomic DNA (Upper set, 50x, 100x, 200x, 400x, 800x dilution of original isolated DNA solution, respectively) and plasmid preparations (Lower set, 50x, 100x, 200x, 400x, 800x dilutionof original isolated DNA solution, respectively).

Figure 3. CYVD pathogenic (lanes 2-5) and non-pathogenic strains (lanes 6-12) detected by multiplex PCR employing primers YV1/4 and a79F/R.

A. 452 bp band amplified by YV1/4

B. 338 bp band amplified by a79F/R

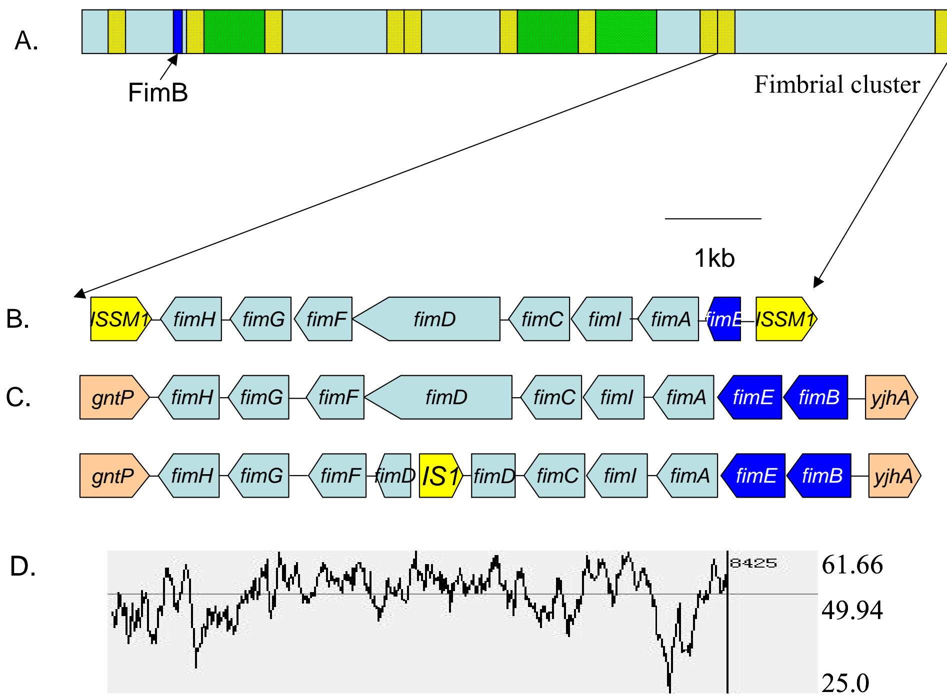

Figure 1.

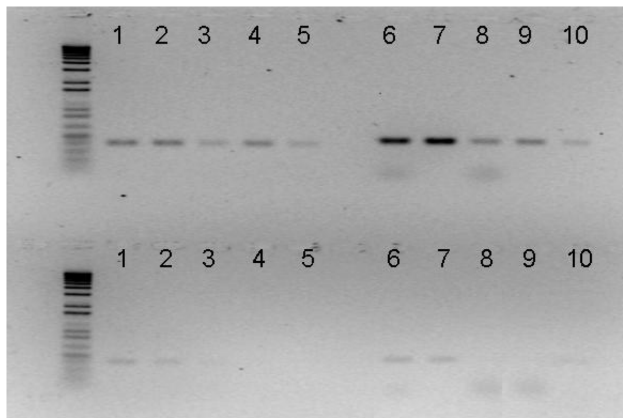

Figure 2.

