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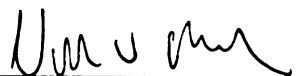
Genetic variation and virulence  
differentiation of *Pantoea stewartii*  
isolates from the eastern United States

presented by

Matthew Ryan Durkin

has been accepted towards fulfillment  
of the requirements for

M.S. degree in Plant Pathology



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**GENETIC VARIATION AND VIRULENCE DIFFERENTIATION OF  
*PANTOEA STEWARTII* ISOLATES FROM THE EASTERN UNITED STATES**

**By**

**Matthew Ryan Durkin**

**A THESIS**

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## ABSTRACT

### GENETIC VARIATION AND VIRULENCE DIFFERENTIATION OF *PANTOEA STEWARTII* ISOLATES FROM THE EASTERN UNITED STATES

By

Matthew Ryan Durkin

Stewart's bacterial wilt is a sporadic disease of sweet corn caused by the bacterium *Pantoea stewartii* and can be responsible for crop losses of up to 100%. Outbreaks of Stewart's bacterial wilt are currently thought to be dependent on the ability of the corn flea beetle (*Chaetocnema pulicaria*) to vector *P. stewartii*. In this research, genomic fingerprints were generated using the repetitive sequence-based polymerase chain reaction (rep-PCR) with the BOX and REP primers to differentiate between 84 isolates of *P. stewartii* that ranged in collection date (1967-2001) and location (Michigan, Illinois, Iowa, Ohio, North Carolina, Kentucky, Indiana). The resulting genomic fingerprints for each isolate were separated into four different strain types. Strain types had a similarity of 94-97% suggesting limited genetic diversity among isolates. These genetic data may be useful when studying future epidemics.

Virulence evaluations of *P. stewartii* isolates representative of each strain were also performed on four sweet corn varieties ranging in susceptibility to Stewart's bacterial wilt from susceptible to resistant. There was no relation between a particular rep-PCR strain type and virulence, but individual isolates were more virulent on certain sweet corn varieties. Pathogen virulence assessment may help with forecasting and prevention of future outbreaks.

Three collection methods for *P. stewartii* isolates (baiting with a susceptible sweet corn variety, sweeping for beetles, and scouting grower's fields) were also compared. During a non-epidemic year, baiting with a susceptible variety was the only method where *P. stewartii* isolates were successfully gathered.

*To my parents, family, and my soon to be wife, Emily.....  
Without you, this wouldn't be possible.....*

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## CHAPTER 1

### INTRODUCTION AND LITERATURE REVIEW

Bacterial plant pathogens are a diverse group of prokaryotic organisms able to infect and kill plants by causing diseases including blights, scabs, wilts, leaf spots, cankers, and soft rots. Each phytopathogenic bacterial species has unique characteristics that can be used to separate it from other organisms. Phytopathogenic bacteria can be either Gram positive or Gram negative and may be aerobic, anaerobic, or facultatively anaerobic (Gerhardt, 1981). The cell shape of phytopathogenic bacteria are generally bacilli, but there are other morphologies, such as cocci, coryneform, and filaments (Gerhardt, 1981). When allowed to grow as single colonies on solid medium, such as nutrient broth agar, colony morphology can be round, wavy edged, concave, convex, irregular, or angular (Schaad et al., 2001). Phytopathogenic bacterial cells can be transparent, or produce pigments, the most common being yellow, white, and red. Other diagnostically useful characteristics of plant pathogenic bacteria include, but are not limited to, their ability to cause a hypersensitive response on tobacco, growth within certain temperature ranges, motility, production of acid, and use of various nutrients (Agrios et al., 2001).

Plant pathogenic bacteria reproduce and grow in different environments. They live as epiphytes on the surface of plants, and are also able to inhabit the phloem or xylem of plants. Phytopathogenic bacteria can also survive as saprophytes in dead or decaying plant tissue (Hirano and Upper, 1983). Bacteria survive in soil, on seed, in the digestive tracts of vertebrates and invertebrates, and in various water sources.

Plants can be infected with bacteria in different ways. Bacteria can be transferred through the use of contaminated farm equipment, enter plants through the stomata, and infiltrate plants through wounds caused by animals or weather (Hartman and Hayward, 1993). Another important way plants are infected with bacteria is by insects. Insects can be vectors of bacterial plant pathogenic diseases by carrying bacteria on external body parts or internally in the digestive system. For example, *Erwinia amylovora* (Burrill), a bacterium which causes fire blight in apple and pear trees, is carried on the body and legs of honey bees (*Apis mellifera*), (Thomson et al., 1992). Honey bees transfer the bacterium to the stigma of apple tree and pear tree flowers when searching for nectar and pollen in the flowers. In contrast, blue-green sharpshooters (*Graphocephala atropunctata*) are xylem feeding suctorial insects which carry *Xylella fastidiosa* in their digestive systems (Purcell and Saunders, 1995). *Xylella fastidiosa* is the causal agent of Pierce's disease in grapevines. Sharpshooters transfer *X. fastidiosa* to grapevines by feeding on grape clusters. Another important insect vector is the corn flea beetle, *Chaetocnema pulicaria* (Melsh), which when feeding on corn plants transfers *Pantoea stewartii* causing Stewart's bacterial wilt (Poos and Elliott, 1935; Poos, 1955; Castor et al., 1975; Adams and Los, 1986).

## **Definitions**

### **The following vocabulary will be used throughout this thesis:**

**Pathogenicity.** The ability for a pathogen to cause disease.

**Phytopathogenic.** The ability for a pathogen to cause disease in a plant.

**Virulence.** The relative capacity of a pathogen to cause disease in a host.

**(more/less) Virulent.** Degree of virulence exhibited by a pathogen on a host.

**Avirulent.** Lacking virulence.

**Isolate.** A pure culture of bacteria that was obtained by subculture of a single colony from a common source (host).

**Strain.** An isolate that has been differentiated based on its (rep-PCR) genomic fingerprint pattern.

**Clonal.** Bacterial populations with little or no genetic change in the evolutionary history of the species.

**Panmictic.** Bacterial populations with genetic changes that have happened frequently during the evolutionary history of the species.

### **Stewart's Bacterial Wilt**

Stewart's bacterial wilt is a disease of field corn and sweet corn (*Zea mays* L.) that is caused by the bacterium *P. stewartii* (Frutche, 1936). The symptoms associated with Stewart's bacterial wilt include stunted plant growth, wilting of leaves, and linear water soaked lesions on the leaf surface. Stewart's bacterial wilt symptoms were first discovered on sweet corn in 1894 by F.C. Stewart in New York. Stewart determined that the cause of the symptoms was due to infection by a bacterium and sent a culture to Erwin F. Smith for taxonomic analysis. In 1897, Smith named the bacterium *Pseudomonas stewartii*, but over the years it has been renamed and is currently *Pantoea stewartii* (Mergaert et al., 1993).

*Pantoea stewartii* is a non-motile, gram negative, facultatively anaerobic, bacillus bacterium (McGee, 1998). On solid media, such as nutrient broth agar or Luria-Bertani agar, the bacterial colonies produce a yellow pigment. The optimal growth is 25-27 °C and it will not grow at temperatures of 37 °C or higher (Schaad et al., 2001). The bacterium produces a positive hypersensitive response when injected into tobacco leaves.

Once infected into the corn plant, *P. stewartii* can spread systemically through the vascular system and has been successfully extracted from leaves, stalks, roots, and seeds (Adams and Los, 1986; Munkvold et al., 1996; Pataky et al., 2000). An exopolysaccharide (EPS) may be responsible for its ability to colonize the vascular system of sweet corn (Braun, 1990; Coplin et al., 1992; Leigh and Coplin, 1992; Denny, 1999). The EPS may act as an anchoring mechanism allowing bacteria to remain in areas of the plants vascular system where nutrients are abundant, resulting in obstruction of water and nutrient pathways causing the plant to wilt and die (Blanco et al., 1979; Leigh and Coplin, 1992). Coplin et al. (1990) suggested that the EPS protects the bacterial cells from plant defense systems. Researchers are also studying plasmids contained in the bacterial cell for other pathogenicity factors (Coplin et al., 1992; Fu et al., 1996, 1997, 1998).

### **Taxonomy**

In 1993, three members of the genus *Erwinia* were classified as belonging to the genus *Pantoea*. Classification of members of the genus *Erwinia* has been under much scrutiny in the last ten years because of the diversity in morphology, genetic make-up, and pathogenicity observed among species classified in this genus (Mergaert et al. 1993). Morphological characteristics that are shared by members of the genus *Erwinia* include being Gram negative, the ability to grow both anaerobically and aerobically, the inability to grow at 37°C, and the lack of ability to produce spores (Schaad et al., 2001). The characteristics that are not shared among all *Erwinia* genus members include bacterial colonies possessing a yellow pigment when grown on certain media, the production and

utilization of pectate lyases and cellulases, and the symptoms produced on plants that include blights, wilts, and soft-rots.

Researchers have worked to better understand, reorganize, and possibly disband the genus *Erwinia* using molecular techniques that include DNA-DNA hybridization, protein profiles, G+C content comparisons, and the sequencing of specific genomic regions (Mergaert et al. 1993; Kwon et al., 1997). Utilizing molecular and morphological results, three subgroups have been loosely defined. Group one is the soft rot group which contains the *carotovora* and *chrysanthemi* species (Kwon et al. 1997). This group has been defined by its ability to utilize pectate lyases and cellulases to degrade cell walls causing soft rot and by the similarity in their G+C content (Mergaert et al. 1993). Group two is the *amylovora* group that includes the *amylovora*, *nigrifluens*, *salicis*, *rubrifacinens*, and *rhapontici* species (Mergaert et al. 1993; Kwon et al., 1997). The *amylovora* group contains bacteria that primarily infect trees and have been grouped based on their carbon utilization and genomic structure (Kwon et al., 1997). The last group that has historically been a part of the genus *Erwinia*, but is now referred to as the genus *Pantoea* contains the species *stewartii*, *ananas*, and *uredovora* (Mergaert et al. 1993). Mergaert et al. (1993) separated these species to the genus *Pantoea* based on DNA-DNA hybridization and protein profiles. *Pantoea stewartii* has been previously classified as; *Pseudomonas stewartii* (1887 E. F. Smith); *Phytomonas stewartii* (1918 E.F. Smith); *Bacillus stewartii* (1920 E.F. Smith); *Xanthomonas stewartii* (1939 E.F. Smith); *Erwinia stewartii* (1963 E.F. Smith); (Pepper, 1967; Mergaert et al. 1993).

The separation and classification of the *Erwinia* genus is still under much debate. Kwon et al. (1997) attempted to resolve some of the ambiguity by analyzing the



relationships between 16 different *Erwinia* species based on their 16S rRNA gene sequences. Six members of the family *Enterobacteriaceae*, of various genera, were also included to help clarify the proposed hypothesis that the genus *Erwinia* is polyphyletic (Kwon et al., 1997). The research performed by Kwon et al. (1997) used an evolutionary distance relationship described by Jukes-Cantor in the form of a neighbor-joining tree to analyze the sequences of the 16S rRNA in each of the 22 species. The conclusion of Kwon et al. (1997) was that the *Erwinia* genus is polyphyletic. More recently, Durkin (unpublished data 2001) reanalyzed the same 16S rRNA sequences using a maximum parsimony character-state approach and a maximum likelihood analysis to show that a polyphyletic relation between members of the *Erwinia* and *Pantoea* species was not statistically more likely than a monophyletic relation. The classification of the genera *Erwinia* and *Pantoea* is currently still under debate.

#### **The Vector: *Chaetocnema pulicaria***

The vector of *P. stewartii* in corn plants is the corn flea beetle, *Chaetocnema pulicaria* (Poos and Elliott, 1935; Pepper, 1967; Adams and Los, 1986). The bacterium is carried in the digestive system of the beetle and transferred to the corn plant when the beetles feed on plant tissue (Poos and Elliott, 1935; Poos, 1955; Adams and Los, 1986). Feeding usually occurs on the leaf surface where the adult beetle chews and removes a superficial layer of the leaf, but feeding on the roots by corn flea beetle larvae has also been observed (Poos and Elliott, 1935; Poos, 1955; Adams and Los et al., 1986). The beetles feed primarily on sweet corn, but have also been known to feed on crabgrass, orchard grass, ryegrass, and fall panicum (Adams and Los, 1986). Because of their minute size (2 mm long), damage caused to the leaf of a corn plant by single beetles is

minimal, wounds are 1 mm wide and 1-30 mm long, but large numbers of beetles on individual plants have the ability to cause extensive structural damage (Poos and Elliott, 1935; Poos, 1955; Adams and Los, 1986). The economic impact from corn flea beetle infestation is the beetle's ability to vector *P. stewartii*.

The life cycle of the corn flea beetle has been studied to better understand and predict when epidemics of Stewart's bacterial wilt may occur. The corn flea beetle over-winters as an adult in the soil or plant debris and re-emerges in the early spring when sweet corn is beginning to emerge (Adams and Los, 1986). Soon after beetles emerge from the ground, they lay their eggs in the soil (Pepper 1967; Adams and Los, 1986). These larvae emerge mid-summer. Adults can continue to transmit *P. stewartii* to uninfected plants after feeding on infected plants (Adams and Los, 1986). In 1934, N. E. Stevens (1934) developed a winter temperature index that predicted the over-wintering probability of corn flea beetles. The index is applied by measuring the sum of the average temperatures ( $^{\circ}\text{C}$ ) during December, January, and February. According to the index, if the sum of the three months is less than  $0.0^{\circ}\text{C}$  then there is a low risk of Stewart's bacterial wilt, but if greater than  $0.0^{\circ}\text{C}$  then the risk of Stewart's bacterial wilt infection is high (Stevens, 1934). It has been shown that only 10-30% of over-wintering adult beetles carry *P. stewartii*, but this winter temperature index makes the assumption that all beetles are carriers of *P. stewartii* (Pepper, 1967). Stevens (1934) also developed a threshold that estimated when beetle populations could be a viable crop problem. The threshold was exceeded if 6 beetles were observed on 100 plants (Stevens, 1934). Other reliable methods of beetle scouting and collection include the use of yellow sticky traps and sweep nets (Adams and Los, 1986). The temperature and threshold indices are

presently the standard tools for predicting epidemics of Stewart's bacterial wilt throughout the eastern United States.

### **Stewart's Bacterial Wilt Epidemics**

Historically, the incidence of Stewart's bacterial wilt in United States sweet corn fields may be described as sporadic. After its discovery in New York by Stewart in 1894, the incidence of Stewart's bacterial wilt in the eastern United States was limited to a few individual sweet corn plants until a severe outbreak occurred in 1932 (Frutchey, 1936). During the 1930's, researchers attempted to answer questions of how and where inoculum levels of *P. stewartii* accumulated to levels that would cause such a severe epidemic (Frutchey, 1936). Researchers directed their studies of *P. stewartii* control towards survival on seed, transmission of bacterium by insects, and breeding for resistance (Stevens, 1934; Poos and Elliott, 1935; Frutchey, 1936). It was concluded that the ability of *P. stewartii* to survive on sweet corn seed was low, less than 3% of infected seed produced plants with Stewart's bacterial wilt symptoms (Frutchey, 1936). More recent studies confirmed that the chance of transmitting *P. stewartii* by seed was minimal (< 5% infected seed produced by infected plants) based on seed infection studies (Block et al., 1998, 1999). Entomological researchers concluded that beetle members of the genus *Chaetocnema*, most importantly the corn flea beetle (*C. pulicaria*), were the primary vectors of *P. stewartii* (Poos and Elliott, 1935). Presently, the corn flea beetle is recognized as the primary vector and source of inoculum for Stewart's bacterial wilt infection (Munkvold et al. 1996). Lastly, Reddy and Holbert (1928) were able to achieve limited control of Stewart's bacterial wilt by testing inbred and hybrid varieties of sweet corn that showed resistant to Stewart's bacterial wilt (Frutchey, 1936). Patakay et al.

(2000) has shown that there are some varieties that are more resistant to Stewart's bacterial wilt infection, but even these varieties can still be killed if infected with high populations ( $10^6$ - $10^8$  colony forming units (CFU) / ml) of *P. stewartii*.

After the Stewart's bacterial wilt outbreak in 1932, the incidence of Stewart's bacterial wilt in the eastern United States was limited to the infection of a few individual sweet corn plants even when high levels of corn flea beetles were observed, until the late 1960's and early 1970's (Pepper, 1967; D. L. Coplin, pers. comm.). During this time, the incidence of Stewart's bacterial wilt of sweet corn again reached epidemic proportion in parts of the eastern United States (D. L. Coplin, pers. comm.). High levels of corn flea beetles vectoring *P. stewartii* were again determined to be the cause, but there was no explanation for the sudden increase in *P. stewartii* inoculum levels.

After the early 1970's, Stewart's bacterial wilt was again only observed sporadically on a few individual plants throughout the eastern United States until another epidemic occurred during the 1989 growing season in New York State, the 1990-1992 growing season in Iowa, the 1995 growing season in Illinois, and the 1996-98 growing seasons in Michigan (Dillard and Kline, 1989; Pataky et al. 1996, Munkvold et al., 1996, W. Kirk, pers. comm.). At this time, researchers shifted away from corn flea beetle population studies and began to explore population structures of the bacterial pathogen, *P. stewartii*. Wilson and Dillard, 1999, utilized the Biolog Inc. (Hayward, CA) Biolog gram-negative microplate which contained 95 carbon sources to determine the phenotypic variability of 124 *P. stewartii* isolates collected from 11 different states in the Eastern United States ranging in collection dates between 1932-1994. It was determined in this study that all of the isolates except one shared the ability to utilize 93% of the

same carbon sources suggesting limited diversity in the phenotype of the different isolates (Wilson and Dillard, 1999). Wilson and Dillard (1999) hypothesized that limited phenotypic diversity may correspond to a limited genotype diversity. Coplin et al. (2002) utilized pulsed field gel electrophoresis (PFGE) to separate restriction fragments produced by two restriction endonucleases and determined that isolates from separate regions could be differentiated at the strain level using this technique. Although it was not the primary objective of their research, Coplin et al. (2002) concluded that the strains analyzed seemed to have a low diversity, based on the large amount of shared restriction fragments. Presently, the genetic population diversity of strains of *P. stewartii* is still uncertain.

### **Bacterial Population Analysis**

Populations of bacterial species have varying levels of genetic diversity (Smith et al., 1993; Spratt and Maiden, 1999). Bacteria reproduce asexually by binary fission, which consists of a mother cell giving rise to two identical daughter cells (Smith et al., 1993). Genetic changes in the chromosome of bacterium most frequently occur through point mutation or recombination by transformation, conjugation, or transduction. There is evidence that recombination and mutation rates may vary broadly between different bacterial populations (Spratt and Maiden, 1999). Therefore, bacterial population structures can range from being clonal to panmictic. Most bacterial populations are somewhere intermediate between the classification of clonal and panmictic. Bacterial populations have also been defined as 'epidemic clonal', which is a panmictic population that sporadically arises as a virulent strain for a short period of time, but survives as an avirulent or less virulent organism over an extended duration. It is theorized that specific

events occur that initiate recombination in bacterial population structures resulting in a pathogen population that is more virulent over a short period of time (Spratt and Maiden, 1999).

Random whole-genome analysis (RWGA), specifically repetitive-sequence-based polymerase chain reaction (rep-PCR), can help to differentiate between members of a bacterial population (Louws et al., 1994). The rep-PCR technique uses primer sets based on conserved repetitive bacterial elements (De Bruijn, 1992; Louws et al., 1999). These primers include the repetitive extragenic palindromic (REP) sequence (35–40 bp) and the BOX element that is made up of boxA, boxB, and boxC (154 bp), (de Bruijn, 1992). When these primers are used in the rep-PCR technique, they are referred to as REP-PCR and BOX-PCR (Louws et al., 1996). PCR amplification by these primer sets can yield genomic fingerprints specific to individual strains of phytopathogenic bacteria. For example, this technique has been used to differentiate within phytopathogenic strains of *Xanthomonas* and *Pseudomonas* that were previously not distinguishable with other classification methods (Louws et al., 1994). Since phytopathogenic strains of *Xanthomonas* and *Pseudomonas* have a wide range of hosts, rep-PCR was shown to be a quick and effective tool which could differentiate these bacteria at the pathovar level. The rep-PCR technique was also used by Louws et al. (1998) to differentiate between five *Clavibacter michiganensis* subspecies that were pathogenic on different hosts (Louws et al., 1998). They also found that rep-PCR could be used as a tool to differentiate between members of the subspecies *C. michiganensis* subsp. *michiganensis*. This subspecies was divided into five rep-PCR strain types based on genomic fingerprint polymorphisms. Although a biological explanation was not concluded, the rep-PCR strain type groupings

also corresponded to strain groupings based on their known virulence (Louws et al., 1998). More recently, Horita and Tsuchiya (2000) assessed the genetic diversity of 74 strains of *Ralstonia solanacearum*, which has an extremely wide host range, and were able to determine that although strains were morphologically similar, they were easily separated into races based on their rep-PCR genomic fingerprints.