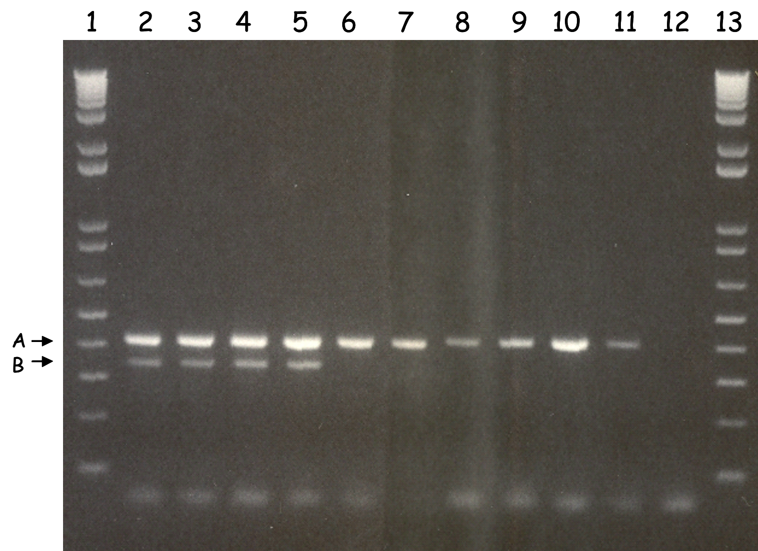

Figure 3.

CHAPTER V

TRANSPOSON MUTAGENESIS OF *SERRATIA MARCESCENS*, THE CAUSAL AGENT OF CUCURBIT YELLOW VINE DISEASE, AND VIRULENCE SCREENING ON *CAENORHABDITIS ELEGANS*

Abstract

Conjugational mutagenesis was conducted on *Serratia marcescens*, the causal agent of cucurbit yellow vine disease, to create insertion mutants. Transformants were identified by antibiotic selection using kanamycin and confirmed by amplification of the kanamycin resistance gene using the polymerase chain reaction. Southern hybridization analysis, however, indicated that the insertion of transposons into the *S. marcescens* chromosome was site-specific rather than random. In this study, we also evaluated the utility of *Caenorhabditis elegans* as an *in vivo* model for the study of *S. marcescens* virulence factors. Both killing assays and feeding inhibition assays showed that *S. marcescens* was only moderately toxic to *C. elegans*. This model, therefore, is not appropriate for large-scale virulence screening.

Introduction

Serratia marcescens is a Gram-negative bacterium that has adapted to various environmental niches, including soil, water, insects and human bodies. This bacterium is also associated with an epidemic plant disease, namely cucurbit yellow vine disease

(CYVD) (3). Symptomatic cucurbits typically show yellowing, wilting and phloem discoloration. Initially identified in central Oklahoma and central Texas, this disease has now been detected in at least ten U.S. states.

Phenotypic and genotypic comparisons of CYVD-associated *S. marcescens* strains with non-phytopathogenic strains from various niches showed significant differences (13) (17). With the complete sequence of the *S. marcescens* insect pathogenic strain db11 available (http://www.sanger.ac.uk/Projects/*S_marcescens*), I would expect that comparative genomics would greatly facilitate our understanding of the evolution of this bacterium. However, to fully understand the pathogenicity mechanisms that lead to CYVD, it is necessary to conduct functional genetic analysis experimentally. Bacterial mutagenesis is an essential tool for this purpose.

Transposon mutagenesis has provided an important tool for analyzing gene structure, function and regulation. Among different types of transposons, Tn5 has been used widely for Gram-negative bacterial mutagenesis because of its high insertion frequency and low specificity (Mobile Element II, 2002). Estepa and Harshey (7) reported the first transposon mutagenesis of a strain of *S. marcescens*, Sm 274, using a broad-host-range conjugative suicide plasmid vector. In their study, multiple transposition events were obtained after elimination of the transferred plasmid. It was speculated that the presence of multiple plasmids was responsible for this phenomenon.

De Lorenzo et al. (5) reported the construction of minitransposons, containing various selectable antibiotic resistance markers, for engineering bacteria. An important feature of minitransposons is that the transposase gene is cognately arranged outside of the transposon's cut-end sequence. As a result, a single transposition of the mintransposon

into the bacterial chromosome occurs, and the insert is stably inherited. Dennis and Zylstra (6) added another feature, self-cloning ability, into minitransposons, and called the resulting constructs plasposons. Together these two features greatly facilitate the rapid genetic analysis of Gram-negative bacterial genomes.

Bruton et al. (4) provided evidence showing that the CYVD bacterium is transmitted by the squash bug, *Anasa tristis*. In that study, cucurbit-derived strains of *S. marcescens* were introduced into greenhouse-grown squash plants by mechanical puncture inoculation and into field-grown squash plants by enclosure with *S. marcescens*-fed squash bugs. Up to 60 percent of the mechanically inoculated plants and up to 17 percent of field plants caged with inoculative squash bugs developed phloem discoloration and tested positive for *S. marcescens* by CYVD-specific PCR. However, the symptoms of plant decline and collapse, normally part of the CYVD syndrome, were not observed in mechanically inoculated plants in the greenhouse. The low percentage of successful inoculations and the inability to obtain full symptom expression following mechanical inoculation makes bacterial mutant pathogenicity screening extremely difficult, especially since thousands of mutants may need to be analyzed in the genetic study. There is a need for development of screening methods that are convenient and reliable.