### **Objectives of this Research**

This research incorporates objectives that will assess both genetic variability and virulence of a range of isolates of *P. stewartii* from the eastern United States. Defining the genetic variability of *P. stewartii* isolates from different temporal and geographic locations may offer Stewart's bacterial wilt researchers an epidemiological tool that can be used when epidemics occur in the future. Also, the development of a procedure that can assess if variation in virulence exists between different isolates of *P. stewartii* may be used as a tool to differentiate *P. stewartii* strain populations that are present during an epidemic.

In order to fulfill these objectives three procedures must be performed:

Specifically;

- 1) Collect *P. stewartii* isolates that represent different geographical locations and different time periods in the eastern United States by:
  - a) contacting researchers of Stewart's bacterial wilt from the eastern United States who maintain *P. stewartii* collections.
  - b) capturing and assaying corn flea beetles for *P. stewartii* from Michigan sweet corn fields
  - c) placing a susceptible variety of sweet corn in Michigan sweet corn fields as bait

- d) scouting sweet corn fields in Michigan for the presence of Stewart's bacterial wilt infection
- 2) Determine if the rep-PCR technique can be used to differentiate between members of a closely related genus (*Erwinia* and *Pantoea*) as well as differentiate between isolates of the same species (*P. stewartii*), and if so use BOX-PCR and REP-PCR as a tool to determine if variability exists between *P. stewartii* isolates.
- 3) If strains of *P. stewartii* can be differentiated using the rep-PCR protocol, determine the variability in virulence between different strains of *P. stewartii* on sweet corn.



## CHAPTER 2

### MATERIALS AND METHODS

**2.1 Isolates Studied.** Eighty-four *P. stewartii* isolates were obtained from both the 2001 growing season and from culture collections (Table 1). Three of the isolates were assayed from Michigan (Ps-II) sweet corn during the 2001 growing season. Fourteen of the isolates assayed (Ps-V; VI; VII) were from sweet corn in three regions of Illinois during the 2001 growing season. Twenty-five isolates (SW 87) were obtained from sweet corn in Iowa and stored by Greg Lamka in 1987 (Lamka, 1991). Twenty-nine isolates (ES 92) were obtained from sweet corn in Iowa and stored by Charles Block in 1992. Fourteen isolates were obtained from Dr. D. Coplin's culture collection at Ohio State University through Dr. C. Block. Two isolates were used as a positive controls: *Pantoea stewartii* – ATCC-29227 (Margaert, 1993) which was obtained from the American Type Culture Collection (ATCC) and Il-Ps 1 which was obtained from Dr. J. Pataky of the University of Illinois, Urbana.

**2.2 Michigan Isolate Collection Techniques.** In 2000, *P. stewartii* was not found in sweet corn plants or corn flea beetles and there were no reports of Stewart's bacterial wilt infection by Michigan sweet corn growers. Therefore, Michigan isolates were collected only over the course of the 2001 growing season (May 15 - Aug 25). Twelve commercial sweet corn fields, ranging in size from 15 to 25 ha, located in the southern half of the Lower Peninsula in Michigan were surveyed on a ten day schedule starting May 15 for Stewart's bacterial wilt infection (Figure 1).

Three techniques were used to find isolates of Stewart's bacterial wilt. Scouting; 15 different rows 100 m long were inspected. All sweet corn plants that exhibited any

**Table 1. Bacterial isolates used in this research for rep-PCR analysis and virulence differentiation on sweet corn.**

| Genus-Species                | Strain <sup>a</sup>  | Source <sup>b</sup> | Location Source <sup>c</sup>                    | Presence (+)<br>Absence (-)<br>of 700 bp<br>band in<br>(BOX-PCR)<br>fingerprint | Presence (+)<br>Absence (-)<br>of 380 bp band<br>in<br>(REP-PCR)<br>fingerprint | PCR<br>fingerprint<br>type <sup>d</sup> |
|------------------------------|----------------------|---------------------|-------------------------------------------------|---------------------------------------------------------------------------------|---------------------------------------------------------------------------------|-----------------------------------------|
| <i>Pantoea<br/>stewartii</i> | ATCC -29227          | ATCC                | United States                                   | +                                                                               | +                                                                               | 1                                       |
|                              | II-Ps 1 <sup>e</sup> | SCP                 | University of<br>Illinois, Urbana<br>Dr. Pataky | -                                                                               | +                                                                               | 3                                       |
|                              | Ps-II-B-CP-1         | SCP                 | Riley Center, MI,<br>(2001)                     | -                                                                               | +                                                                               | 3                                       |
|                              | Ps-II-B-CP-3         | SCP                 | Riley Center, MI,<br>(2001)                     | -                                                                               | +                                                                               | 3                                       |
|                              | Ps-II-B-CP-4         | SCP                 | Riley Center, MI,<br>(2001)                     | -                                                                               | +                                                                               | 3                                       |
|                              | Ps-V-A-CP-1-1        | SCP                 | Champaign, IL-<br>Dr. Pataky Lab<br>(2001)      | -                                                                               | +                                                                               | 3                                       |
|                              | Ps-V-A-CP-1-2        | SCP                 | Champaign, IL-<br>(2001)                        | -                                                                               | +                                                                               | 3                                       |
|                              | Ps-V-A-CP-3-1        | SCP                 | Champaign, IL-<br>(2001)                        | -                                                                               | +                                                                               | 3                                       |
|                              | Ps-V-A-CP-3-2        | SCP                 | Champaign, IL-<br>(2001)                        | -                                                                               | +                                                                               | 3                                       |
|                              | Ps-V-A-CP-4-1        | SCP                 | Champaign, IL-<br>(2001)                        | -                                                                               | +                                                                               | 3                                       |
|                              | Ps-V-A-CP-4-2        | SCP                 | Champaign, IL-<br>(2001)                        | -                                                                               | +                                                                               | 3                                       |
|                              | Ps-VI-A-CP-1-1       | SCP                 | Mendota, IL<br>(2001)                           | -                                                                               | +                                                                               | 3                                       |
|                              | Ps-VI-A-CP-1-2       | SCP                 | Mendota, IL<br>(2001)                           | -                                                                               | +                                                                               | 3                                       |
|                              | Ps-VI-A-CP-1-3       | SCP                 | Mendota, IL<br>(2001)                           | -                                                                               | +                                                                               | 3                                       |

| Genus-Species            | Strain <sup>a</sup> | Source <sup>b</sup> | Location Source <sup>c</sup>       | Presence (+)<br>Absence (-)<br>of 700 bp<br>band in<br>(BOX-PCR)<br>fingerprint | Presence (+)<br>Absence (-)<br>of 380 bp band<br>in<br>(REP-PCR)<br>fingerprint | PCR<br>fingerprint<br>type <sup>d</sup> |
|--------------------------|---------------------|---------------------|------------------------------------|---------------------------------------------------------------------------------|---------------------------------------------------------------------------------|-----------------------------------------|
| <i>Pantoea stewartii</i> | Ps-VI-A-CP-2-1      | SCP                 | Mendota, IL (2001)                 | -                                                                               | +                                                                               | 3                                       |
|                          | Ps-VI-A-CP-2-3      | SCP                 | Mendota, IL (2001)                 | -                                                                               | +                                                                               | 3                                       |
|                          | Ps-VI-A-CP-4-1      | SCP                 | Mendota, IL (2001)                 | -                                                                               | +                                                                               | 3                                       |
|                          | Ps-VIII-A-CP-3-1    | SCP                 | Dixon Springs, IL (2001)           | -                                                                               | +                                                                               | 3                                       |
|                          | Ps-VIII-A-CP-3-2    | SCP                 | Dixon Springs, IL (2001)           | -                                                                               | +                                                                               | 3                                       |
|                          | SW 1                | SCP                 | OSU (Ohio) (1974)                  | +                                                                               | -                                                                               | 2                                       |
|                          | SW 2                | SCP                 | OSU (Ohio) (1974)                  | +                                                                               | -                                                                               | 2                                       |
|                          | SW 13               | SCP                 | OSU (Indiana) (1974)               | +                                                                               | +                                                                               | 1                                       |
|                          | SW 14               | SCP                 | OSU (Indiana) (1974)               | +                                                                               | +                                                                               | 1                                       |
|                          | SW 19               | SCP                 | OSU (Kentucky) (1975)              | +                                                                               | +                                                                               | 1                                       |
|                          | SW 36               | SCP                 | OSU (Tennessee) (1975)             | +                                                                               | +                                                                               | 1                                       |
|                          | SW 39               | SCP                 | OSU (North Carolina) (1975)        | +                                                                               | +                                                                               | 1                                       |
|                          | SW 45               | SCP                 | OSU Illinois (1975)                | +                                                                               | +                                                                               | 1                                       |
|                          | MU 51               | SCP                 | OSU (acapsular virulent mutant)    | +                                                                               | -                                                                               | 2                                       |
|                          | GAL E8              | SCP                 | OSU (spontaneous avirulent mutant) | +                                                                               | -                                                                               | 2                                       |

| Genus-Species | Strain <sup>a</sup>                                                              | Source <sup>b</sup> | Location Source <sup>c</sup>                                   | Presence (+)<br>Absence (-)<br>of 700 bp<br>band in<br>(BOX-PCR)<br>fingerprint | Presence (+)<br>Absence (-)<br>of 380 bp<br>band in<br>(REP-PCR)<br>fingerprint | PCR<br>fingerprint<br>typed <sup>d</sup> |
|---------------|----------------------------------------------------------------------------------|---------------------|----------------------------------------------------------------|---------------------------------------------------------------------------------|---------------------------------------------------------------------------------|------------------------------------------|
|               | DC 160                                                                           | SCP                 | OSU<br>(New York) (1976)                                       | +                                                                               | -                                                                               | 2                                        |
|               | DC 283                                                                           | SCP                 | OSU<br>(spontaneous<br>SS104- mutant)                          | +                                                                               | -                                                                               | 2                                        |
|               | Rif 9a                                                                           | SCP                 | OSU<br>(rifampicin resistant<br>strain-derived from<br>DC 283) | +                                                                               | -                                                                               | 2                                        |
|               | SW 87-1 –SW 87-5<br>SW 87-7 – SW 87-13<br>SW 87-15-16; 18<br>SW 87-20-23; 25; 26 | SCP                 | ISU<br>(1987)                                                  | +                                                                               | +                                                                               | 1                                        |
|               | SW 87-6; 17; 19; 24                                                              | SCP                 | ISU<br>(1987)                                                  | +                                                                               | -                                                                               | 2                                        |
|               | ES 9201                                                                          | SCP                 | Washington, IA                                                 | +                                                                               | +                                                                               | 1                                        |
|               | ES 9203                                                                          | SCP                 | Lonetree Iowa                                                  | +                                                                               | +                                                                               | 1                                        |
|               | ES 9204                                                                          | SCP                 | Washington Iowa                                                | +                                                                               | +                                                                               | 1                                        |
|               | ES 9205                                                                          | SCP                 | Nevada, Iowa                                                   | +                                                                               | +                                                                               | 1                                        |
|               | ES 9206                                                                          | SCP                 | Nevada, Iowa                                                   | -                                                                               | +                                                                               | 3                                        |
|               | ES 9207                                                                          | SCP                 | Story City Iowa                                                | +                                                                               | +                                                                               | 1                                        |
|               | ES 9208                                                                          | SCP                 | Boone, Iowa                                                    | +                                                                               | +                                                                               | 1                                        |
|               | ES 9209                                                                          | SCP                 | Boone, Iowa                                                    | +                                                                               | +                                                                               | 1                                        |
|               | ES 9210                                                                          | SCP                 | Boone, Iowa                                                    | +                                                                               | +                                                                               | 1                                        |
|               | ES 9211                                                                          | SCP                 | Slater, Iowa                                                   | +                                                                               | +                                                                               | 1                                        |
|               | ES 9212                                                                          | SCP                 | Story City Iowa                                                | +                                                                               | -                                                                               | 2                                        |
|               | ES 9213                                                                          | SCP                 | Slater, Iowa                                                   | -                                                                               | +                                                                               | 3                                        |
|               | ES 9221                                                                          | SCP                 | Washington, IA                                                 | +                                                                               | +                                                                               | 1                                        |

| Genus-Species            | Strain <sup>a</sup> | Source <sup>b</sup> | Location Source <sup>c</sup> | Presence (+)<br>Absence (-)<br>of 700 bp<br>band in<br>(BOX-PCR)<br>fingerprint | Presence (+)<br>Absence (-)<br>of 380 bp<br>band in<br>(REP-PCR)<br>fingerprint | PCR<br>fingerprint<br>type <sup>d</sup> |
|--------------------------|---------------------|---------------------|------------------------------|---------------------------------------------------------------------------------|---------------------------------------------------------------------------------|-----------------------------------------|
| <i>Pantoea stewartii</i> | ES 9225             | SCP                 | Washington, IA               | +                                                                               | +                                                                               | 1                                       |
|                          | ES 9222             | SCP                 | Washington, IA               | +                                                                               | +                                                                               | 1                                       |
|                          | ES 9230             | SCP                 | Maxwell, IA                  | +                                                                               | -                                                                               | 2                                       |
|                          | ES 9243             | SCP                 | Slater, Iowa                 | +                                                                               | +                                                                               | 1                                       |
|                          | ES 9244             | SCP                 | Slater, Iowa                 | +                                                                               | +                                                                               | 1                                       |
|                          | ES 9245             | SCP                 | Nevada, Iowa                 | -                                                                               | -                                                                               | 4                                       |
|                          | ES 9246             | SCP                 | Coon Rapids, IA              | +                                                                               | +                                                                               | 1                                       |
|                          | ES 9247             | SCP                 | Slater, Iowa                 | +                                                                               | +                                                                               | 1                                       |
|                          | ES 9248             | SCP                 | Madrid, IA                   | +                                                                               | +                                                                               | 1                                       |
|                          | ES 9249             | SCP                 | Story City Iowa              | +                                                                               | +                                                                               | 1                                       |
|                          | ES 9250             | SCP                 | Madrid, IA                   | +                                                                               | +                                                                               | 1                                       |
|                          | ES 9253             | SCP                 | Schaller, IA                 | +                                                                               | +                                                                               | 1                                       |
|                          | ES 9256             | SCP                 | Coon Rapids, IA              | +                                                                               | -                                                                               | 2                                       |
|                          | SS 104              | ICPB                | Illinois (1967)              | +                                                                               | -                                                                               | 2                                       |
|                          | ES 9257             | SCP                 | Grundy Center, IA            | -                                                                               | +                                                                               | 3                                       |
| <i>P. anannus</i>        | DC 130              | SCP                 | OSU<br>New York              | na <sup>f</sup>                                                                 | na                                                                              | na                                      |
| <i>Erwinia amylovora</i> | 10                  | AT                  | Michigan                     | na                                                                              | na                                                                              | na                                      |
|                          | 321                 |                     | France                       | na                                                                              | na                                                                              | na                                      |
|                          | MRI                 | R                   | Michigan                     | na                                                                              | na                                                                              | na                                      |

| Genus-Species          | Strain <sup>a</sup> | Source <sup>b</sup> | Location Source <sup>c</sup> | Presence (+)<br>Absence (-)<br>of 700 bp band in<br>(BOX-PCR)<br>fingerprint | Presence (+)<br>Absence (-)<br>of 380 bp band in<br>(REP-PCR)<br>fingerprint | PCR<br>fingerprint<br>type <sup>d</sup> |
|------------------------|---------------------|---------------------|------------------------------|------------------------------------------------------------------------------|------------------------------------------------------------------------------|-----------------------------------------|
| <i>E. amylovora</i>    | PSLN                |                     | Michigan                     | na                                                                           | na                                                                           | na                                      |
|                        | CFB Pr              | uk                  | uk                           | na                                                                           | na                                                                           | na                                      |
| <i>E. carotovora</i>   | Uk <sup>g</sup>     | uk                  | uk                           | na                                                                           | na                                                                           | na                                      |
| <i>E. chrysanthami</i> | uk                  | uk                  | uk                           | na                                                                           | na                                                                           | na                                      |

<sup>a</sup>The designation given to each strain. For Michigan and Illinois strains Es=*Erwinia stewartii*, Ps=*Pantoea stewartii* the roman-numeral=the region, the letter = the field, the CP=potted sweet corn plant, and the number indicates the order in which it was collected. For the Iowa strains the ES or the SW = *Pantoea stewartii* and Stewart's Wilt respectively. The first two digits equal the year and the last two digits indicate the order in which they were collected.

-Strains Ps-II- Matthew Durkin, Michigan State University

-Strains Ps-V, VI, VIII – Dr. J Pataky University of Illinois, Urbana

-Strains Es 92 – Dr. Charles Block's Culture Collection, Iowa State University

-Strains SW 87 – Dr. Greg Lamka's Culture Collection, Iowa State University

-Strains from OSU- Dr. D. Coplin Culture Collection, Ohio State University

-*E. amylovora* strains – Dr. Al Jones Culture Collection, Michigan State University

<sup>b</sup> Source: ATCC – American Type Culture Collection; SCP – Sweet Corn Plant Tissue; ICPB – International Collection of Phytopathogenic Bacteria, University of California, Davis; AT – Apple Tree; R – Raspberry.

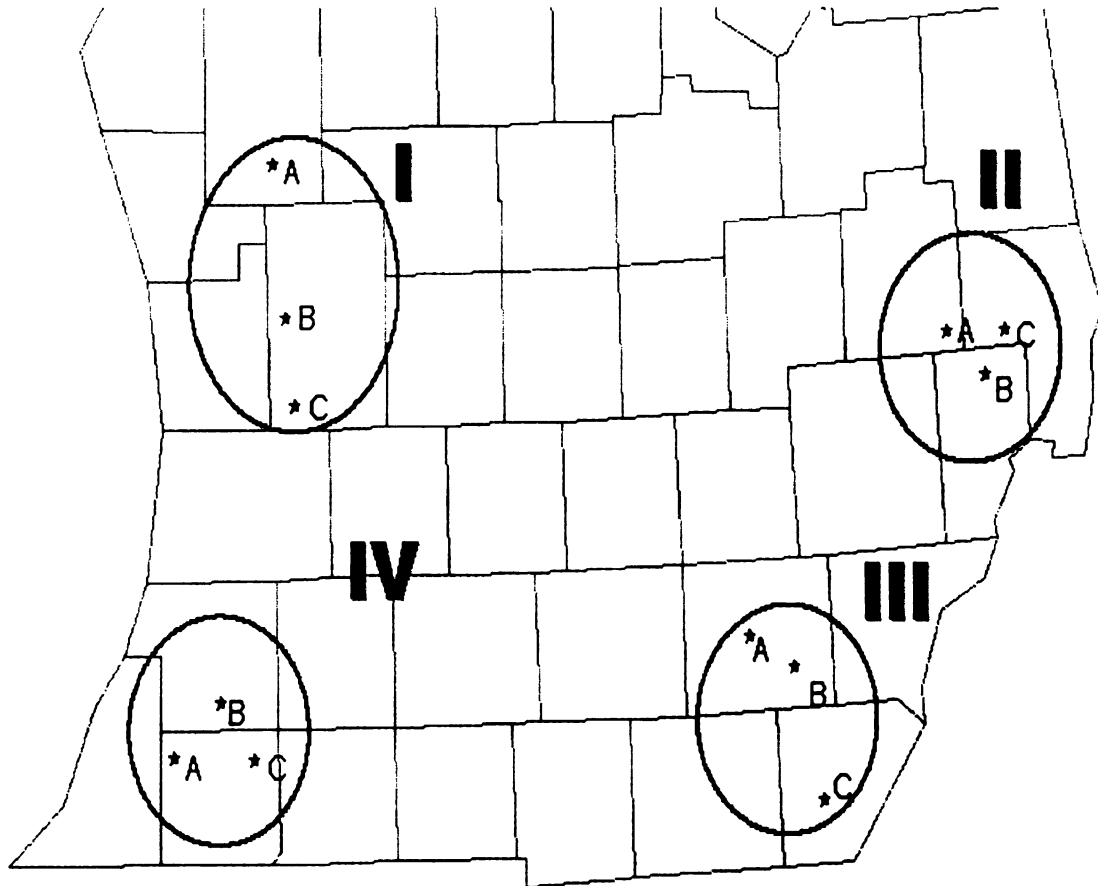
<sup>c</sup> City, state, or country where the isolate was sampled from (if known).

<sup>d</sup> Designation given to a strain based on its genomic fingerprints from rep-PCR using the BOX and REP primers.