Tan and Ausubel (16) first used *Caenorhabditis elegans,* a free-living nematode well known for its genetically tractable traits, convenience and economical maintenance, to investigate host-pathogen interactions. *C. elegans* died within 24 h when fed on a lawn of *Pseudomonus aeruginosa,* a bacterium capable of causing diseases in both plants and animals. Another cosmopolitan bacterium, *Burkholderia pseudomallei*, also causes the

mortality of *C. elegans* (10). More recently, Kurz et al. (9) extended the utility of *C. elegans* as an *in vivo* model for the study of *S. marcescens* virulence factors.

In early studies, killing assays relied on the exposure of *C. elegans* to bacterial pathogens grown on solid media and the visual scoring of nematode mortality. This test was especially useful in testing the mechanisms of toxin-based paralysis. Smith et al. (15) developed a liquid-based feeding inhibition assay for the assessment of bacterial virulence, which allowed the effects of bacterial pathogens on *C. elegans*, including food evading, to be quantified more precisely. In this study we evaluated the capability of *S. marcescens* to cause disease in *C. elegans* by both a killing assay and a feeding inhibition test.

Materials and Methods

Bacterial strains, nematodes and culture conditions. Bacteria and nematodes used in this study are presented in Table 1. *E. coli* JM109 (pTnMod-KmO) and *E. coli* DH5α (pRK2013) were obtained from Carol L. Bender (Oklahoma State University, OK). *S. marcescens* Z01-A, isolated originally from zucchini squash with CYVD, has been described (3). Bacteria were stored, -80ºC, in 1.5 ml Luria-Bertani (LB) (14) broth aliquots containing 15% glycerol. For use in experiments, bacteria were streaked onto LB agar or inoculated into LB broth and incubated at 28ºC for 24 h. *E. coli* was grown at 37ºC in LB broth.

C. elegans wild type strain N2 was used for killing assay, and *C. elegans phm-2*, a mutant strain with aberrant food grinding apparatus (2), was used for feeding inhibition

assay. The nematode culture methods, nematode growth medium (NGM) and K-medium have been described (8).

Selection of Z01-A rifampcin resistant spontaneous mutants. CYVD strain Z01-A was grown overnight at 28ºC in LB broth. A quantity of 100 µl of the bacterial suspension was added to 5 ml LB broth containing 15 µg /ml rifampicin, and allowed to grow for 48 h at 28ºC. One hundred µl was subsequently transferred to 30 µg /ml rifampicin and grown as before. A quantity of 100 µl of these cells was plated onto LB agar containing 30 µg/ml rifampicin. One colony, designated Z01-RR1, was selected and transferred to LB broth, grown to log phase and stored frozen in aliquots as described above (Rascoe, unpublished data).

To determine whether the rifampicin resistance phenotype of Z01-RR1 is stably maintained a loopful of Z01-RR1 stock solution from a frozen aliquot was streaked onto LB agar without rifampicin. Each day, an additional streak was made from the previous bacterial culture. After 20 generations of streaks, a loopful of bacterial cells was streaked onto LB agar containing 30 µg/ml rifampicin to determine whether antibiotic resistance had been retained.

Determination of minimum inhibitory concentration (MIC) of antibiotics. Antibiotics used for selection in these experiments included kanamycin and rifampicin. A series of two-fold dilutions of the antibiotic stock solutions were prepared in LB broth. Around 100 bacterial cells were inoculated into each dilution of antibiotic broth culture

and shaken for 24 hours at 28ºC (*S. marcescens* Z01-A) or 48 h at 37ºC (*E. coli* JM109, DH5α). Growth was assessed by the turbidity of the culture medium at 550nm.