<sup>e</sup> Population of strains maintained on greenhouse sweet corn plants. The population originated 10 to 15 years ago and was assayed from a sweet corn plant collected in Urbana, Illinois by Dr. J Pataky. The population has been added to each year with populations from infected leaf tissue collected in Urbana, Illinois.

<sup>f</sup> Not applicable.

<sup>g</sup> Unknown.



**Figure 1. Southern portion of Michigan's Lower Peninsula showing the twelve locations where isolates of *Pantoea stewartii* were sought. The roman numerals represent the regions and the lettered stars represent the closest city to the fields. I-A-Fremont; I-B-Grand Rapids; I-C-Byron City; II-A-Romeo; II-B-Almont; II-C-Riley Center; III-A-Chelsea (field 1) III-B-Chelsea (field 2); III-C-Monroe; IV-A-Dowagiac; IV-B-Decatur; IV-C-Marcellus.**

evidence of Stewart's bacterial wilt in the grower's fields were harvested and assayed for *P. stewartii*. Bait; 25 potted (pot size = 20.5 cm) Jubilee sweet corn plants were placed as bait in weeds around the margins of the fields until mid-June, after the grower's sweet corn reached the 3-5 leaf stage, when they were then placed between sweet corn plants in the field. The pots were placed in five separate rows, five pots per row with 5 m spaces between each pot. Plants with symptoms of Stewart's bacterial wilt or corn flea beetle feeding scars (1-30 mm lines 1 mm wide where the epidermis had been removed on the leaf tissue) were assayed for *P. stewartii*. Sweeping; corn flea beetles were captured with an insect sweep net (38 cm diameter rim and polyester net bag). Four sweeps were performed per visit in the sweet corn field and in the weeds that surrounded the field. Single sweeping events were conducted over 100 m sweeping the net approximately 150 times. The contents of the net bag were emptied into a 1 L plastic Ziploc® bag from which corn flea beetles were removed, identified, and assayed (described in section 2.3) for *P. stewartii*.

## **2.3 Isolation Techniques.**

**2.3.1 Sweet Corn Plants.** Each sweet corn sample that was suspected to be infected with Stewart's bacterial wilt was assayed for the presence of *P. stewartii* by removing a 1.5 cm<sup>2</sup> section of leaf tissue from a symptomatic sweet corn sample. The section was placed in 0.4 ml of buffered saline (0.01 M potassium phosphate, 0.8% sodium chloride, pH 7.0) in a sterile porcelain mortar and macerated using a sterile porcelain pestle for 10 seconds (Ivanoff, 1933). The macerated solution was then streaked using a 1 µl sterile loop on a plate (100 x 15 mm) containing nutrient broth agar (1.5% agar, 8 g nutrient broth/ L



Difco-NBA). The plates were incubated in the dark for 48 hours at 23°C. Single colonies that had the general characteristics of *P. stewartii* (yellow pigment, < 3 mm in size) were picked and re-streaked on NBA plates. The procedure was repeated 3 consecutive times to ensure the purity of the culture so a frozen stock sample could be preserved. A frozen stock sample was prepared by removing a single colony from the final culture, which was transferred to a 12 x 150 mm screw cap culture tube containing 5 ml of nutrient agar, and incubated for 24 hours at 23 °C on a shaker at 200 rpm. A 1.0 ml sample of the solution was placed in 0.5 ml of 50% glycerol and stored in 1.5 ml centrifuge tubes at -80 °C in a freezer.

**2.3.2 Corn Flea Beetle.** Corn flea beetles were macerated and assayed according to the previous method (Section 2.3.1). The beetles were first placed in a -20 °C freezer for 10 minutes to slow their activity. Each beetle was evaluated individually.

#### **2.4 rep-PCR differentiation between members of the genus *Erwinia* and genus**

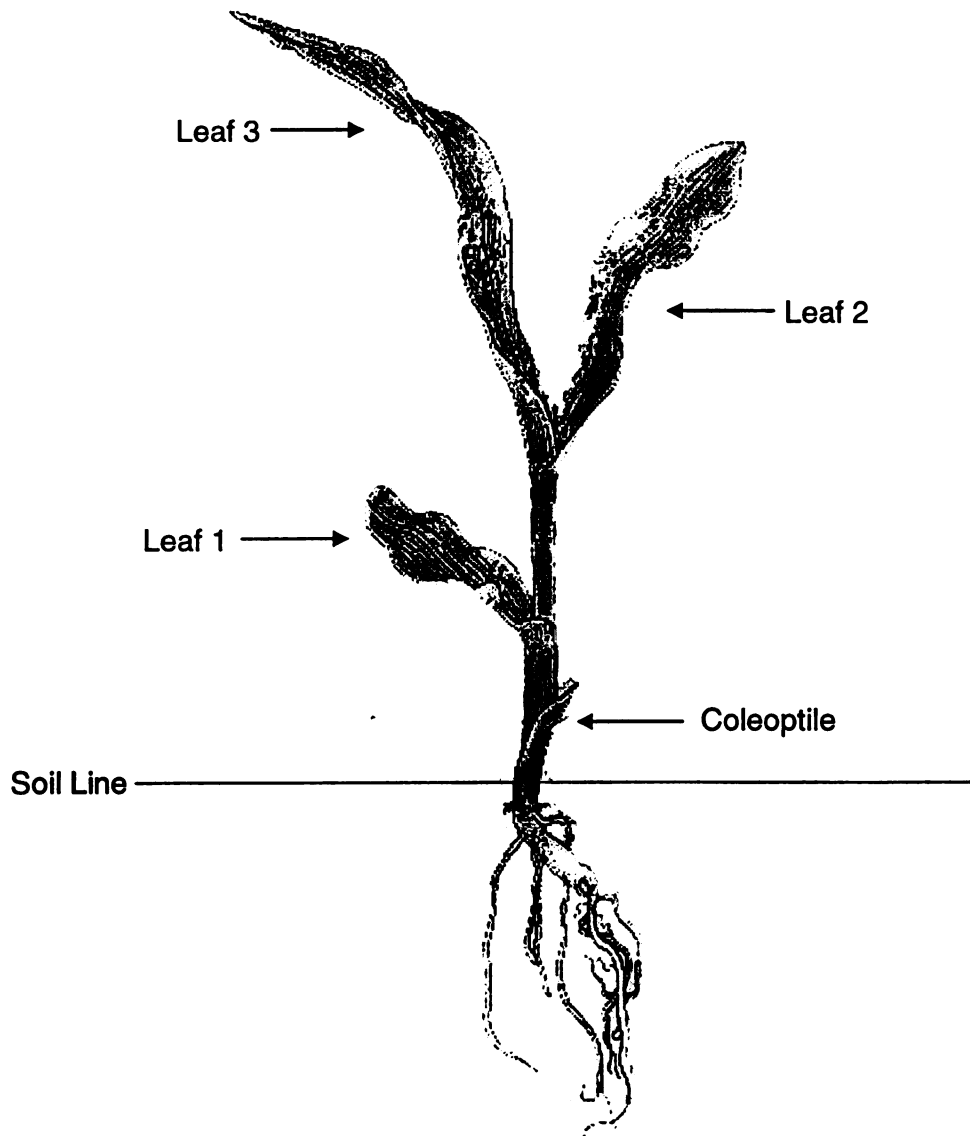
*Pantoea*. Since it was not known if rep-PCR could differentiate between members of the genera *Pantoea* and *Erwinia* (previously in the same genus) (Mergaert et al., 1993), a preliminary rep-PCR run using the BOX primer (Versalovic et al., 1994) was completed. Five strains of *Erwinia amylovora* (10, 321, MRI, PSLN, CFB Pr), one strain of *Erwinia chrysanthemi*, one strain of *Erwinia carotovora*, and two strains of *P. stewartii* [ATCC 29227 and Illinois control-(Il-Ps 1)] were amplified (Table 1). The BOX-PCR procedure was performed as described by Versalovic et al. (1994) with some adjustments; mineral oil was not added to all rep-PCR reactions because the thermal cycler used (GeneMate Magnum Techne, Princeton, NJ, USA) had a heated hood that prevented evaporation; the

BOX primer was synthesized by IDT-Integrated DNA Technologies, INC, Coralville, IA, USA; the electrophoresis procedure followed methods from Louws et al. (1998).

**2.5 Identification of Isolates (Gram Stain, Pathogenicity, BOX-PCR).** Samples were identified as *P. stewartii* isolates by Gram stain, pathogenicity on a susceptible variety of sweet corn (Jubilee), and comparison of genomic fingerprints using BOX-PCR.

The modified Gram stain protocol was performed as described by Schaad et al. (2001). Bacterial samples were prepared by streaking single colonies on a NBA (8 g/L, 1.5% agar) plate (100 x 15 mm) and incubating them in the dark for 72 hours. A single colony was transferred to a sterile screw cap vial (15 x 60 mm) containing 10 ml of nutrient broth and incubated for 24 hours at 23 °C on a shaker at 200 rpm.

The pathogenicity of each isolate was determined by inoculating Jubilee sweet corn plants at the 2-3 leaf stage (Figure 2) and assessing the potential of the bacterium to cause Stewart's bacterial wilt (Blanco et al., 1977). The sweet corn was sown in 20.5 cm black plastic pots and grown in a greenhouse with an average temperature of 29 °C ( $\pm$  3.2 °C), 14 hours daylight, 80% ( $\pm$  10.2%) relative humidity. Inoculum was prepared by streaking frozen stock samples of isolates on NBA plates and incubating at 23 °C for 48 hours. A single colony was transferred from the NBA culture to 5 ml of sterile nutrient broth in a sterile screw cap vial (15 x 60 mm). The vial was incubated at 23 °C on a shaker for 24 hours at 200 rpm. The absorbance ( $A_{600nm}$ ) of each inoculation isolate was between 0.39 and 0.41 (approximately  $1 \times 10^8$  cfu/ml). In all experiments, inoculum concentrations were determined by standard diluting and plating techniques. The plants were inoculated using a 5 ml syringe with a 30.5 gauge needle. The first step in the inoculation technique was to load 1.0 ml of inoculum solution into the syringe. The



**Figure 2. Sweet corn plant at the 2-3-leaf growth stage. The needle was inserted at the arrow marked by the coleoptile label.**

syringe plunger was then depressed to initiate flow of inoculum prior to injection of the plant tissue. The stem was then injected through the coleoptile sheath (approximately 1 cm from the soil surface) four times in 2 seconds while the inoculum was flowing from the needle.

The BOX-PCR procedure was used as a tool to construct genetic fingerprints of each isolate (protocol described in section 2.6).

**2.6 rep-PCR using BOX-PCR and REP-PCR.** The rep-PCR procedure, using the BOX primer, was performed as described by Versalovic et al. (1994) with slight adjustments. The amount of H<sub>2</sub>O, per BOX-PCR reaction, was 13.34 µl and the amount of primer was 1.16 µl (0.3 µg/µl). Preliminary trials were performed to determine the optimal BOX-PCR reaction for *P. stewartii* (example can be seen in Appendix A, Figure 15).

The rep-PCR procedure, using the repetitive extragenic palindromic (REP) primer, was performed as described by Versalovic et al. (1994) with slight adjustments. The amount of H<sub>2</sub>O, per REP-PCR reaction, was 13.64 µl. Preliminary trials were performed to determine the optimal REP-PCR reaction for *P. stewartii* (example can be seen in Appendix B, Figure 16). The REP primer was synthesized by IDT-Integrated DNA Technologies, INC, Coralville, IA, USA.

The electrophoresis procedure followed methods from Louws et al. (1998). The ethidium bromide stained gels for the rep-PCR reactions (BOX-PCR and REP-PCR) were photographed with a Polaroid camera and Type 57 High Speed Polaroid Film (Polaroid Corporation, Cambridge, MA, U.S.A). The photographs were transferred to computer images using a Hewlett-Packard Scanjet 4C digital scanner (Hewlett-Packard

Company, Palo Alto, CA, U.S.A.) and saved as TIFF files. Images were enhanced by inverting them and adjusting the brightness and contrast using Adobe Photoshop 6.0 (Adobe Systems Incorporated, San Jose, CA, U.S.A). Gel fingerprints were analyzed based on the presence or absence of fragments between the size of 350 base pairs (bp) and 3000 bp for each isolate.

Genetic fingerprints of the 84 isolates of *P. stewartii* were produced using both BOX-PCR and REP-PCR. BOX-PCR and REP-PCR were repeated four times on each of the isolates. Bands were considered to be present if they were observed in all four of the fingerprint patterns. A pairwise similarity matrix was constructed using *p*-distances by first determining the proportion (*p*) of bands differing between any particular pair of BOX-PCR or REP-PCR fingerprint patterns. The equation for *p*-distance used was;

$$p = \frac{n_d}{n}$$

where  $n_d$  = the number of differences, and  $n$  = the total number of bands. Distances were generated using PAUP\* v 4.0b4a (Swofford, 2002), and an index of similarity (*s*) was then calculated using the equation;

$$s = 1 - d$$

where  $d$  = *p*-distance.

**2.7 Virulence Assessment Protocol (Leaf Symptom Analysis).** Four varieties of sweet corn that differed in susceptibility to Stewart's bacterial wilt from susceptible to resistant (Table 2) were inoculated with fifteen *P. stewartii* strains (Table 3), as described above. The varieties of sweet corn were chosen based on ratings performed by Pataky et al. (2000). The sweet corn varieties included two resistant, one moderate, and one

Table 2. Varieties of sweet corn used in virulence assessment protocol.

| <b>Variety</b> | <b>SW-rating<sup>a</sup></b> | <b>Kernel color</b> | <b>Endosperm type</b> | <b>Seed Source</b> |
|----------------|------------------------------|---------------------|-----------------------|--------------------|
| Jubilee        | 9                            | yellow              | su <sup>b</sup>       | Rogers             |
| Native Gem     | 5                            | bi-color            | se <sup>c</sup>       | Mesa-Maize         |
| Buckeye        | 2                            | bi-color            | se                    | Mesa-Maize         |
| Ambrosia       | 1                            | bi-color            | se                    | Crookham           |

<sup>a</sup> The susceptibility rating of the sweet corn variety to Stewart's bacterial wilt infection. The rating scale was developed by Dr. Jerald K. Pataky, Department of Crop Sciences, University of Illinois, Urbana, IL, 61801 (Pataky, 2000). The rating scale is from 0-9 (0=resistant and 9=susceptible).

<sup>b</sup> Sugary.

<sup>c</sup> Sugary enhanced.

Table 3. *Pantoea stewartii* strain types used as inoculum in virulence assessment protocol.

| <b><i>P. stewartii</i><sup>a</sup><br/>strain</b> |
|---------------------------------------------------|
| Negative Control                                  |
| ATCC-29227                                        |
| Es 9211                                           |
| Es 9208                                           |
| SW 45                                             |
| SW 87-2                                           |
| SS104                                             |
| Es 9230                                           |
| SW 87-6                                           |
| SW 87-19                                          |
| SW 1                                              |
| II Ps-1                                           |
| II-B-CP-1                                         |
| V-A-CP-1-1                                        |
| VI-A-CP-1-1                                       |
| ES 9245                                           |

<sup>a</sup>Strain information can be seen in Table 1.

susceptible to Stewart's bacterial wilt (Table 2). The sweet corn was sown as described in section 2.5. Four replicates of each treatment were positioned on top of 1 m high benches in a random complete block design (RCBD). The experiment was performed twice; starting dates were 8 Feb and 12 Feb, 2002. The *P. stewartii* strains were selected based on strain types defined by the rep-PCR protocol. The isolates were incubated on NBA (8 g/1L, 1.5% agar) plates (100 x 15 mm) for 72 hours, and a single colony was transferred to a sterile screw cap vial (15 x 60 mm) containing 10 ml of nutrient broth. The vials were incubated at 23°C on a shaker for 24 hours at 200 rpm. The absorbance of the inoculum was between 0.39-0.41 (approximately  $1 \times 10^8$  cfu/ml) and was determined based on the optical density at 600 nm. The plants were inoculated, as described in section 2.5, 12 days after planting.

Forty-eight hours after inoculation, each plant was visually assessed for signs or symptoms of infection (bacterial ooze, water soaked lesions, wilting, yellow streaking). The first, second, and third leaf were rated based on a 0-3 scale (0-no signs or symptoms; 1- slight loss of rigidity in leaf, water soaked lesions less than 1 cm in length; 2- drooping of leaf, slight loss of rigidity in leaf, water soaked lesions greater than 1cm in length but covering less than 50% of plant surface; 3- bacterial ooze, drooping of leaf, water soaked lesions cover more than 50% of plant surface, general necrosis). Each leaf, 1, 2, 3 (Figure 2), was evaluated on each replicate plant every 24 hours for four days after first assessment.

## **2.8 Virulence Assessment Protocol (Colony Forming Units in Second Leaf Analysis).**

Six days after inoculation, infection was quantitatively assessed based on the number of colony forming units (CFU) recovered from the second leaf blade. The amount of



bacteria was determined by removing three (7 mm diameter) leaf disks from the second leaf blade of individual plants six days after inoculation. The leaf disks were taken from the base, middle, and tip of the leaf. The leaf disks were placed in a 1.5 ml centrifuge tube with sterile buffered saline (0.3 ml, 0.01 M potassium phosphate, 0.8% sodium chloride, pH 7.0). The leaf disks were macerated for approximately 10 seconds using a tissue grinder. An aliquot (0.2 ml) of the 'saline/leaf disk' solution was removed from the centrifuge tube and placed in a single well of a 96 well cell culture cluster (Corning Incorporated 3593). The aliquot was serially diluted to  $10^{-1}$  by transferring 0.02 ml of the 'saline/leaf disk' solution to a well containing 0.18 ml of sterile buffered saline. The process was repeated seven times, producing a  $10^{-7}$  dilution, by transferring each newly diluted solution to separate wells containing 0.18 ml of sterile buffered saline. After the dilution was complete, 0.1 ml of the  $10^{-7}$  dilution solution was evenly spread on a NBA (8 g / 1.0 L, 1.5% agar) plate (100 x 15 mm) for a final dilution of  $10^{-8}$ . The plate was incubated for 48 hours at 23°C. CFU on each plate that had the morphology of *P. stewartii* were then counted and recorded. Colonies were confirmed as *P. stewartii* by randomly selecting 15 NBA plates that contained each of the 15 different bacterial isolates previously inoculated into the sweet corn plants. The bacterial isolates were positively identified as the original strains inoculated in the sweet corn plant by comparing their genomic fingerprints produced by using the BOX-PCR technique (Appendix C, Figure 17).

**2.9 Data analysis (Leaf Symptom Analysis).** Statistical analyses were performed using SigmaStat statistical software package (SPSS Inc., Chicago, IL, U.S.A.). The area under the disease progress curve (AUDPC) data were analyzed by two-way analysis of

variance (ANOVA) and means compared at  $p = 0.05$  level of significance by a multiple range comparison of means (Tukey, SigmaStat). The AUDPC was calculated by adding the area under the linear progression of symptom ratings (rating scale section 2.7) between each successive rating of symptom severity from two days after inoculation to six days after inoculation.

The AUDPC was estimated using the formula

$$AUDPC = \sum_{i=1}^{n-1} (T_{i+1} - T_i) * \left( \frac{D_{i+1} + D_i}{2} \right)$$

where;  $n$  was the number of ratings,  $T$  was the time in days since inoculation and  $D$  was the disease symptom rating (Campbell and Madden, 1990). The experiment was performed two separate times and the data were combined because the variation between treatments in the two experiments was not different. The AUDPC was calculated on; leaf 1 (AUDPC<sub>L1</sub>), leaf 2 (AUDPC<sub>L2</sub>), leaf 3 (AUDPC<sub>L3</sub>), and for combinations of leaves; leaf 1, 2, 3 (AUDPC<sub>L1-3</sub>), leaf 2, 3 (AUDPC<sub>L23</sub>).

**2.9.1 Data analysis (Colony Forming Units in Second Leaf Analysis).** Statistical analyses were performed using the SigmaStat statistical software package (SPSS Inc., Chicago, IL, U.S.A.) The number of colony forming units per leaf sample was not normally distributed so the data were normalized by a  $\log_{10}$  transformation. A two-way analysis of variance was performed. The experiment was performed two separate times and the data were combined because the variation between treatments in the two experiments was not different.

## CHAPTER 3

### RESULTS

**3.1 Michigan isolate collection.** Over the course of the 2001 summer, a collection of 137 yellow-pigmented bacterial colonies were isolated and stored in frozen stock from corn flea beetles, potted susceptible varieties, and growers' sweet corn from the 12 sweet corn fields in the southern half of the Lower Peninsula of Michigan (Figure 1; Table 4). Of the 137 colonies, three were found to be strains of *P. stewartii* based on Gram staining, pathogenicity tests on Jubilee, and BOX-PCR genetic fingerprinting. These three isolates were assayed from different pots containing a susceptible variety of sweet corn (Jubilee), but were found in the same field, region II field B (Figure 1). No other isolates were identified as *P. stewartii* during the 2001 growing season and there were no reports of infection in Michigan sweet corn fields.