Conjugational mutagenesis. *S. marcescens* Z01-RR1, *E. co*li JM109 (pTnMod) and *E. coli* DH5α (pRK2013) were individually grown in LB broth to mid-log phase. Three hundred and thirty microliters of each culture were added to a single tube, vortexed and centrifuged at 5000x g for 1 min. The supernatant was discarded and the pellet resuspended in 150 µl LB broth and plated on a non-selective LB plate. The mating plate was incubated for 14 h at 28ºC. The bacterial cells were then scraped from the agar surface and resuspended in 1 ml 10% sterile glycerol. The resultant bacterial cell culture was plated on LB plates containing 75µg/ml kanamycin and 100µg/ml rifampicin. Ten colonies were selected in each mating experiment.

PCR amplification. 16S rDNA sequence was amplified by polymerase chain reaction (PCR) from *S. marcescens* Z01-RR1using primers YV1 (5'-GGGAGCTTGCTCCCCGG-3') and YV4 (5'-AACGTCAATTGATGAACGTATTAAGT-3'). PCR amplification was performed in 25 µl reaction mixtures containing a bacterial colony, 1x Gitshier buffer (16.6 mM $[NH_4]_2SO_4$; 67 mM Tris-HCl, pH 8.8; 6.7 mM $MgCl_2$; 6.7 mM EDTA; 30 mM β-mercaptoethanol), 100 µM of each dNTP, 0.5 µM each of primers YV1 and YV4, and 2 U *Taq* DNA polymerase (Promega, Madison, WI, USA). This mixture was placed in a GeneAmp PCR system 9600 (Perkin-Elmer Cetus) thermal cycler and subjected to a 5 min denaturation at 95 °C followed by 30 cycles at 95 °C for 30 s, 55 °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% agarose gel. The kanamycin resistance gene marker was amplified from a JM109 or DH5α colony using primers km1 (5'-CCGAGGCAGTTCCATAGGATG-3') and km2 (5'-TTTATATGGGTATAAATGGGCTCGC-3'). The program for amplification of the kanamycin resistance gene was the same as that described above except that the annealing temperature was raised to 64 °C. Z01-A transformants were subjected to two independent PCR amplifications using primer pairs YV1/4 and Km1/2, respectively.

Southern hybridization. Genomic DNA was extracted using a hexadecyl trimethyl ammonium bromide (CTAB) method (1). The sample of each strain was prepared for electrophoresis by digesting 2 μg of genomic DNA with *Eco*RI restriction enzymes in a total of 20 μl, and the DNA fragments were separated in a 0.8% agarose gel. The gels were blotted onto nylon membranes (Amersham Biosciences, Piscataway, NJ, USA) following the manufacturer's instructions. The probe was made from PCR product amplified from the kanamycin resistance gene and labeled with the DIG High Prime DNA labeling kit (Roche, Switzerland). Prehybridization and hybridization were done with DIG Easy Hyb (Roche, Switzerland) at 42°C in a hybridization bag incubated in a water bath. The digoxigenin-labeled probe was detected with CSPD (Roche, Switzerland), and exposures were from 30 min to 3 h.

Killing assay of *C. elegans*. Nematode growth medium (NGM) was poured into 5.5 cm petri plates, covered and allowed to dry at room temperature for 2 days. Ten

microliters of an overnight culture of a test bacterial strain was spread on a NGM plate and incubated at 37°C for 24 hours. Optimum growth of the bacterial culture was then obtained by incubating the plates at room temperature for another 8-24 hours depending on the growth rate of different bacteria. Each plate was seeded with 20 L4-stage *C. elegans* worms (post-embryonic development of *C. elegans* involves growth through four larvae stages, L1 to L4, before the final molt to the adult stage). Plates were scored for nematode survival every 8 hours, and three replicates per trial were performed. A worm was considered dead when it no longer moved in response to stimulation. Worms that dried out on the sidewall of the plate were excluded from the analysis.

***C. elegans* feeding inhibition assay.** The feeding inhibition assay was carried out using the method by Smith et al. (15), except for minor changes. Briefly, a bacterial pellet was resuspended in K-medium to an OD_{550} of approximately 1.2. *C. elegans* mutant *phm-2* worms grown on an *E. coli* lawn were washed with K-medium (2) and brought to a concentration of 10 worms/µl by removing the supernatant after 20 min incubation. Experiments were conducted in 24 well polystyrene cell culture trays (Costar, Corning, NY, USA). Aliquots of 1.7 ml of bacterial suspension, 200 µl of evenly mixed worms and 100 µl of K-medium were added to each well. For the feeding inhibition control, the K-medium was replaced with 100 µl 2 M sodium salicylate solution, which inhibits the feeding of *C. elegans*. To measure the natural degradation of the bacteria in K-medium, a well without nematodes was also prepared. Every 24 h during the incubation, 1 ml samples were transferred to sterile disposable cuvettes and the OD_{550}s were measured after the worms settled to the bottom.