**3.2 rep-PCR (BOX-PCR) differentiation between members of the genus *Erwinia* and genus *Pantoea*.** The genetic fingerprint of each *Erwinia* and *Pantoea* sample was unique, and the differences found among members of the same species were minimal (Figure 3). Each of the eight isolates contained 18-25 bands between the 350 to 4000-bp range, but very few bands were shared among species. Among the two isolates of *P. stewartii*, there were multiple bands that were the same size, but there was also a polymorphism that differentiated the two isolates (ATCC-29227 and Il-Ps 1). Based on the genomic fingerprints obtained by using the rep-PCR procedure, it was determined that using the rep-PCR procedure could differentiate between closely related *Pantoea* and *Erwinia* species as well as within species of *P. stewartii*.

Table 4. *Pantoea stewartii* isolates collected from Michigan during the 2001 growing season.

|                                        | Total number of specimens inspected <sup>a</sup> | Specimens Assayed <sup>b</sup> | Yellow Bacteria Isolated <sup>c</sup> | Gram-stain negative | Pathogenicity on Jubilee <sup>d</sup> | rep-PCR (BOX) Identification <sup>e</sup> |
|----------------------------------------|--------------------------------------------------|--------------------------------|---------------------------------------|---------------------|---------------------------------------|-------------------------------------------|
| Scouting of Growers Field <sup>f</sup> | ca. 1 x 10 <sup>6</sup>                          | 30                             | 12                                    | 12                  | 0                                     | 0                                         |
| Bait (Potted Jubilee) <sup>g</sup>     | ca. 3000                                         | 5                              | 4                                     | 4                   | 3                                     | 3                                         |
| Corn Flea Beetle Sweep <sup>h</sup>    | ca. 480                                          | 320                            | 121                                   | 95                  | 0                                     | 0                                         |
| Total                                  | -                                                | 355                            | 137                                   | 111                 | 3                                     | 3                                         |

- <sup>a</sup> Scouting – 15 rows, 100 m, plants were spaced approximately 15 cm, 10 inspections during the 2001 growing.  
Baiting – 25 potted plants per inspection, 10 pot replacements during the 2001 growing season.  
Sweeping- 4 sweeps per inspection. 10 inspections during the 2001 growing season.
- <sup>b</sup> The number of visually infected sweet corn plants from grower's Michigan sweet corn fields, Jubilee sweet corn plants that were placed in fields as bait and were visually infected, and corn flea beetles that were captured during sweeps.
- <sup>c</sup> Number of bacterial colonies (yellow pigment, < 3 mm in diameter) extracted from assayed specimens onto nutrient broth agar (NBA).
- <sup>d</sup> Number of isolates that were able to cause symptoms of Stewart's bacterial wilt when inoculated into the sweet corn variety Jubilee.
- <sup>e</sup> Number of isolates with genomic fingerprints similar to ATCC-29227 and II-Es1 using BOX-PCR.
- <sup>f</sup> Scouting of fields consisted of walking through sweet corn fields and looking for signs or symptoms of Stewart's bacterial wilt on the sweet corn.
- <sup>g</sup> Potted Jubilee sweet corn plants (susceptible to Stewart's bacterial wilt) were placed around the margins and in sweet corn fields.
- <sup>h</sup> Corn flea beetles were captured in sweet corn fields using an insect sweep net.

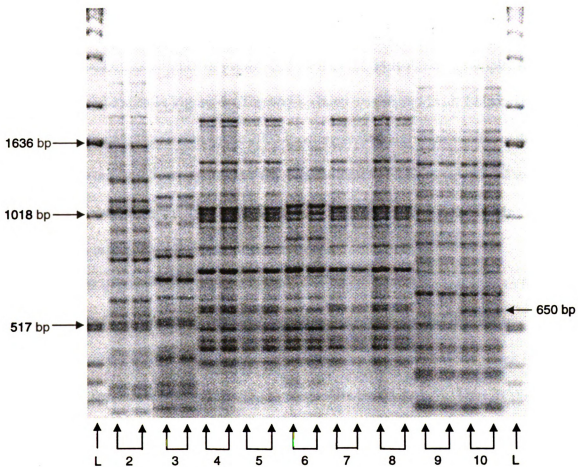


Figure 3. BOX-PCR generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea* and *Erwinia* species. The numbered brackets below indicate the isolate of *Pantoea* or *Erwinia* that is being analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The two lanes labeled with an 'L' contain a 1 Kb DNA Ladder. 2-*Erwinia chrysanthemi*; 3-*Erwinia carotovora*; 4-*Erwinia amylovora* (Es 110); 5-*E. amylovora* (Es 321); 6-*E. amylovora* (Es MR1); 7-*E. amylovora* (PSLN); 8-*E. amylovora* (CFB Pr). Lanes, 9-*Pantoea stewartii* (Il-Ps 1) and 10-*Pantoea stewartii* (ATCC-29227) are differentiated by a 650 bp band that is absent in lane 9, but present in lane 10.

**3.3 BOX-PCR genetic variation of *P. stewartii* strains.** When the BOX primer was used in the rep-PCR procedure, unique genomic fingerprints were found between the 84 isolates of *P. stewartii* which were collected from different geographical locations (Appendix D, Figures 18-33). Each isolate shared 18-19 fragments between the 350 to 4000-bp range. Only one band (approximately 650-bp in size) differentiated the collection based on its absence or presence in each of the 84 isolates. Examples of the BOX-PCR can be seen in Figure 4. The 650-bp band was absent in all isolates collected from Michigan and Illinois during the 2001 growing season and were similar to three isolates collected from Iowa in 1992 by Dr. Charles Block (Es 9206; 45; 57). Two of these isolates were from the same city (Nevada, Iowa), but from different fields. All other isolates in this study had the 650-bp fragment.

**3.4 REP-PCR genetic variation of *P. stewartii* strains.** REP-PCR was used to further differentiate the 84 strains of *P. stewartii* based on their genomic fingerprints in combination with the BOX-PCR fingerprints (Appendix E, Figure 34-50). Each isolate shared 16-17 fragments between the 350 to 4000-bp range (Figure 5). Like BOX-PCR, only one band (approximately 380-bp in size) differentiated the isolates based on its absence or presence in each of the 84 isolates. The 380-bp fragment was absent from isolates collected from Ohio in 1974 (SW 1, SW 2), SS104 (Illinois-1967), DC 160 (New York-1976), DC 283 (spontaneous mutant of SS104), MU 51 (capsular avirulent mutant), GAL E8 (EPS spontaneous mutant), four of the 25 isolates collected by Greg Lamka in 1987 (SW 87-6; 17; 19; 24), and two of the 28 isolates collected by Dr. Charles Block in 1992 (Es 9212, Es 9256). All other isolates in this study had the 380-bp fragment.

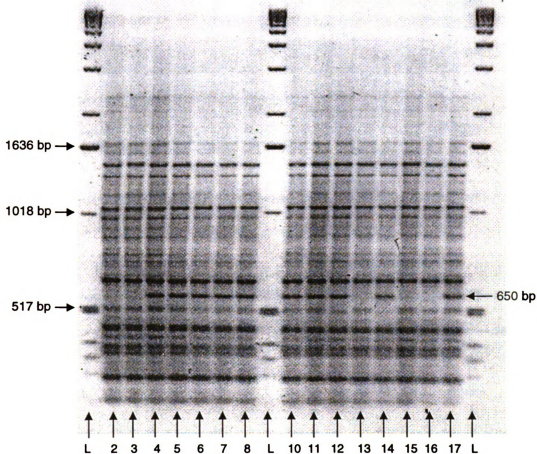


Figure 4. BOX-PCR generated genomic fingerprint, on a 1.5% agarose gel, of 15 *Pantoea stewartii* strains that were used in the virulence trial. The numbered lines with arrows below indicate the isolate of *P. stewartii* that is being analyzed. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Strains missing the 650-bp band - Lane 2-Illinois (Ps-VI-A-CP-1-1); 3-Illinois (Ps-V-A-CP-1-1); 13-Iowa (Es 9245); 15- Michigan (Ps-II-B-CP-1); 16- Illinois (II-Ps 1). Strains with the 650-bp band- Lane 4-Iowa (SW 87-2); 5-Illinois (SW 45); 6-Ohio (SW 1); 7-Iowa (SW 87-19); 8-Iowa (SW 87-6); 10-Iowa (Es 9208); 11-Iowa (Es 9211); 12-Iowa (Es 9230); 14-Illinois (SS 104); 17-ATCC-29227

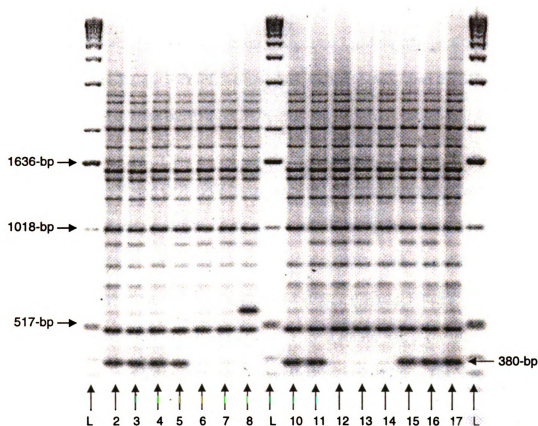


Figure 5. REP-PCR generated genomic fingerprint, on a 1.5% agarose gel, of 15 *Pantoea stewartii* strains that were used in the virulence trial. The numbered lines with arrows below indicate the isolate of *P. stewartii* that is being analyzed. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Strains missing the 380-bp band - Lane 6-Ohio (SW1); 7-Iowa (SW 87-19); 8-Iowa (SW 87-6); 12-Iowa (Es 9230); 13-Iowa (Es 9245); 14-Illinois (SS 104). Strains with the 380-bp band- 2-Illinois (Ps-VI-A-CP-1-1); 3-Illinois (Ps-V-A-CP-1-1); 4-Iowa (SW 87-2); 5-Illinois (SW 45); 10-Iowa (Es 9208); 11-Iowa (Es 9211); 15- Michigan (Ps-II-B-CP-1); 16-Illinois (II-Ps 1); 17-ATCC-29227



**3.5 Determination of strains based on BOX-PCR and REP-PCR.** It was determined that the 84 isolates could be separated into four different strain types, designated strain type 1 through 4, based on their BOX-PCR and REP-PCR fingerprints (Table 1). The four strains were determined by the absence or presence of the 650-bp band in the BOX-PCR fingerprint and the 380-bp band in the REP-PCR fingerprint (Figure 6). Strains designated 1 had both of the bands. Strains designated 2 had the 650-bp BOX-PCR band, but did not have the 380-bp REP-PCR band. Strains designated 3 had the 380-bp REP-PCR band, but did not have the 650-bp BOX-PCR band. Strains designated 4 did not have either band.

The REP-PCR and BOX-PCR fingerprint data were combined to determine the  $p$ -distances between the strain types 1 through 4 based on the presence of 34-36 bands. Type 1 strains and Type 3 strains had  $p$ -distances of 0.02857, with a corresponding similarity of 0.97143. Type 2 strains and Type 4 strains also had  $p$ -distances of 0.02857 and similarities of 0.97143. Type 1 strains and Type 3 strains had  $p$ -distances of 0.5714 from Type 2 strains and Type 4 strains, and similarities of 0.94286. The low  $p$ -distances and high similarities between strain types reflect the fact that there were only two band differences in the BOX-PCR and REP-PCR fingerprint patterns.

**3.6 Virulence Assessment (Leaf Symptom Analysis).** Leaf symptoms of Stewart's bacterial wilt on leaves 1, 2 and 3 progressed over 3-6 days after initial inoculation with 15 different strains of *P. stewartii* (sweet corn varieties combined, Figure 7). On leaf 1, 2, and 3, the maximum disease symptom rating six days after inoculation was 0.09 (SW 87-19), 1.23 (Es 9230), and 2.69 (Es 9230), respectively. Seven out of the 15 bacterial strains (ATCC-29227, II-B-JCP-1, Es 9211, Es 9208, SW 87-6, SW 45, SW 87-2) did not

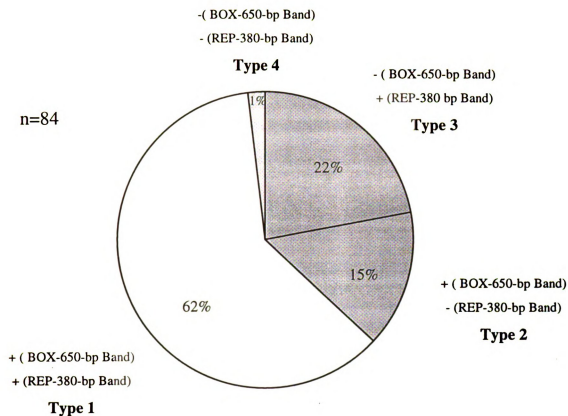
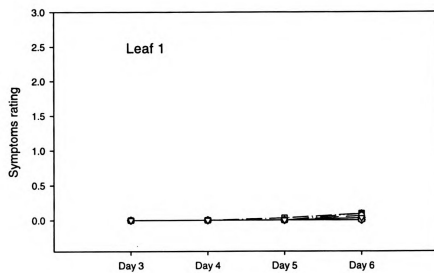


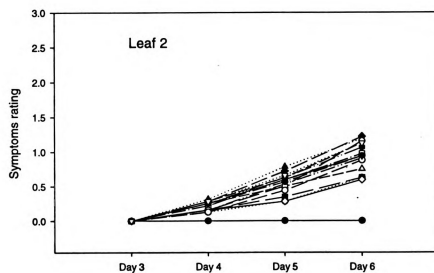
Figure 6. The rep-PCR strain type (1, 2, 3, 4) distribution of 84 *Pantoea stewartii* isolates from the eastern United States (Table 1).

Figure 7 (A-C). Progression of Stewart's bacterial wilt symptoms (scale 0-3 as described in text; briefly 0= no symptoms, 3= complete necrosis of leaf) on individual leaves (Figure A-C, leaf position 1-3) from four varieties of sweet corn [Jubilee, Native Gem, Buckeye, Ambrosia (individual points represent the average severity score of varieties combined)] after inoculation with selected isolates of *P. stewartii* from Eastern USA (listed in Table 3). —●— Negative control (No bacteria) ; —▼— II Es1 ; —▽— II-B-CP-1 ; —■— SS104 ; —□— Es 9245 ; —◆— Es 9230 ; —◇— Es 9211 ; —▲— Es 9208 ; —△— SW 87-6 ; —●— SW 87-19 ; —○— SW 1 ; —●— SW 45 ; —○— SW 87-2 ; —▼— V-A-CP-1-1 ; —▽— VI-A-CP-1-1 .

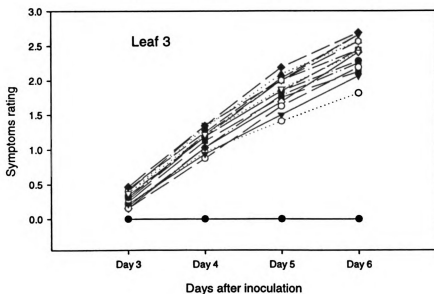
A)



B)



C)



cause disease symptoms on leaf 1. The disease progression (measured by the AUDPC) of Stewart's bacterial wilt on sweet corn was significantly different ( $p < 0.05$ ) between leaves 1, 2 and 3 [leaf 3 > 2 > 1 (Figure 8)]. When the varieties of sweet corn were analyzed individually, the disease symptom ratings on leaf 1, leaf 2, and leaf 3 for each sweet corn variety followed a similar trend (leaf 3 > leaf 2 > leaf 1).

The AUDPC<sub>L23</sub> were; Jubilee (3.8), Native Gem (2.4), Buckeye (1.8), Ambrosia (2.5). These differences were significant ( $p < 0.05$ ) among the four varieties of sweet corn (Figure 9).

When all four varieties of sweet corn were combined, there were no significant differences ( $p < 0.05$ ) in AUDPC<sub>L1</sub> or AUDPC<sub>L2</sub> between the 15 *P. stewartii* strains, but there were significant differences in AUDPC<sub>L3</sub> (Table 5). AUDPC<sub>L3</sub> of strain Es 9230 was significantly greater (more virulent) than strains ATCC-29227, SW 87-2, and V-A-CP-1-1. There were also significant differences in AUDPC<sub>L1-3</sub> and AUDPC<sub>L23</sub> among isolates (Table 5). Since the analysis of leaves 1, 2, and 3 combined (AUDPC<sub>L1-3</sub>) and leaves 2 and 3 (AUDPC<sub>L23</sub>) combined resulted in similar differences among bacterial isolates, it was decided that because infection on leaf 1 was minimal or not infected (e.g., Buckeye) that leaf 1 should be excluded from subsequent analyses. AUDPC<sub>L23</sub> was therefore selected as the best indicator of Stewart's bacterial wilt. AUDPC<sub>L23</sub> type 2 strain Es9230 was significantly more virulent than type 1 strains Es 9211, Es 9208, and SW 87-2, and type 2 strain SS104 (Table 5).

When the AUDPC<sub>L23</sub> for the 15 isolates of *P. stewartii* were compared within the individual varieties of sweet corn (Jubilee, Native Gem, Buckeye, Ambrosia) there were significant differences ( $p < 0.05$ ) found between the isolates (Table 6). Within each sweet

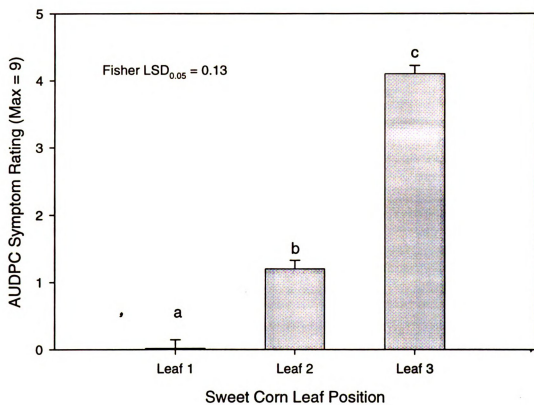


Figure 8. Estimate of the area under the disease progress curve on leaves 1, 2, and 3 ( $AUDPC_{L1}$ ,  $AUDPC_{L2}$ ,  $AUDPC_{L3}$ ) of the combined disease symptoms caused by 15 separate *Pantoea stewartii* strains on four different varieties of sweet corn.

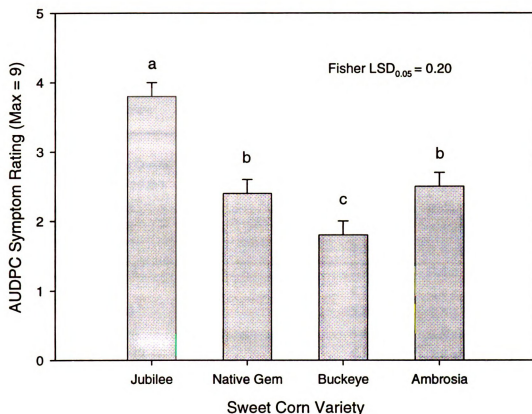


Figure 9. Estimate of the area under the disease progress curve of leaves 2 and 3 (AUDPC<sub>L23</sub>) for the combined disease symptoms caused by 15 separate *Pantoea stewartii* strains on four individual varieties of sweet corn which were inoculated near the coleoptile and allowed to incubate for six days. Bars followed by the same letter were not significantly different at the  $p=0.05$  level of probability.