Results and Discussion

To further the genetic analysis of *S. marcescens*, the causal agent of CYVD, mutagenesis was conducted on this bacterium to create a pool of mutants. *S. marcescens* is an enterobacterium that exhibits very low efficiency of transformation (11), but successful transfer of a plasmid into *S. marcescens* by conjugation has been reported (12). In the current study we used *E. co*li JM109 (pTnMod-OKm) as the donor strain and *E. coli* DH5α (pRK2013) as the helper strain for transconjugation. To facilitate selecting conjugational transformants from triparents, a phenotypic marker was obtained by selecting a spontaneous rifampicin resistant Z01-A mutant. The mutant showed resistance even after 20 passages of selection on LB without rifampicin, demonstrating that the antibiotic resistance phenotype was stably inherited. The spontaneous mutant, designated Z01-RR1, was used for conjugational mutagenesis.

The MICs of kanamycin and rifampicin for each of the triparental strains were determined before conjugational mutagenesis (Table 2). The final kanamycin concentration used for selecting conjugational transformants was 75µg/ml, which is slightly lower than the MIC for JM109 and DH5α. The final concentration of rifampicin, 100 µg/ml, on the other hand, was higher than the MIC of JM109 and DH5α but lower than that of Z01-RR1.

Selected transformants were confirmed by two independent PCRs. The first PCR, using primer YV1 and YV4, amplified a 452 bp segment of 16S rDNA sequence that is specific to recipient, Z01-RR1. The second PCR, using primers km1 and km2*,* amplified a 750 bp segment of the kanamycin resistance gene, which is absent in the recipient strain. The resulting products from both reactions were mixed and electrophoresed. Two

bands, 452 bp and 750 bp, were detected in all ten randomly selected conjugational transformants (Figure 1). As a comparison, a single band of 452 bp was amplified from the recipient strain Z01-A and a band of 750 bp was amplified from the donor strain JM109 and the helper strain DH5α. These results confirmed that all selected colonies were true transformants.

The Southern hybridization experiment result again showed that a single band was recognized in all six randomly selected transformants by a kanamycin resistance gene specific probe (Figure 2). However, all the bands were of approximately the same size, which indicates that the insertion of transposons into the Z01-A chromosome was site specific rather than random. An alternative explanation is that the transposon preferably inserted into a Z01-A plasmid that has no *Eco*RI specific restriction site. In either case the transformants obtained in this study would not be appropriate for use in large scale virulence screening.

Because screening for loss of pathogenicity of CYVD strains on the host plant is so difficult, other screening methods were investigated to study pathogenicity defective mutants. Preliminary experiments by others (Luo and Fletcher, personal communication) have demonstrated the unsuitability of using other plant species, such as onion, carrot, and tobacco, for virulence screening. In this study we investigated the capability of CYVD wild type strains and their derivatives to cause mortality of *C. elegans* N2. In the positive control, 90% of the worms fed on *S. marcescens* db11 died within 4 days (Figure 3), whereas half of the worms fed on *E. coli* OP50 were still alive after 6 days. Overall, a slightly lower percentage of *C. elegans* survived when fed on CYVD wild type strains W01-A, Z01-A and the rifampicin resistant derivative Z01-RR, as compared to worms

fed on *E. coli* OP50. These results illustrate that CYVD of *S. marcescens* strains are only moderately toxic to *C. elegans.* Similar results were obtained in the feeding inhibition assay, in which a *C. elegans* mutant was used. The mutant *C. elegans phm-2* has a malfunctional grinder. Due to the aberrant grinder, increased numbers of bacteria can pass through the nematode pharynx into the gut (2). In the control treatment, the optical density of the bacterial suspensions from which the worms had settled down fell over time as the nematodes fed on *E. coli* OP50. In the first 24 h of incubation the rate of decrease in optical density was similar for all treatments (Table 3). However, in the incubation period from 24 h to 48 h there were differences among treatments. When *C. elegans phm-2* was incubated with *S. marcescens* Z01-RR1 or db11, the rate of reduction of OD_{550} was higher than when the salicylic acid inhibitor was added to the worms incubated with *E. coli* OP50, but lower than when worms were incubated with only *E. coli*, indicating that Z01-RR1 was only moderately toxic to the *C.elegans* worms. This data, however, is not statistically significant as the experiment was conducted only once. In a separate experiment, Ewbank et al. (unpublished data) found that all the worms fed on W01-A died within four days. This is clearly in contrast to the findings in this study. In the future, replicate experiments with more sampling time intervals should be conducted to assess the virulence of CYVD strains on *C. elegans*.