Table 5. Area under the disease progress curve (AUDPC) for Stewart's bacterial wilt symptoms on individual leaves (AUDPC<sub>L1</sub>, AUDPC<sub>L2</sub>, AUDPC<sub>L3</sub>), on leaves 2 and 3 (AUDPC<sub>L23</sub>), and leaves 1, 2, and 3 (AUDPC<sub>L1-3</sub>) for four varieties (combined data) of sweet corn which were rated for 4 days starting 3 days after inoculation at the 3-leaf stage.<sup>†</sup>

| Rep-PCR<br>Strain<br>Type   | <i>P. stewartii</i><br>Isolate | AUDPC <sub>L1</sub> <sup>u</sup> | AUDPC <sub>L2</sub> <sup>v</sup> | AUDPC <sub>L3</sub> <sup>w</sup> | AUDPC <sub>L23</sub> <sup>x</sup> | AUDPC <sub>L1-3</sub> <sup>y</sup> |
|-----------------------------|--------------------------------|----------------------------------|----------------------------------|----------------------------------|-----------------------------------|------------------------------------|
|                             | Negative Control <sup>z</sup>  | 0.0 a                            | 0.0 b                            | 0.0 c                            | 0.0 d                             | 0.0 d                              |
| 1                           | ATCC-29227                     | 0.0 a                            | 0.8 ab                           | 3.7 b                            | 2.2 c                             | 1.5 c                              |
| 1                           | Es 9211                        | 0.0 a                            | 0.8 ab                           | 4.0 ab                           | 2.4 c                             | 1.6 c                              |
| 1                           | Es 9208                        | 0.0 a                            | 1.7 a                            | 4.9 ab                           | 3.3 ab                            | 2.2 ab                             |
| 1                           | SW 45                          | 0.0 a                            | 1.3 a                            | 4.3 ab                           | 2.8 abc                           | 1.9 abc                            |
| 1                           | SW 87-2                        | 0.0 a                            | 1.0 ab                           | 3.7 b                            | 2.4 c                             | 1.6 c                              |
| 2                           | SS104                          | 0.0 a                            | 0.8 ab                           | 4.1 ab                           | 2.4 c                             | 1.6 c                              |
| 2                           | Es 9230                        | 0.0 a                            | 1.7 a                            | 5.2 a                            | 3.5 a                             | 2.3 a                              |
| 2                           | SW 87-6                        | 0.0 a                            | 1.2 a                            | 4.4 ab                           | 2.8 abc                           | 1.9 abc                            |
| 2                           | SW 87-19                       | 0.0 a                            | 1.5 a                            | 4.1 ab                           | 2.8 abc                           | 1.9 abc                            |
| 2                           | SW 1                           | 0.0 a                            | 1.3 a                            | 4.6 ab                           | 2.9 abc                           | 1.9 abc                            |
| 3                           | II Es 1                        | 0.0 a                            | 1.5 a                            | 4.8 ab                           | 3.2 ab                            | 2.1 ab                             |
| 3                           | II-B-CP-1                      | 0.0 a                            | 1.1 ab                           | 4.7 ab                           | 2.9 abc                           | 1.9 abc                            |
| 3                           | V-A-CP-1-1                     | 0.0 a                            | 1.3 a                            | 3.7 b                            | 2.5 abc                           | 1.7 abc                            |
| 3                           | VI-A-CP-1-1                    | 0.0 a                            | 1.4 a                            | 4.4 ab                           | 2.9 abc                           | 2.0 ab                             |
| 4                           | ES 9245                        | 0.1 a                            | 1.3 a                            | 4.6 ab                           | 3.0 ab                            | 2.0 ab                             |
| Degrees of freedom          |                                | 511                              | 511                              | 511                              | 1023                              | 1535                               |
| Fishers LSD <sub>0.05</sub> |                                | 0.055                            | 0.64                             | 0.70                             | 0.71                              | 0.61                               |

<sup>†</sup> ANOVA was performed on combined data of two trials (start dates-8 Feb and 12 Feb, 2002); means followed by the same letter were not significantly different at p=0.05 level of probability (Tukey multiple comparison).

<sup>u</sup> Symptom rating analysis on Leaf 1; Max AUDPC = 9.

<sup>v</sup> Symptom rating analysis on Leaf 2; Max AUDPC = 9.

<sup>w</sup> Symptom rating analysis on Leaf 3; Max AUDPC = 9.

<sup>x</sup> Combined symptom rating analysis of Leaf 2 and Leaf 3; Max AUDPC= 9.

<sup>y</sup> Combined symptom rating analysis of Leaf 1, Leaf 2, and Leaf 3; Max AUDPC= 9.

<sup>z</sup> Inoculation with sterile nutrient broth.



Table 6. Area under the disease progress curve (AUDPC, max = 9) for Stewart's bacterial wilt symptoms on the combination of leaves 2 and 3 (AUDPC<sub>L23</sub>) on four separate varieties of sweet corn which were rated for 4 days starting 3 days after inoculation.<sup>a</sup>

| Rep-PCR<br>Strain<br>type   | Isolates of<br><i>P. stewartii</i> | Native Gem |                   |           | Buckeye              |           |      | Ambrosia             |      |  |
|-----------------------------|------------------------------------|------------|-------------------|-----------|----------------------|-----------|------|----------------------|------|--|
|                             |                                    | Jubilee    |                   |           | AUDPC <sub>L23</sub> |           |      | AUDPC <sub>L23</sub> |      |  |
|                             | Negative Control <sup>w</sup>      | 0.0 f      |                   | 0.0 g     |                      | 0.0 g     |      | 0.0 g                |      |  |
| 1                           | ATCC-29227                         | 3.4 cde    | (LV) <sup>x</sup> | 2.0 f     | (LV)                 | 1.4 f     | (LV) | 2.2 def              | (LV) |  |
| 1                           | Es 9211                            | 3.3 de     | (LV)              | 2.4 cdef  | (LV)                 | 1.8 def   | (LV) | 2.2 cdef             | (LV) |  |
| 1                           | Es 9208                            | 4.7 a      | (MV) <sup>y</sup> | 2.9 abcd  | (MV)                 | 2.2 abcd  | (MV) | 3.2 abc              | (MV) |  |
| 1                           | SW 45                              | 3.9 abcd   | (MV)              | 3.0 abc   | (MV)                 | 1.7 def   | (LV) | 2.7 abcde            | (MV) |  |
| 1                           | SW 87-2                            | 4.3 abc    | (MV)              | 1.8 f     | (LV)                 | 1.5 ef    | (LV) | 1.8 ef               | (LV) |  |
| 2                           | SS104                              | 2.8 e      | (LV)              | 2.5 bcdef | (LV)                 | 1.8 def   | (LV) | 2.7 abcde            | (MV) |  |
| 2                           | Es 9230                            | 4.3 abc    | (MV)              | 3.3 a     | (MV)                 | 2.8 a     | (MV) | 3.4 ab               | (MV) |  |
| 2                           | SW 87-6                            | 3.6 bcd    | (IV) <sup>z</sup> | 2.3 def   | (LV)                 | 1.8 cdef  | (LV) | 3.5 a                | (MV) |  |
| 2                           | SW 87-19                           | 4.3 abc    | (MV)              | 2.2 ef    | (LV)                 | 1.6 def   | (LV) | 2.9 abcd             | (MV) |  |
| 2                           | SW 1                               | 4.7 a      | (MV)              | 2.4 cdef  | (LV)                 | 1.5 ef    | (LV) | 3.1 abcd             | (MV) |  |
| 3                           | II-Ps 1                            | 4.5 ab     | (MV)              | 3.2 ab    | (MV)                 | 2.5 ab    | (MV) | 2.5 abcde            | (MV) |  |
| 3                           | II-B-CP-1                          | 3.9 abcd   | (MV)              | 2.2 f     | (LV)                 | 2.4 abc   | (MV) | 3.2 abcd             | (MV) |  |
| 3                           | V-A-CP-1-1                         | 4.3 abc    | (MV)              | 2.3 def   | (LV)                 | 1.8 def   | (LV) | 1.6 f                | (LV) |  |
| 3                           | VI-A-CP-1-1                        | 4.5 a      | (MV)              | 2.9 abcde | (MV)                 | 2.0 bcdef | (LV) | 2.3 bcdef            | (LV) |  |
| 4                           | ES 9245                            | 4.5 a      | (MV)              | 2.4 cdef  | (LV)                 | 2.1 abcde | (MV) | 2.9 a                | (MV) |  |
| Degrees of freedom          |                                    | 255        |                   | 255       |                      | 255       |      | 255                  |      |  |
| Fishers LSD <sub>0.05</sub> |                                    | 1.00       |                   | 0.69      |                      | 0.70      |      | 1.07                 |      |  |

<sup>a</sup> ANOVA was performed on combined data of two trials (start dates-8 Feb and 12 Feb, 2002); means followed by the same letter were not significantly different at p=0.05 level of probability (Tukey multiple comparison).

<sup>w</sup> Combined symptom rating analysis of Leaf 2, and Leaf 3; Max AUDPC= 9.

<sup>x</sup> Inoculation with sterile nutrient broth.

<sup>y</sup> MV – More Virulent (not significantly different (p=0.05 level of probability) from the isolate with the highest AUDPC<sub>L23</sub>).

<sup>z</sup> LV – Less Virulent (not significantly different (p=0.05 level of probability) from the isolate with the lowest AUDPC<sub>L23</sub>).

<sup>\*</sup> IV Intermediately Virulent (not significantly different (p=0.05 level of probability) from the isolates with the lowest or highest AUDPC<sub>L23</sub>).

corn variety, the 15 isolates of *P. stewartii* were designated more virulent (MV), less virulent (LV), or intermediately virulent (IV) based on their statistical relation (not significantly different at the  $p=0.05$  level of probability) to the isolates with the highest and lowest AUDPC<sub>L23</sub>. The isolates that were consistently MV across all varieties of sweet corn were Es 9208 (strain type 1), Es 9230 (strain type 2) and Il-Ps 1 (strain type 3). The isolates that were consistently LV across all varieties of sweet corn were ATCC-29227 (strain type 1) and Es 9211 (strain type 1). All other isolates were not consistently designated LV or MV on the four different varieties of sweet corn. On the most susceptible variety of sweet corn (Jubilee), only ATCC-29227 (strain type 1), Es 9211 (strain type 1), SS 104 (strain type 2), and SW-87-6 (strain type 2) were significantly LV than all other isolates. On the resistant variety of sweet corn (Buckeye), members of strain type 1; ATCC-29227, Es 9211, SW 45, SW 87-2, members of strain type 2; SS 104, SW 87-6, SW 87-19, SW 1, and members of strain type 3; VI-A-CP-1-1, V-A-CP-1-1 were significantly LV than the other isolates. The *P. stewartii* isolates ATCC-29227 (strain type 1), Es 9211 (strain type 1), SW 87-2 (strain type 1), V-A-CP-1-1 (strain type 3) were LV on the two moderately susceptible sweet corn varieties, Ambrosia and Native Gem, while Es 9208 (strain type 1), SW 45 (strain type 1), Es 9230 (strain type 2) and Il Ps-1 (strain type 3) were designated MV. The other seven strains were not consistently MV or LV on Ambrosia and Native Gem.

**3.7 Virulence Assessment (Colony Forming Units in Second Leaf Analysis)** Mean CFU / 115 mm<sup>2</sup> (leaf 2) were significantly higher ( $p<0.05$ ) in the Jubilee variety of sweet corn than in Ambrosia, Native Gem, or Buckeye (Figure 10). Mean CFU / 115 mm<sup>2</sup> (leaf 2) in sweet corn varieties (Ambrosia, Native Gem, and Buckeye) were not

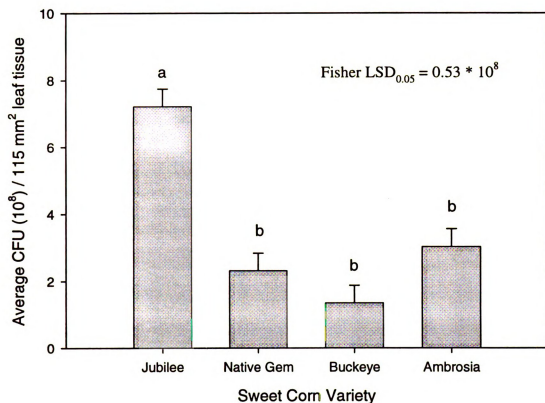


Figure 10. Average CFU (10<sup>8</sup>) from a 115 mm<sup>2</sup> section of leaf 2 (samples were serially diluted to 10<sup>-8</sup>) for each of the four varieties of sweet corn six days after inoculation with 15 different isolates of *P. stewartii*. When inoculated with isolates of *P. stewartii*, plants were in a random complete block design with eight replications. Bars followed by the same letter were not significantly different at the p=0.05 level of probability.

significantly different between strains (Figure 10). Jubilee, plants inoculated with Es 9208 had a significantly higher mean CFU / 115 mm<sup>2</sup> in leaf 2 (Figure 11). No other strains were significantly different in terms of CFU / 115 mm<sup>2</sup> in leaf 2 (Figure 12, 13, 14). There were no significantly different mean CFU / 115 mm<sup>2</sup> in leaf 2 within Native Gem, Buckeye, or Ambrosia.

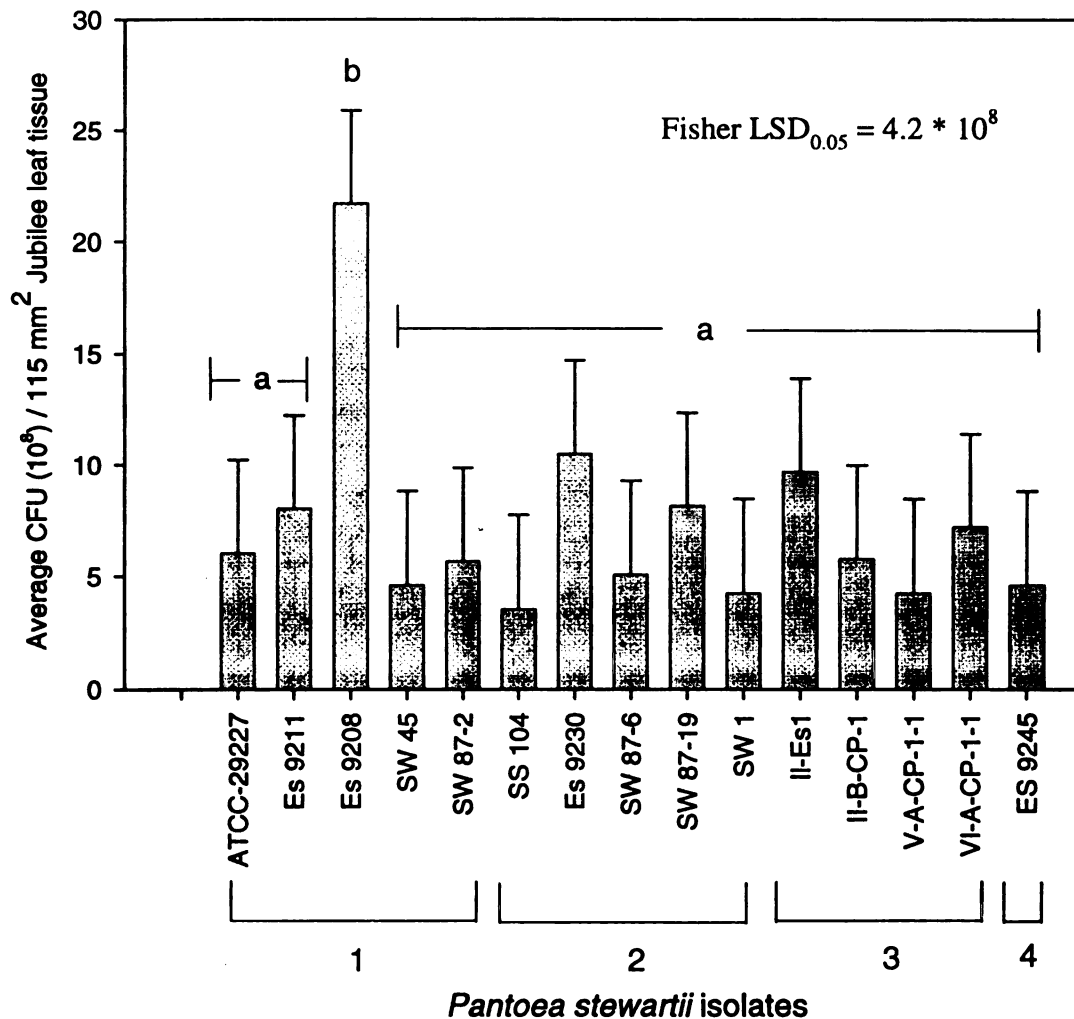


Figure 11. Average CFU ( $10^8$ ) / 115 mm<sup>2</sup> of leaf tissue (samples were serially diluted to  $10^{-8}$ ) in leaf two of Jubilee sweet corn plants six days after inoculation with 15 different isolates of *P. stewartii*. Brackets below (1-4) indicate the rep-PCR strain type of each isolate. The same bracketed letters above bars were not significantly different at  $p < 0.05$  level of probability.

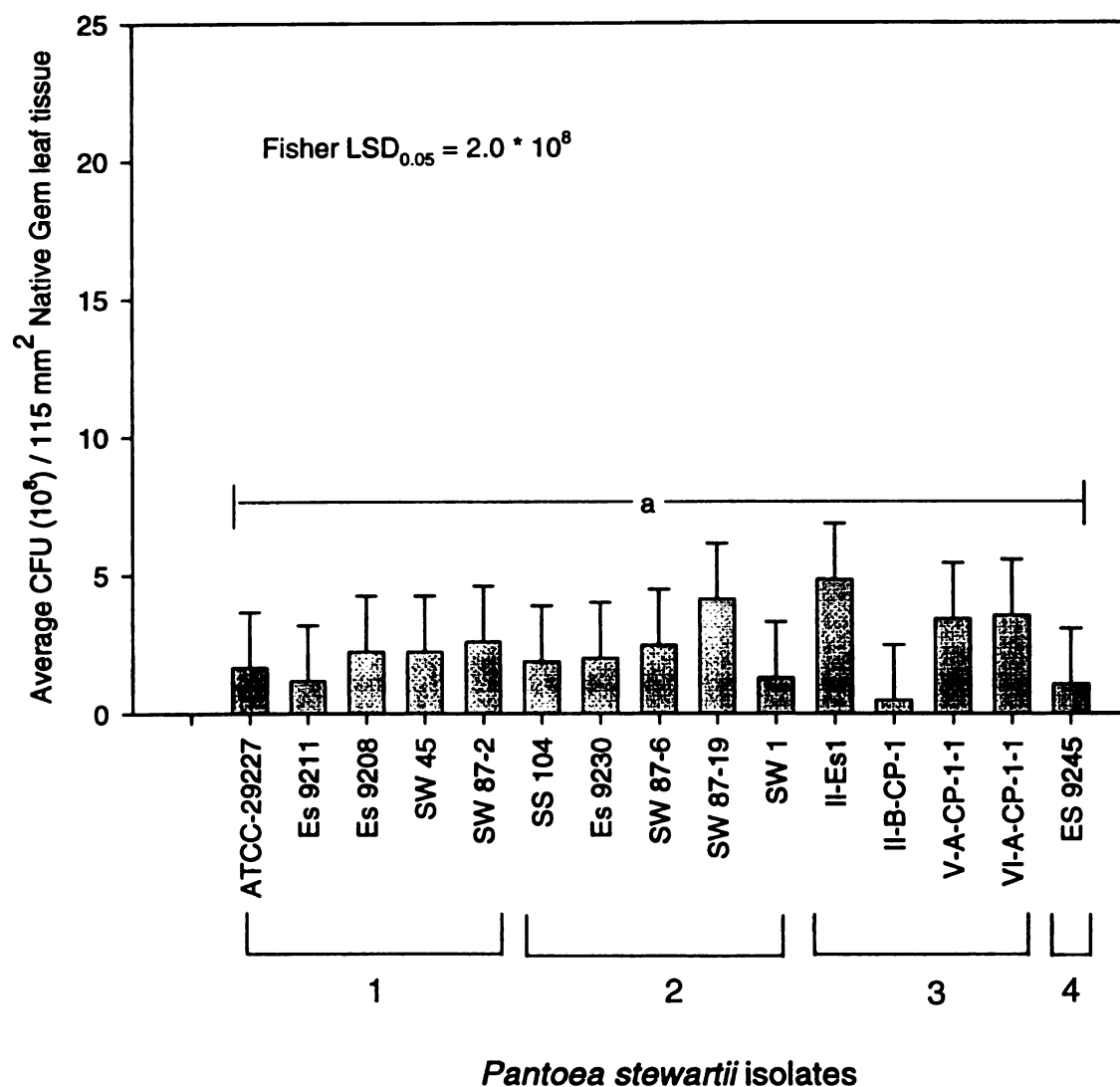


Figure 12. Average CFU ( $10^8$ ) / 115 mm<sup>2</sup> of leaf tissue (samples were serially diluted to  $10^{-8}$ ) in leaf two of Native Gem sweet corn plants six days after inoculation with 15 different isolates of *P. stewartii*. Brackets below (1-4) indicate the rep-PCR strain type of each isolate. The same bracketed letters above bars were not significantly different at  $p < 0.05$  level of probability.

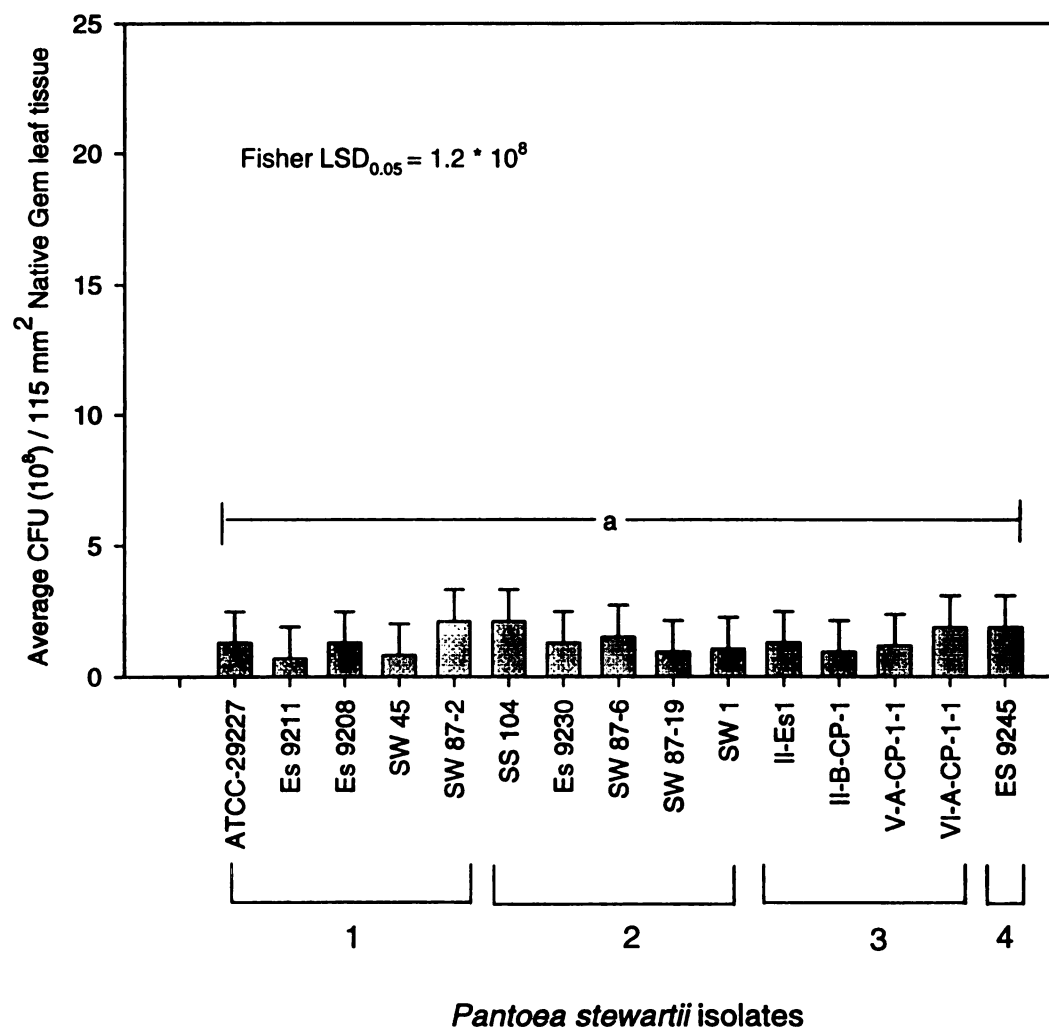


Figure 13. Average CFU ( $10^8$ ) / 115 mm<sup>2</sup> of leaf tissue (samples were serially diluted to  $10^{-8}$ ) in leaf two of Buckeye sweet corn plants six days after inoculation with 15 different isolates of *P. stewartii*. Brackets below (1-4) indicate the rep-PCR strain type of each isolate. The same bracketed letters above bars were not significantly different at  $p < 0.05$  level of probability.

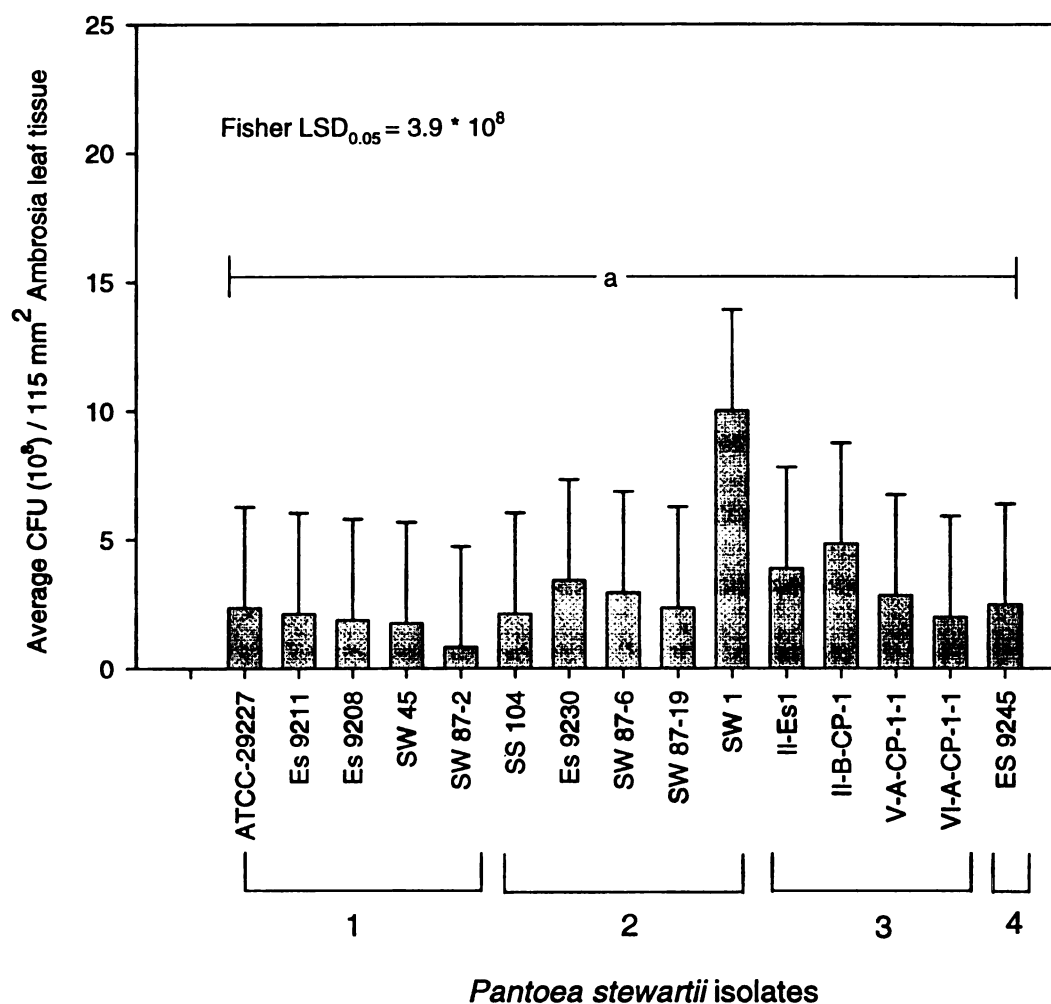


Figure 14. Average CFU (10<sup>6</sup>) / 115 mm<sup>2</sup> of leaf tissue (samples were serially diluted to 10<sup>-8</sup>) in leaf two of Ambrosia sweet corn plants six days after inoculation with 15 different isolates of *P. stewartii*. Brackets below (1-4) indicate the rep-PCR strain type of each isolate. The same bracketed letters above bars were not significantly different at p<0.05 level of probability.



## CHAPTER 4

### DISCUSSION

In this study, it was shown that the repetitive extragenic sequence, BOX, is present in the genome of *Pantoea* and *Erwinia* isolates, and that the BOX-PCR protocol can be used to differentiate these two genera. It was also demonstrated that the REP-PCR and BOX-PCR protocols, referred to as rep-PCR collectively, could be used to differentiate between isolates of *Pantoea stewartii*. Eighty-four *P. stewartii* isolates were divided into four strain types (1, 2, 3, 4) based on the presence of 34-36 bands in rep-PCR (BOX-PCR and REP-PCR) generated DNA fingerprints. Type 1 strains and Type 3 strains had *p*-distances of 0.02857, with a corresponding similarity of 0.97143. Type 2 strains and Type 4 strains also had *p*-distances of 0.02857 and similarities of 0.97143. Type 1 strains and Type 3 strains had *p*-distances of 0.5714 from Type 2 strains and Type 4 strains, and similarities of 0.94286. This research also explored three different collection methods of *P. stewartii* isolates in and around sweet corn fields. Lastly, this study demonstrated a rapid method for virulence assessment of the 84 *P. stewartii* isolates. This method can also be used to determine sweet corn susceptibility to Stewart's bacterial wilt infection.

A preliminary evaluation was conducted to confirm that two closely related genera, *Pantoea* and *Erwinia*, could be differentiated from each other using the BOX-PCR protocol. Previously, phytopathogenic species of *Pseudomonas*, *Xanthomonas*, *Clavibacter*, and *Ralstonia* have been differentiated using the rep-PCR protocol, but members of the genera *Pantoea* and *Erwinia* have not (Louws et al., 1994, 1998; Horita

and Tsuchiya, 2001). The main objective of comparing *Erwinia* and *Pantoea* isolates was to verify that the rep-PCR protocol, specifically BOX-PCR, could be used as a tool to differentiate between these genera. The unique genomic fingerprint patterns observed may also be a useful taxonomic tool in addition to methods described by Mergaert et al., (1993), (DNA-DNA hybridization and protein profiling) and Kwon et al., (1997), (16S rRNA gene sequences). In this study, the small number of *Erwinia* and *Pantoea* isolates used would not allow for a complete taxonomic analysis. This preliminary trial was also conducted to demonstrate that the rep-PCR protocol could also be used to differentiate between two isolates (ATCC-29227 and Il-Ps1) of *P. stewartii*.

The rep-PCR procedure was also used to differentiate 84 *P. stewartii* isolates into four strain types based on their rep-PCR produced genomic fingerprints using the BOX element and repetitive extragenic palindromic (REP) primers, referred to as BOX-PCR and REP-PCR respectively. The isolates ranged in collection dates from 1967-2001 and collection locations (Iowa, Illinois, New York, Kentucky, Ohio, North Carolina, Michigan). Each of the 84 isolates shared 18-19 bands (BOX-PCR) or 16-17 bands (REP-PCR) in their rep-PCR genomic fingerprints. The isolates were placed into strain type, (1, 2, 3, 4) based on the two banding pattern differences. Type 1 strains and type 3 strains had a similarity of 97.143%. Type 2 strains and type 4 strains also had a similarity of 97.143%. Type 1 strains and type 3 strains had a similarity of 94.286% with type 2 strains and type 4 strains. The highly conserved relation between isolates of *P. stewartii* was consistent with carbon utilization (BIOLOG) research conducted by Wilson et al. (1999). Wilson et al. (1999) determined that a collection *P. stewartii* isolates that ranged in collection dates from 1932-1994 and collection locations

(Delaware, Connecticut, Missouri, Iowa, Illinois, New York, Kentucky, Ohio, North Carolina, Michigan) had a similarity of 0.93 to 1.0 based on the isolates ability to utilize carbon sources. Five isolates that were collected during 1974 and 1975 from Ohio, Indiana, Kentucky, and Tennessee were in both the Wilson et al. (1999) study and this study; ATCC 29227, SW1, SW 13, SW 19, and SW 36. Wilson et al. (1999) determined that ATCC 29227, SW 19, SW 13, and SW 36 had a similarity of 0.96 to 1.0, while SW 1 had a similarity of 0.94 to 0.96 to these four isolates. These results are in agreement with this study where ATCC 29227, SW 13, SW 19, and SW 36 had a similarity of 1.0, and SW 1 had a similarity of 0.94286 with the other isolates. Wilson et al. (1999) hypothesized that the limited variability among strains of *P. stewartii* could be explained by its narrow host range, corn flea beetle digestive tract and maize (*Z. mays*), (Wilson et al., 1999). Alternative hosts, such as grasses and weeds, have been shown to sustain *P. stewartii* populations, but it is possible that other hosts exist (Pepper, 1967; Wilson, 1999). Although an alternative host has not been identified, the proposed limited habitat of *P. stewartii* favors the concept that a clonal existence is more likely than genetic strain heterogeneity. Opportunities to introduce and sustain genomic changes caused by recombination and mutations may be restricted because epidemics, when bacterial populations are elevated, are infrequent. The infrequent nature of Stewart's bacterial wilt epidemics may also be caused by an 'epidemic clonal' bacterial population. The *P. stewartii* isolates in this study were collected during or within three years after the occurrence of a Stewart's bacterial wilt epidemic. Spratt and Maiden (1999) theorize that prior to an epidemic, some bacterial populations survive as avirulent or weakly virulent pathogens, but experience some type of recombinational event that for a short period of

time alters the population structure causing it to be more virulent. This may be a possible explanation for the sporadic nature of Stewart's bacterial wilt epidemics.

The rep-PCR protocol was used by Louws et al. (1998) to detect genetic variation in strains of *Clavbacter michiganensis* subsp. *michiganensis*. They were able to divide this subspecies into four distinct groups (A, B, C, D) based on differences found in their rep-PCR genomic fingerprint patterns. Louws et al. (1998) also observed that 30% of the members designated 'A' were nonpathogenic on tomato plants.

The variation in virulence among the strains of *P. stewartii* used in this research had never been defined. Therefore, a leaf symptom analysis procedure was developed that would rapidly assess virulence of *P. stewartii* strains on four varieties of sweet corn seedlings at the 3 leaf growth stage. The strains of *P. stewartii* for the virulence assessment were selected to represent each rep-PCR defined strain type (1, 2, 3, 4). This was done to explore the possibility that there may be a relation between rep-PCR strain-type and virulence on sweet corn. The four varieties of sweet corn used in this study ranged in susceptibility from susceptible to resistant. When the data from all four varieties of sweet corn were combined, it was determined that the area under the disease curve for leaf 1 and leaf 2 ( $AUDPC_{L1}$ ;  $AUDPC_{L2}$ ), individually, were not good indicators of virulence because there were no significant differences observed between *P. stewartii* isolates. The  $AUDPC_{L1}$  was 0.0 for all of the isolates except Es 9245, which had an  $AUDPC_{L1}$  of 0.1. There were significant differences between the AUDPC of strains on leaf 3 ( $AUDPC_{L3}$ ), but more significant differences between isolates were found when the AUDPC of leaf 2 and 3 or leaf 1, 2, and 3 ( $AUDPC_{L23}$ ;  $AUDPC_{L1-3}$ ) were combined. Since the  $AUDPC_{L1}$  was 0.0 or very close to 0 and may underestimate the amount of

disease, the AUDPC<sub>L23</sub> was determined to be the best indicator of isolate virulence. There were significant differences in the virulence of individual strains, (Es 9230, II Es 1, Es 9245, Es 9208 were significantly more virulent than ATCC-29227, Es 9211, SW 87-2, SS 104), but since the more virulent isolates represent all strain types (1, 2, 3, 4) rep-PCR fingerprints likely do not relate to virulence. The variation in virulence observed between individual isolates may be attributed to phenotypic differences that are not genotypically detectable by REP-PCR and BOX-PCR. When the virulence of the isolates was analyzed on sweet corn varieties individually, there were significant differences between the isolates of *P. stewartii*. However, there was no clear trend for isolates from one rep-PCR strain type, temporal, or geographic location to be more virulent than another on all varieties of sweet corn. Therefore, a range of sweet corn varieties that vary in susceptibility should be used to properly assess the virulence differentiation between isolates of *P. stewartii*.

This virulence technique also showed that there were significant differences in the ability of the *P. stewartii* isolates evaluated to cause disease symptoms on the four varieties of sweet corn (Jubilee, Native Gem, Buckeye, and Ambrosia). The susceptibility assessment conducted by Pataky et al. (2000) rated; Jubilee as susceptible; Native Gem as moderately susceptible; Buckeye as resistant; and Ambrosia as resistant. The results from this study were consistent with the ratings performed by Pataky, 2000, except in the case of Ambrosia. It was shown that the susceptibility of Ambrosia was more closely related to Native Gem which would make Ambrosia a moderately resistant variety rather than resistant.

The virulence assessment of *P. stewartii* isolates was rapid and therefore, may be useful for the fine screening of sweet corn varieties to determine susceptibility to Stewart's bacterial wilt at the seedling stage. In addition, this technique could be used to determine the aggressiveness of *P. stewartii* isolates present in fields during future epidemics. It could also enable the rapid analysis of chemical treatments, e.g. insecticides to control corn flea beetles, used to control Stewart's bacterial wilt infection.

The assessment of colony forming units (CFU) in leaf 2 did not differentiate between strains of *P. stewartii*. There were no significant differences found among the moderate and resistant varieties of sweet corn (Native Gem, Buckeye, Ambrosia) analyzed and although there were significantly higher amounts of CFU in the susceptible variety (Jubilee) than in the moderate and resistant, only one isolate evaluated on the variety Jubilee had significantly higher CFU (Es 9208) in leaf 2. A relation between virulence and the production of CFU from leaf 2 is not feasible. Although Es 9208 produced more CFU than any other isolate in Jubilee, in the combined sweet corn variety leaf symptom virulence assessment it was not significantly more virulent than 11 out of the 15 isolates. Determining CFU in leaf 2 was extremely labor intensive and was not a useful tool to find any differences in the ability of strains to systemically infect sweet corn with Stewart's bacterial wilt.

The difficulty with which the 2001 Michigan isolate collection was assembled raised important issues about the disease presence and importance of Stewart's bacterial wilt. After an entire summer of intensively searching for the casual organism of Stewart's bacterial wilt (*P. stewartii*) only three isolates were obtained and there were no reports of Stewart's bacterial wilt in Michigan grower's fields. Scouting the grower's

fields for symptoms of Stewart's wilt and catching corn flea beetles proved to be an unsuccessful method of collection during a year in which the incidence of Stewart's bacterial wilt was minimal. Baiting with a susceptible variety of sweet corn was the only technique that was successful, but even this method only resulted in a 1.0% rate of return. Pepper (1967) estimated that as much as 20% of spring emerging beetles can be vectors of *P. stewartii*. In epidemic years, the starting population of vectoring beetles may be about 10%-20% (Pepper, 1967), but in years when epidemics do not occur the starting population may be much lower, e.g. <1.0%. In such years, population density may increase and late in the season corn flea beetles may pick up inoculum that is enough to initiate a more severe epidemic the following year. Mature plants, when infected with Stewart's bacterial wilt, may not show symptoms that are typical of the seedling stage of the disease. The sporadic pattern of Stewart's bacterial wilt outbreaks, which may be separated by up to 30 years in Michigan, is of significant epidemiological interest and should continue to be monitored to determine early indicators of epidemic initiation. Until a putative alternative host or location in which bacteria are able to persist for long periods of time without genetic change is found that can better explain the clonal populations of *P. stewartii* found in the eastern United States, prediction methods associated with the corn flea beetle's ability to survive and carry *P. stewartii* should be pursued.

## Summary

Epidemic risk assessment could be based on a model that combines; a) estimation of corn flea beetle population size; b) percentage of beetles carrying *P. stewartii*; c) corn flea beetle prediction models that evaluate soil temperatures to better predict the number

of corn flea beetles emerging in the spring that are carrying *P. stewartii* (W. Kirk, pers. comm.). The combination of these variables may help seed companies and sweet corn growers decide if insecticide seed treatments or foliar insecticide treatments are warranted. The 'epidemic clonal' bacterial population theory by Spratt and Maiden (1999) should also be explored. Sweet corn, the digestive tract of the corn flea beetle, and other possible hosts such as weeds and grasses should be examined for the existence of an avirulent or weakly virulent form of *P. stewartii* during non-epidemic growing seasons. The rep-PCR procedure, along with the leaf symptom virulence assessment may be used as tools to rapidly identify *P. stewartii* isolates. Should another series of Stewart's bacterial wilt epidemics occur, the virulence levels of *P. stewartii* isolates can be compared to isolates that have caused epidemics in the past. Until another Stewart's bacterial wilt epidemic occurs or epidemics become more frequent, exploring other genetic methods of bacterial strain differentiation may not be worthwhile because it appears there is very little genetic diversity among *P. stewartii* isolates present in the eastern United States.