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Table 1. Test bacteria and nematodes used in this study.

Organism	Source	Notes	Reference
Bacterial strains			
Z01-A	J. Fletcher	CYVD strain	(3)
Z01-RR1	This study	Rif $^{+}$	
E. coli JM109	C.L. Bender	Donor strain, Kan^{+}	(6)
E. coli DH5α	C.L. Bender	Helper strain, Kan^{+}	(6)
E. coli OP50	CGC [a]	Natural food source for *C. elegans*	(2)
Caenorhabditis elegans			
N2	CGC [a]	Wild type	(2)
Phm-2	CGC [a]	Mutation in allele ad597	(2)

[a] CGC: Caenorhabditis Genetics Center (250 Biological Sciences Center, University of Minnesota, MN).

Table 2. Minimum inhibitory concentration of antibiotics for experimental bacterial strains.

Antibiotic	MIC (μg/ml^{-1})		
	E. coli JM109	*E. coli* DH5α	*S. marcescens* Z01-RR1
Kanamycin	>100	100	75
Rifampicin	75	50	200

Table 3. *Caenorhabditis elegans* feeding inhibition.

Treatment	OD_{550}		
	0h	24h	48h
E. coli OP50+ worms	1	0.828	0.694
E. coli + worms + inhibitor	1	0.831	0.819
Z01-RR1 + worms	1	0.817	0.720
Z01-RR1	1	0.841	0.837
Db11 + worms	1	0.880	0.776
Db11	1	0.831	0.830

Figure 1. Z01-A transformants (lanes 1-10), Z01-A (lane 11), JM109 (lane12) and DH5α (lane 13) tested by two independent PCRs. A: 750 bp band amplified by Km1/Km2; B: 452 bp band amplified by YV1/YV4.

Figure 2. Southern hybridization analysis of *Eco*RI-digested Z01-RR1 transformants (Lanes 1-3, 5-7) and *Escherichia coli* JM109 (lane 4) probed with a DIG-labeled PCR product of the kanamycin resistance gene. The PCR product of the kanamycin resistance gene was loaded as control (lane 8).

Figure 3. Survival of *Caenorhabditis elegans* N2 fed on *Escherichia coli* OP50, *Serratia marcescens* Db11 and CYVD strains.

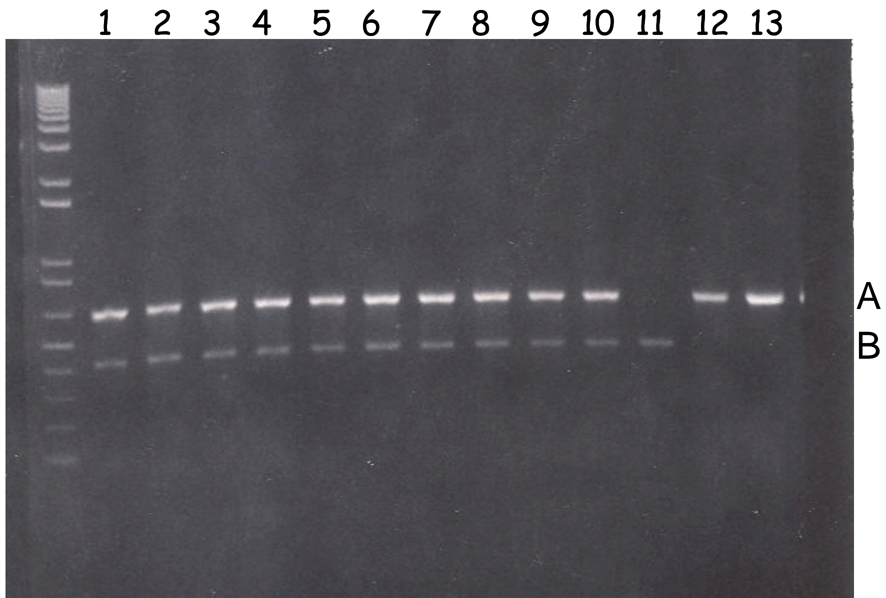

Figure 1.