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## **APPENDICES**

## **APPENDIX A**

### **Optimization of BOX primer for rep-PCR (Example)**

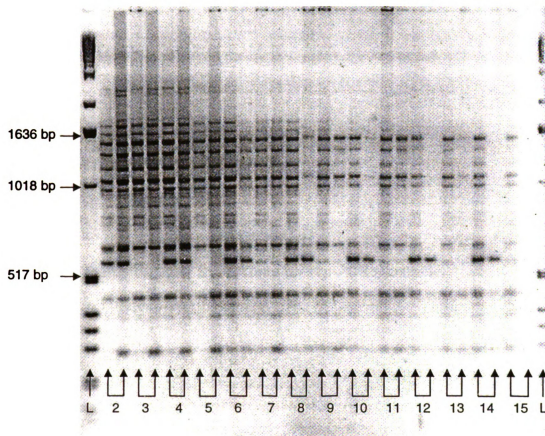


Figure 15. Optimization of Rep-PCR using the BOX primer generated genomic fingerprint of *Pantoea stewartii* strains (ATCC-29227 and II-Ps 1). The numbered brackets below indicate the strain of *P. stewartii* and levels of H<sub>2</sub>O that is being analyzed. Each strain and H<sub>2</sub>O alteration is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2-II-Ps 1 (12.25  $\mu$ l of H<sub>2</sub>O); 3-ATCC-29227 (12.25  $\mu$ l of H<sub>2</sub>O); 4-II-Ps 1 (12.30  $\mu$ l of H<sub>2</sub>O); 5-ATCC-29227 (12.30  $\mu$ l of H<sub>2</sub>O); 6-II-Ps 1 (12.35  $\mu$ l of H<sub>2</sub>O); 7-ATCC-29227 (12.35  $\mu$ l of H<sub>2</sub>O); 8-II-Ps 1 (12.40  $\mu$ l of H<sub>2</sub>O); 9-ATCC-29227 (12.40  $\mu$ l of H<sub>2</sub>O); 10-II-Ps 1 (12.45  $\mu$ l of H<sub>2</sub>O); 11-ATCC-29227 (12.45  $\mu$ l of H<sub>2</sub>O); 12-II-Ps 1 (12.50  $\mu$ l of H<sub>2</sub>O); 13-ATCC-29227 (12.50  $\mu$ l of H<sub>2</sub>O); 14-II-Ps 1 (12.55  $\mu$ l of H<sub>2</sub>O); 15-ATCC-29227 (12.55  $\mu$ l of H<sub>2</sub>O).

## **APPENDIX B**

### **Optimization of REP primer for rep-PCR (Example)**



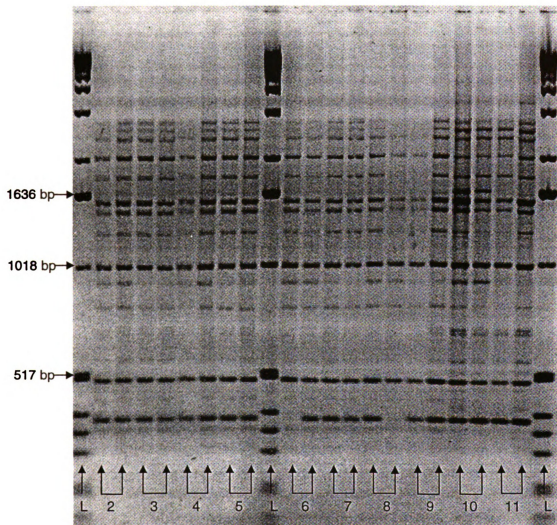


Figure 16. Optimization of Rep-PCR using the REP primer generated genomic fingerprint of *Pantoea stewartii* strains (ATCC-29227 and II-Ps 1). The numbered brackets below indicate the strain of *P. stewartii* and levels of H<sub>2</sub>O that are being analyzed. Each strain and H<sub>2</sub>O alteration is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lane 2-ATCC-29227 (12.45  $\mu$ l of H<sub>2</sub>O); 3-II-Ps 1 (12.45  $\mu$ l of H<sub>2</sub>O); 4 ATCC-29227 (12.50  $\mu$ l of H<sub>2</sub>O); 5-II-Ps 1 (12.50  $\mu$ l of H<sub>2</sub>O); 6-ATCC-29227 (12.55  $\mu$ l of H<sub>2</sub>O); 7-II-Ps 1 (12.55  $\mu$ l of H<sub>2</sub>O); 8-ATCC-29227 (12.60  $\mu$ l of H<sub>2</sub>O); 9-II-Ps 1 (12.60  $\mu$ l of H<sub>2</sub>O); 10-ATCC-29227 (12.65  $\mu$ l of H<sub>2</sub>O); 11-II-Ps 1 (12.65  $\mu$ l of H<sub>2</sub>O).

## **APPENDIX C**

### **Identification of CFU using Rep-PCR and the BOX primer**

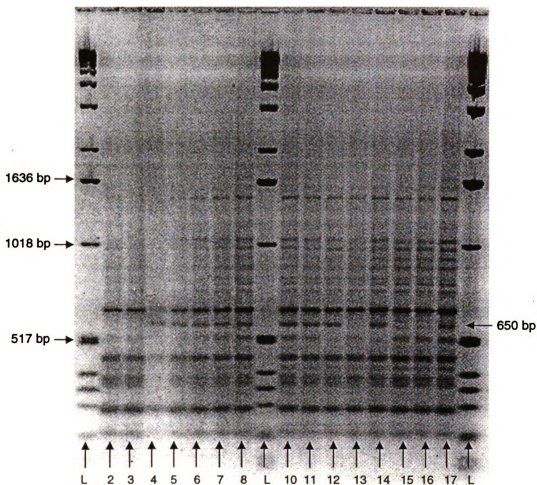


Figure 17. rep-PCR using the BOX primer generated genomic fingerprint, on a 1.5% agarose gel, of 15 *Pantoea stewartii* strains that were randomly selected as checks to confirm that the colonies counted in the virulence trial were *P. stewartii*. The numbered lines with arrows below indicate the isolate of *P. stewartii* that is being analyzed. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Strains missing the 650-bp band - Lane 2-Illinois (Ps-VI-A-CP-1-1); 3-Illinois (Ps-V-A-CP-1-1); 13-Iowa (Es 9245); 15- Michigan (II-B-CP-1); 16- Illinois (II-Ps 1). Strains with the 650-bp band- Lane 4-Iowa (SW 87-2); 5-Illinois (SW 45); 6-Ohio (SW 1); 7-Iowa (SW 87-19); 8-Iowa (SW 87-6); 10-Iowa (Es 9208); 11-Iowa (Es 9211); 12-Iowa (Es 9230); 14-Illinois (SS 104); 17-ATCC-29227

## **APPENDIX D**

**Rep-PCR using the BOX primer gels with 84 isolates of *P. stewartii***

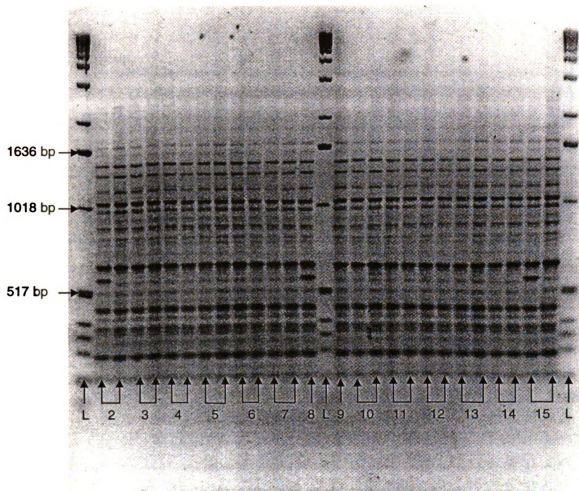


Figure 18. Rep-PCR using the BOX primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- ATCC-29227 and Illinois-control (II-Ps-1). Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (II-Ps-1), respectively. 3-Illinois (VIII-A-CP-3-1); 4-Illinois (VI-A-CP-2-1); 5-Illinois (V-A-CP-4-2); 6-Illinois (VIII-A-CP-3-2); 7-Michigan (II-B-CP-1); 10-Illinois (V-A-CP-1-2); 11-Illinois (VI-A-CP-2-3); 12-Illinois (VI-A CP-1-3); 13-Illinois (VI-A-CP-1-2); 14-Illinois (VI-A-CP-1-1).

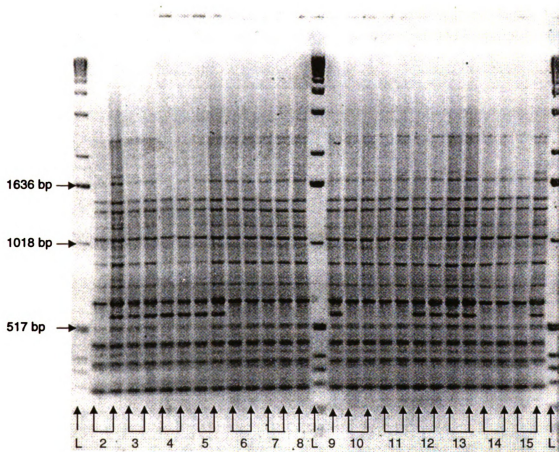


Figure 19. Rep-PCR using the BOX primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- Illinois-control (Il-Ps-1) and ATCC-29227. Lane 8 and 9 also contain strain Illinois-control (Il- Ps-1) and ATCC-29227, respectively. 3-Iowa (SW 87-1); 4-Iowa (SW 87-2); 5-Ohio (SW1); 6-Illinois (V-A-CP-1-1); 7-Illinois (V-A-CP-3-2); 10-Illinois (V-A-CP-3-1); 11-Illinois (V-A-CP-4-1); 12-Iowa (SW 87-3); 13-Iowa (SW 87-4); 14-Illinois (VI-A-CP-4-1).

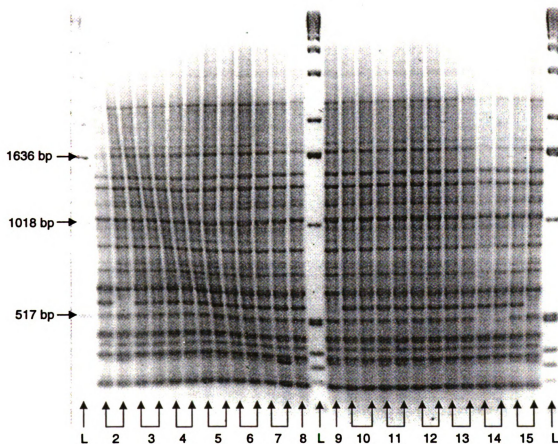


Figure 20. Rep-PCR using the BOX primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- ATCC-29227 and Illinois-control (Il-Ps-1). Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (Il-Ps-1), respectively. 3-Iowa (SW 87-23); 4-Iowa (SW-87-22); 5-Iowa (SW-87-21); 6-Iowa (SW-87-20); 7-Iowa (SW-87-19); 10-Iowa (SW-87-18); 11-Iowa (SW-87-9); 12-Iowa (SW-87-7); 13-Iowa (SW-87-25); 14-Iowa (87-24).

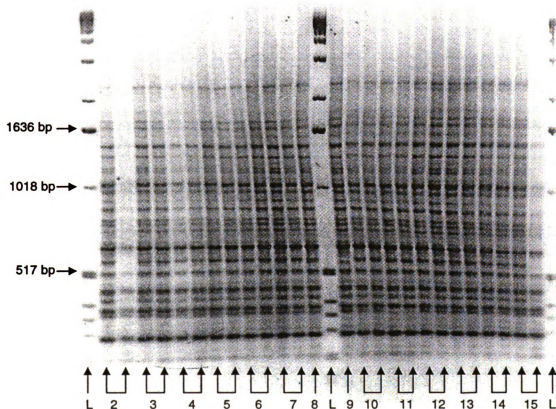


Figure 21. Rep-PCR using the BOX primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- ATCC-29227 and Illinois-control (II-Ps-1). Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (II-Ps-1), respectively. 3-Iowa (SW-87-17); 4-Iowa (SW-87-16); 5-Iowa (SW-87-15); 6-Iowa (SW-87-13); 7-Iowa (SW 87-12); 10-Iowa (SW 87-11); 11-Iowa (SW 87-10); 12-Iowa (SW-87-8); 13-Iowa (SW-87-5); 14-Iowa (SW-87-6).



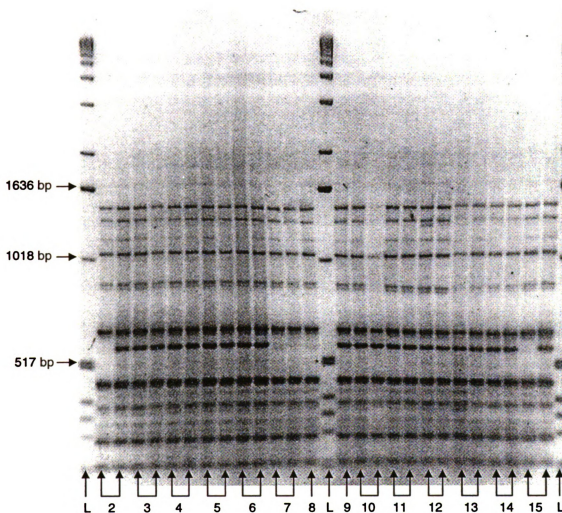


Figure 22. Rep-PCR using the BOX primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- ATCC-29227 and Illinois-control (II-Ps-1). Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (II-Ps-1), respectively. 3-Iowa (Es 9225); 4-Iowa (Es 9230); 5-Iowa (Es 9243); 6-Iowa (Es 9244); 7-Iowa (Es 9245); 10-Iowa (Es 9222); 11-Iowa (Es 9221); 12-Iowa (Es 9203); 13-Iowa (Es 9204); 14-Iowa (Es 9205).

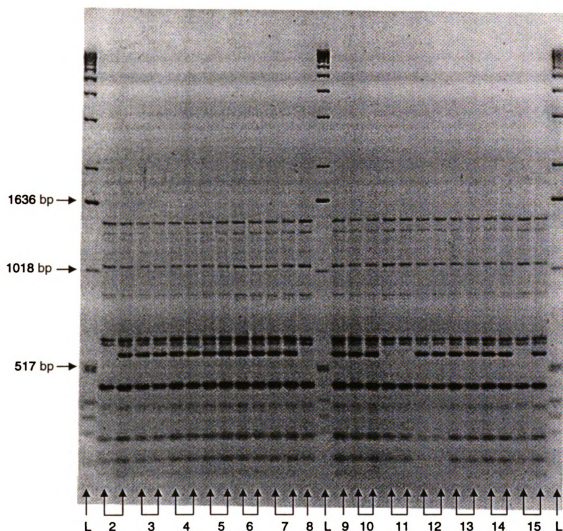


Figure 23. Rep-PCR using the BOX primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls-Illinois-control (II- Ps-1) and ATCC-29227. Lane 8 and 9 also contain strain Illinois-control (II- Ps-1) and ATCC-29227, respectively. 3-Illinois (SS 104) . 4-North Carolina (SW 39); 5-Iowa (Es 9250); 6-Iowa (Es 9256); 7-Tennessee (SW 36); 10-Iowa (Es 9253); 11-Iowa (Es 9245); 12-Indiana (SW 14); 13-Iowa (Es 9247); 14-Illinois (SW 45).

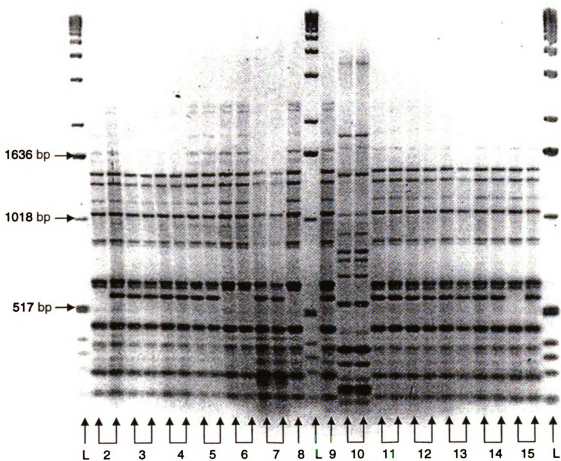


Figure 24. Rep-PCR using the BOX primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls - Illinois-control (Il-Ps-1) and ATCC-29227. Lane 8 and 9 also contain strain Illinois-control (Il-Ps-1) and ATCC-29227, respectively. 3-Indiana (SW 13); 4-Ohio (SW 2); 5-Ohio (Rif EsA); 6-Iowa (Es 9257); 7-Ohio (SW 1); 10-Iowa (Es 9254); 11-Kentucky (SW 19); 12-Iowa (Es 9248); 13-Iowa (Es 9249); 14-Iowa (9246).

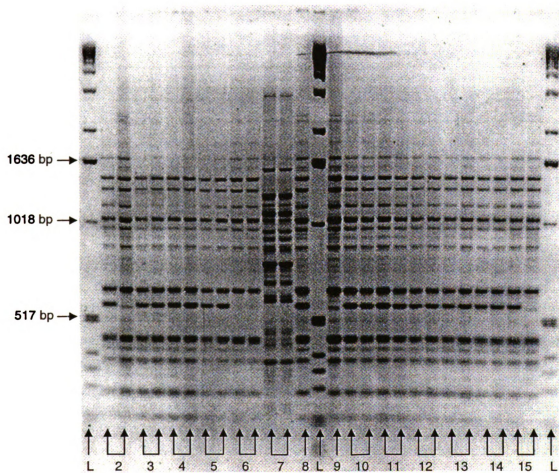


Figure 25. Rep-PCR using the BOX primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- ATCC-29227 and Illinois-control (II-Ps-1), respectively. 3-Iowa (Es 9203); 4-Iowa (Es 9204); 5 Iowa (Es 9205); 6-Iowa (Es 9206); 7-Iowa (Es 9207); 10-Iowa (Es 9208); 11-Iowa (Es 9209); 12-Iowa (Es 9211); 13-Iowa (Es 9210); 14-Iowa (Es 9201).

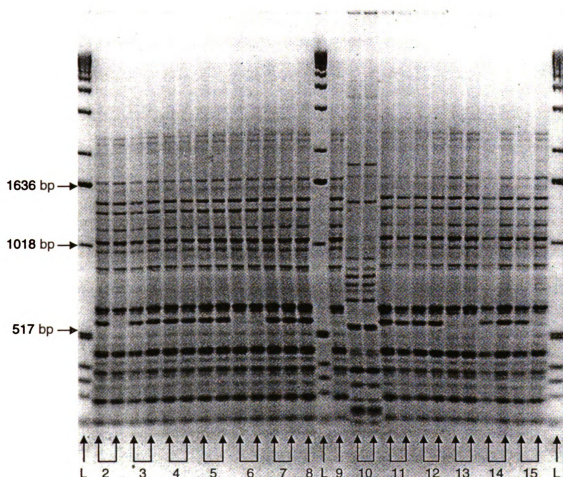


Figure 26. Rep-PCR using the BOX primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- ATCC-29227 and Illinois-control (II-Ps-1). Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (II-Ps-1), respectively. 3-Iowa (Es 9223); 4-Iowa (Es 9222); 5-Iowa (Es 9221); 6-Iowa (Es 9213); 7-Iowa (Es 9212); 10-Iowa (Es 9264); 11-Iowa (Es 9244); 12-Iowa (Es 9243); 13-Iowa (Es 9246); 14-Iowa (Es 9230).

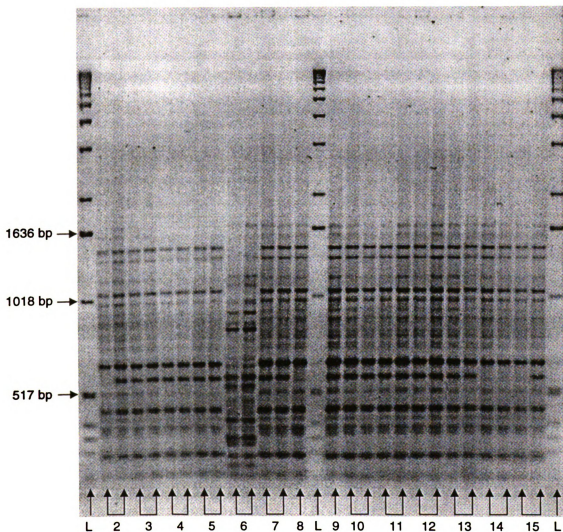


Figure 27. Rep-PCR using the BOX primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- Illinois-control (II-Ps-1) and ATCC-29227. Lane 8 and 9 also contain strain Illinois-control (II-Ps-1) and ATCC-29227, respectively. 3-Illinois (SW 45); 4-Kentucky (SW 19); 5-Ohio (GAL E8); 6-Ohio (DC 130); 7-Ohio (DC 160); 10-Ohio (MU 51); 11-Indiana (SW 13); 12-Indiana (SW 14); 13-Ohio (DC 283); 14-Michigan (II-B-CP-7).



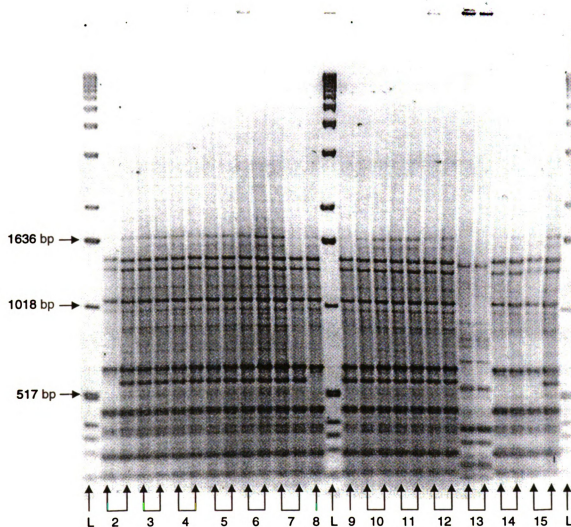


Figure 28. Rep-PCR using the BOX primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls-ATCC-29227 and Illinois-control (Il-Ps-1). Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (Il-Ps-1), respectively. 3-Iowa (Es 9249); 4-Iowa (Es 9247); 5-Iowa (Es 9248); 6-Iowa (Es 9250); 7-Iowa (Es 9253); 10-Iowa (Es 9256); 11-Ohio (Rif Es 9A); 12-Illinois (SS 104); 13-Iowa (Es 9264); 14-Iowa (Es 9257).