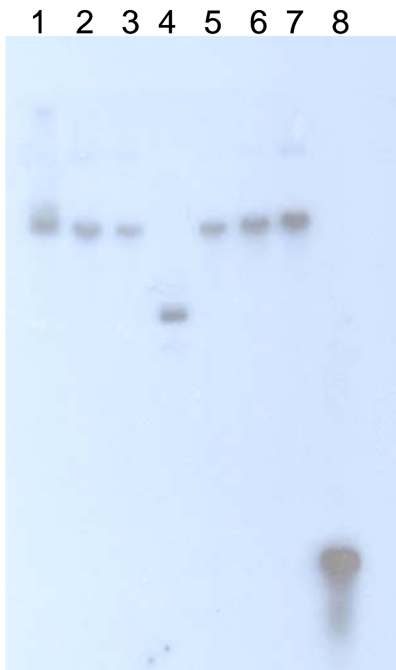

Figure 2.

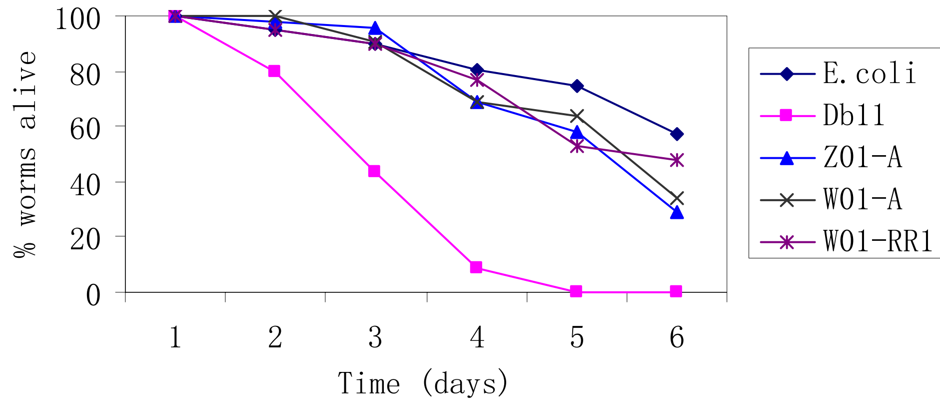

Figure 3.

APPENDIX I. SUMMARY OF FOSU1

ORF	Position	Strand	Top BLASTX Match - Organism	P_value
1	164..688	F	Endonuclease - *Wigglesworthia brevipalpis*	4e-46
2	1188..1568	F	Unknown	
3	2056..2750	F	ISSM1-*Serratia marcescens*	5e-06
4	2914..3174	F	Hypotheical protein - *Klebsiella pneumoniae*	4e-22
5	3216..3776	F	Hypothetical protein - *Klebsiella pneumoniae*	1e-90
6	3813..4256	F	Hypothetical protein - *Escherichia coli*	4e-50
7	4272..4604	F	Hypothetical protein LV103 - *Klebsiella pneumoniae*	2e-23
8	4655..5146	F	Hypothetical protein LV102a - *Klebsiella pneumoniae*	1e-79
9	5267..5849	F	FimB - *Escherichia coli*	1e-21
10	6076..6810	R	ISSM1 - *Serratia marcescens*	1e-131
11	7019..7819	R	IstB protein - *Escherichia coli*	1e-124
12	7819..8967	R	IstA protein - *Escherichia coli*	0.0
13	9105..9758	F	ISSM1 unknown - *Serratia marcescens*	1e-123
14	9900..10571	R	RecA protein - *Pseudomonas aeruginosa PA01*	6e-56
15	11774..12634	R	Putative cytoplasmic protein	e-129
16	12718..13365	R	Putative resolvase (RlgA) - *Salmonella typhimurium*	3e-39
17	13778..14158	F	Plasmid partitioning protein - *Yersinia*	6e-59

			enterocolitica	
18	14228..14527	F	ISSM1 - *Serratia marcescens*	1e-46
19	14662..16441	F	ISSM1 - *Serratia marcescens*	1e-131
20	17345..17968	R	Partition protein A - *Xanthomonas axonopodis*	4e-77
21	18140..18904	R	Orf81 - *Yersinia enterocolitica*	5e-68
22	20188..20487	F	ISSM1 - *Serratia marcescens*	1e-46
23	20750..21707	R	IstB protein - *Escherichia coli*	4e-52
24	21707..22855	R	IstA protein - *Escherichia coli*	0.0
25	22953..23264	R	ISSM1 - *Serratia marcescens*	9e-45
26	23323..23580	R	Transposase - *Proteus vulgaris*	1e-24
27	23977..24090	F	ISSM1 unknown - *Serratia marcescens*	1e-11
28	24270..25130	F	Hypothetical protein LV077 - *Klebsiella pneumoniae*	1e-156
29	25169..25903	R	ISSM1 - *Serratia marcescens*	1e-129
30	27255..27635	R	FimH- *Klebsiella pneumoniae*	3e-50
31	28243..28746	R	FimG -*Escherichia coli*	5e-54
32	28759..29283	R	FimF - *Escherichia coli*	3e-44
33	29305..31897	R	FimD - *Escherichia coli*	8e-75
34	32015..32686	R	FimC - *Escherichia coli*	2e-84
35	32811..33350	R	FimI - *Shigella flexneri*	8e-64
36	33420..33968	R	FimA - *Escherichia coli*	2e-54
37	34610..34840	F	ISSM1 - *Serratia marcescens*	5e-21

Note: the sequence and open reading frames (ORF) were entered into Artemis program (http://www.sanger.ac.uk/Software/Artemis/)
and then each ORF was compared to the GenBank protein database using Blastp. ORFs greater than 80 amino acids (or 240 nucleotides) were presented here.

APPENDIX II. SUMMARY OF FOSU2

ORF	Position	Strand	Top BLASTX Match - Organism	P_value
1	350..841	F	Hypothetical protein STM0909 - Phage Fels-1	2e-66
2	834..2927	F	Hypothetical protein - Phage Gifsy-2	0.0
3	3133..4653	F	Hypothetical protein STM0911 - Phage Fels-1	0.0
4	4586..6631	F	Fels-1 prophage protease subunits - Phage Fels-1	0.0
5	6712..7089	F	Hypothetical prtein in prophage CP-933U - *Escherichia coli*	8e-14
6	7086..7403	F	Hypothetical protein STM0913.2N - Phage Fels-1	1e-17
7	7369..7929	F	Phage tail component - Phage Fels-1	1e-46
8	8037..8327	F	Gp12 - Bacteriophage N15	8e-33
9	8338..8841	F	Tail fiber component V of prophage CP-933U - *Escherichia coli*	2e-65
10	8857..9246	F	Putative tail component of prophage CP-933K - *Shigella flexneri*	1e-18
11	9556..12531	F	Phage tail component H- like protein - *Salmonella typhimurium*	0.0
12	12566..12862	F	Phage tail component M - like protein- *Salmonella typhimurium*	2e-26
13	12903..13601	F	Fels-1 prophage minor tail protein - Phage Fels-1	5e-72
14	13617..14345	F	Putative tail component of prophage - *Escherichia coli*	6e-92
15	14249..14896	F	Tail assembly protein-Phage Gifsy-2	4e-51
16	14915..18988	F	Putative tail component of prophage -	0.0

			Escherichia coli	
17	18988..19365	F	Unknown	
18	20588..21505	F	Tail fiber - Bacteriophage HK97	3e-36
19	21558..22106	F	Tail fiber protein of prophage CP-933X - *Escherichia coli*	2e-29
20	22120..23277	F	Tail fiber protein - *Yersinia pestis*	2e-34
21	23602..23925	R	Putative cytoplasmic protein - *Salmonella typhimurium*	1e-15
22	26199..26453	F	Putative phage integrase - *Yersinia pestis*	1e-36
23	28117..28812	F	Unknown	
24	30142..30378	F	Unkown	
25	30950..31693	F	Ant2 - *Enterobacteria* phage	6e-21
26	31690..31995	F	Unknown	
27	32084..32452	F	Unknown	
28	32452..33084	F	Hypothetical protein - Bacteriophage P27	7e-18
29	33094..33450	F	Unknown	

Note: the sequence and open reading frames (ORF) were entered into Artemis program (http://www.sanger.ac.uk/Software/Artemis/)

and then each ORF was compared to the GenBank NR protein database using Blastp. ORFs greater than 80 amino acids (or 240 nucleotides) were presented here.

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