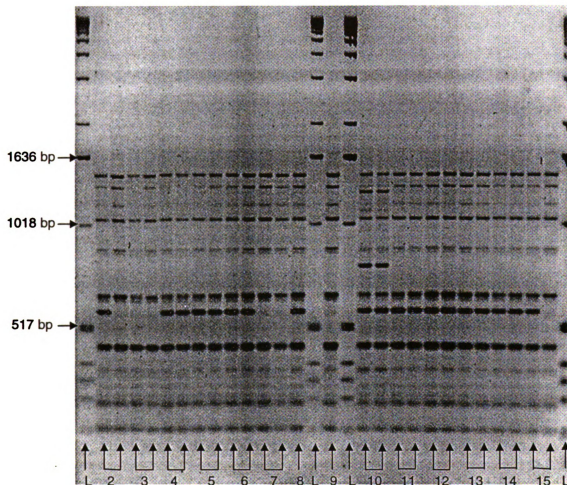
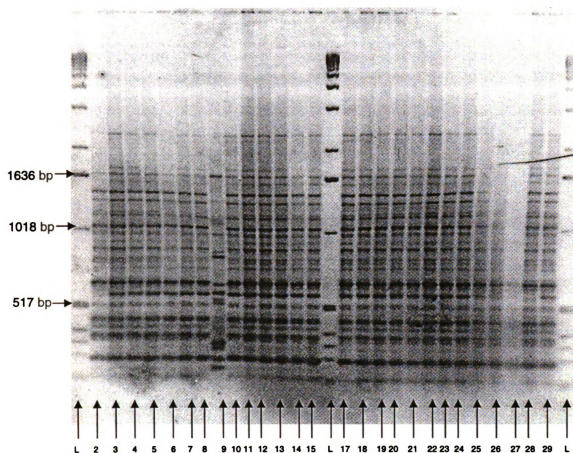


Figure 29. Rep-PCR using the BOX primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- ATCC-29227 and Illinois-control (Il-Ps-1), respectively. Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (Il-Ps-1), respectively. 3-Iowa (Es 9213); 4-Iowa (Es 9212); 5-Iowa (Es 9211); 6-Iowa (Es 9210); 7-Iowa (Es 9206); 10-Iowa (Es 9207); 11-Iowa (Es 9208); 12-Iowa (Es 9209); 13-Iowa (SW 87-26); 14-Iowa (Es 9201).





**Figure 30.** Rep-PCR using the BOX primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbers below the arrows indicate the strain of *Pantoea stewartii* that was analyzed. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 3 contain the positive controls- Illinois-control (Il-Ps-1) and ATCC-29227, respectively. Lane 17 contains the ATCC-29227 control. Lane 28 and 29 also contain strain Illinois-control (Il-Ps-1) and ATCC-29227, respectively. 4-Iowa (SW 87-11); 5-Iowa (SW 87-12); 6-Iowa (SW 87-13); 7-Iowa (SW 87-15); 8-Iowa (SW 87-1); 9-Ohio (DC 130); 10-Ohio (DC 160); 11-North Carolina (SW 39); 12-Illinois (SW 45); 13-Tennessee (SW 36); 14-Kentucky (SW 19); 15-Iowa (SW 87-16); 18-Iowa (SW 87-17); 19-Iowa (SW 87-18); 20-Iowa (SW 87-19); 21-Iowa (SW 87-20); 22-Iowa (SW 87-21); 23-Iowa (SW 87-26); 24-Iowa (SW 87-25); 25-Iowa (SW 87-24); 26-Iowa (SW 87-23); 27-Iowa (SW 87-22).

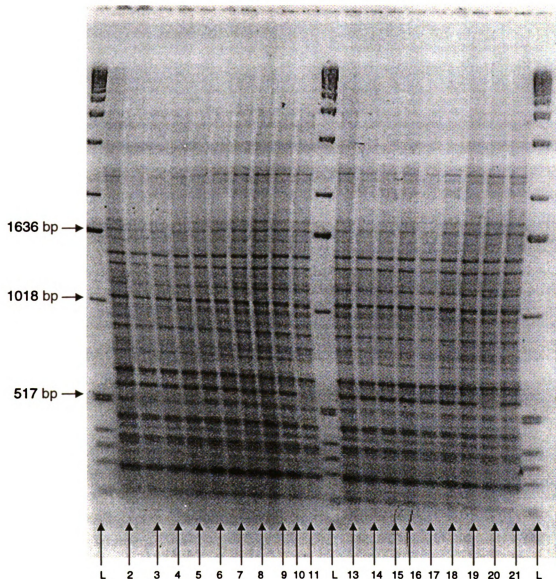


Figure 31. Rep-PCR using the BOX primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbers below the arrows indicate the strain of *Pantoea stewartii* that was analyzed. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lane 2 contains the positive control- ATCC-29227. Lane 13 contains the Illinois control (Il Ps-1). Lane 21 contains ATCC-29227. 3-Ohio (DC 283); 4-Ohio (SW 1); 5-Ohio (MU 51); 6-Ohio (GAL E8); 7-Indiana (SW 14); 8-Indiana (SW 13); 9-Ohio (SW 2); 10-Iowa (SW 87-2); 11-Iowa (SW 87-3); 14-Iowa (SW 87-4); 15-Iowa (SW 87-5); 16-Iowa (SW 87-6); 17-Iowa (SW 87-7); 18-Iowa (SW 87-8); 19-Iowa (SW 87-9); 20-Iowa (SW 87-10).

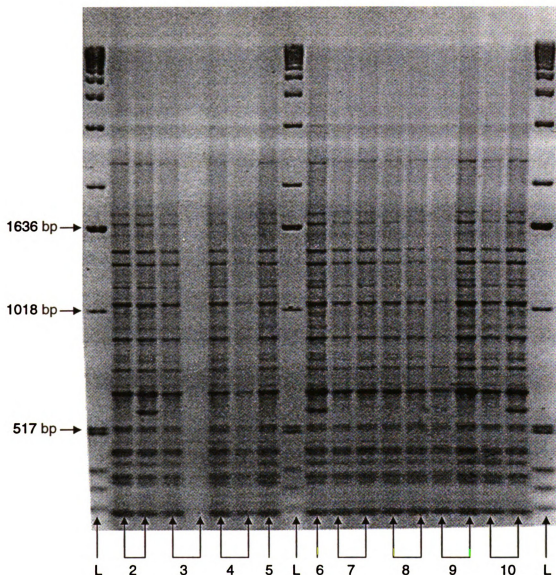


Figure 32. Rep-PCR using the BOX primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 10 contain the positive controls- Illinois-control (II-Ps-1) and ATCC-29227. Lane 5 and 6 also contain strain Illinois-control (II-Ps-1) and ATCC-29227, respectively. 3-Illinois (V-A-CP-4-1); 4-Illinois (VIII-A-CP-3-2); 7-Illinois (VIII-A-CP-3-1); 8-Illinois (VI-A-CP-2-1); 9-Illinois (VI-A-CP-4-1).

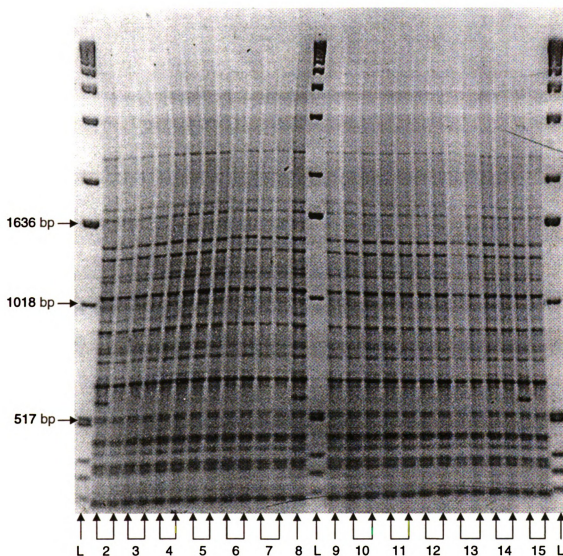


Figure 33. Rep-PCR using the BOX primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- ATCC-29227 and Illinois-control (II-Ps-1). Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (II-Ps-1), respectively. 3-Illinois (VI-A-CP-1-2); 4-Illinois (VI-A-CP-1-3); 5-Illinois (V-A-CP4-2); 6-Michigan (II-B-CP-5); 7-Illinois (VI-A-CP-1-3); 10-Illinois (V-A-CP-3-2); 11-Illinois (V-A-CP-1-1); 12-Illinois (V-A-CP-3-1); 13-Illinois (V-A-CP-1-2); 14-Illinois (VI-A-CP-1-1).

## **APPENDIX E**

**Rep-PCR using the REP primer gels with 84 isolates of *P. stewartii***

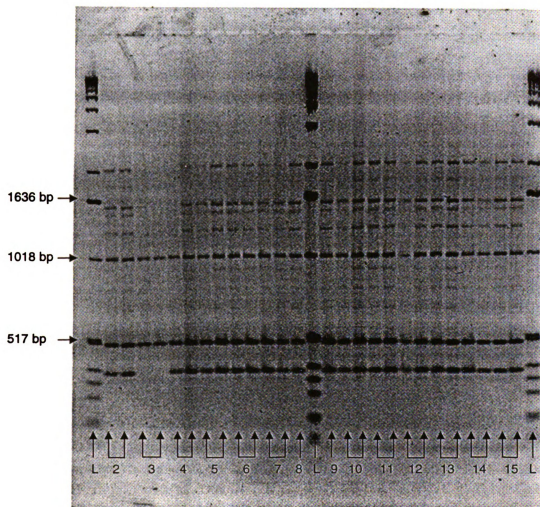


Figure 34. Rep-PCR using the REP primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls-ATCC-29227 and Illinois-control (II-Ps 1). Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (II-Ps 1), respectively. 3-Illinois (VIII-A-CP-3-1); 4-Illinois (VI-A-CP-2-1); 5-Illinois (V-A-CP-4-2); 6-Illinois (VIII-A-CP-3-2); 7-Michigan (II-B-CP-1); 10-Illinois (V-A-CP-1-2); 11-Illinois (VI-A-CP-2-3); 12-Illinois (VI-A-CP-1-3); 13-Illinois (VI-A-CP-1-2); 14-Illinois (VI-A-CP-1-1).



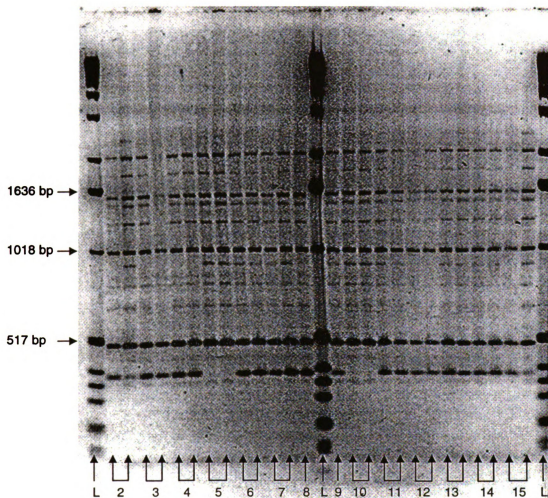


Figure 35. Rep-PCR using the REP primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls-ATCC-29227 and Illinois-control (Il-Ps 1), respectively. 3-Iowa (SW 87-1); 4-Iowa (SW-87-2); 5-Ohio (SW 1); 6-Illinois (V-A-CP-1-1); 7-Illinois (V-A-CP-3-2); 10-Illinois (V-A-CP-3-1); 11-Illinois (V-A-CP-4-1); 12-Iowa (SW-87-3); 13-Iowa (SW-87-4); 14-Illinois (VI-A-CP-4-1).

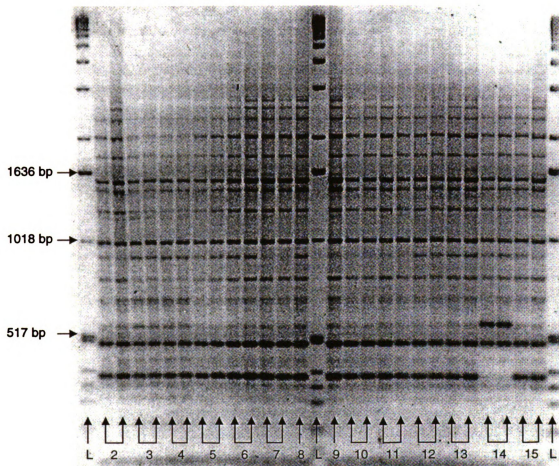


Figure 36. Rep-PCR using the REP primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- ATCC-29227 and Illinois-control (II-Ps 1). Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (II-Ps 1), respectively. 3-Iowa (SW 87-23); 4-Iowa (SW-87-22); 5-Iowa (SW-87-15); 6-Iowa (SW 87-13); 7-Iowa (SW-87-12); 10-Iowa (SW-87-11); 11-Iowa (SW 87-10); 12-Iowa (SW-87-8); 13-Iowa (SW-87-5); 14-Iowa (SW 87-6).



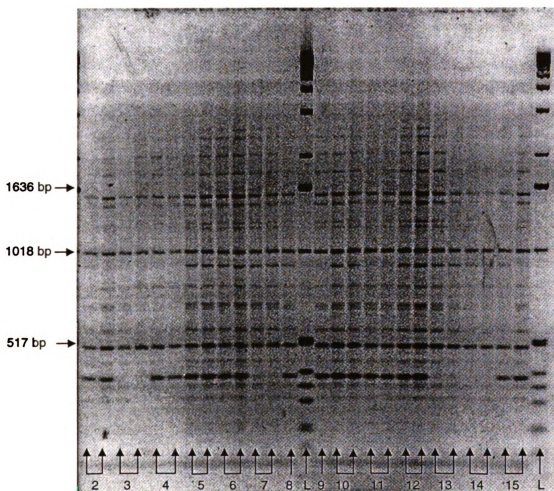


Figure 37. Rep-PCR using the REP primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- ATCC-29227 and Illinois-control (Il-Ps 1). Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (Il-Ps 1), respectively. 3-Iowa (SW 87-17); 4-Iowa (SW-87-16); 5-Iowa (SW-87-21); 6-Iowa (SW 87-20); 7-Iowa (SW-87-19); 10-Iowa (SW-87-18); 11-Iowa (SW 87-9); 12-Iowa (SW-87-7); 13-Iowa (SW-87-24); 14-Iowa (SW 87-25).

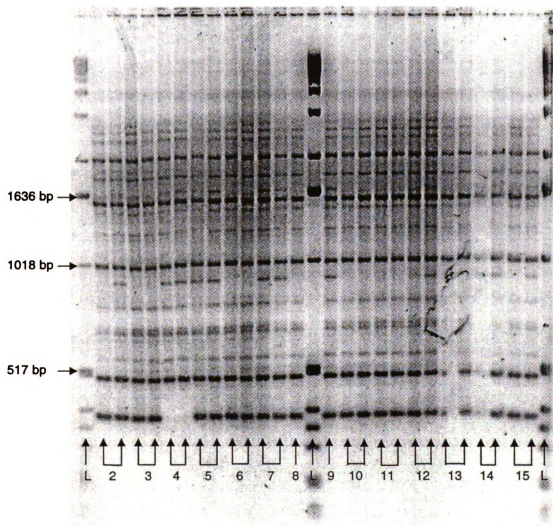


Figure 38. Rep-PCR using the REP primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- ATCC-29227 and Illinois-control (II-Ps 1). Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (II-Ps 1), respectively. 3-Iowa (Es 9257); 4-Iowa (Es 9256); 5-Iowa (Es 9253); 6-Iowa (Es 9250); 7-Iowa (Es 9249); 10-Iowa (Es 9248); 11-Iowa (Es 9247); 12-Kentucky (SW 19); 13-Indiana (SW 2); 14-Indiana (SW 13).

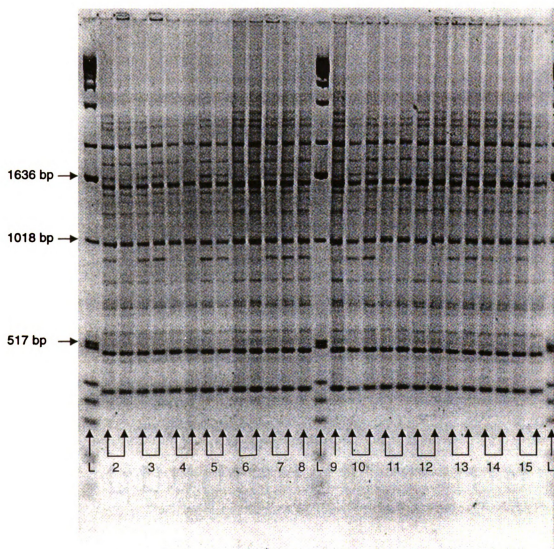


Figure 39. Rep-PCR using the REP primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- ATCC-29227 and Illinois-control (Il-Ps 1). Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (Il-Ps 1), respectively. 3-Iowa (Es 9213); 4-Iowa (Es 9212); 5-Iowa (Es 9211); 6-Iowa (Es 9210); 7-Iowa (Es 9206); 10-Iowa (Es 9207); 11-Iowa (Es 9208); 12-Iowa (Es 9209); 13-Iowa (SW 87-26); 14-Iowa (Es 9201).

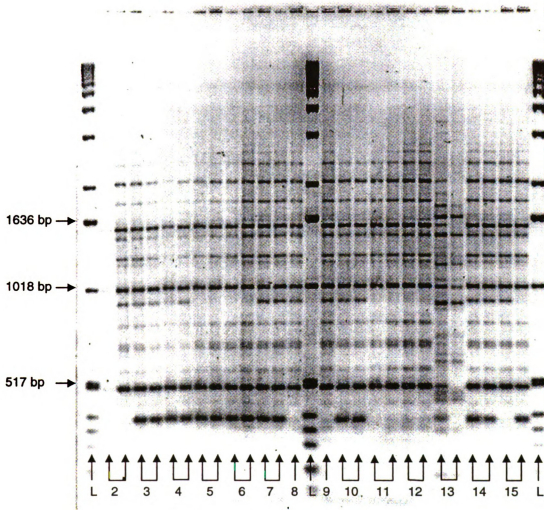


Figure 40. Rep-PCR using the REP primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- ATCC-29227 and Illinois-control (II-Ps 1). Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (II-Ps 1), respectively. 3-Iowa (Es 9249); 4-Iowa (Es 9247); 5-Iowa (Es 9248); 6-Iowa (Es 9250); 7-Iowa (Es 9253); 10-Iowa (Es 9256); 11-Ohio (Rif Es 9A); 12-Illinois (SS 104); 13-Iowa (Es 9264); 14-Iowa (Es 9257).

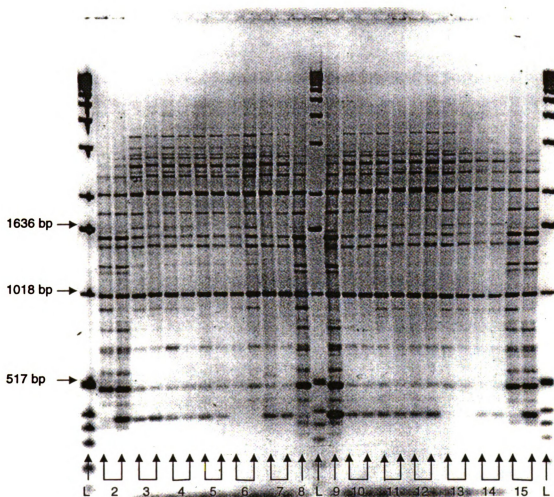


Figure 41. Rep-PCR using the REP primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- ATCC-29227 and Illinois-control (II-Ps 1). Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (II-Ps 1), respectively. 3-Iowa (Es 9243); 4-Iowa (Es 9240); 5-Iowa (Es 9225); 6-Iowa (Es 9230); 7-Iowa (Es 9222); 10-Iowa (Es 9221); 11-Iowa (Es 9213); 12-Iowa (Es 9244); 13-Iowa (Es 9245); 14-Iowa (Es 9246).



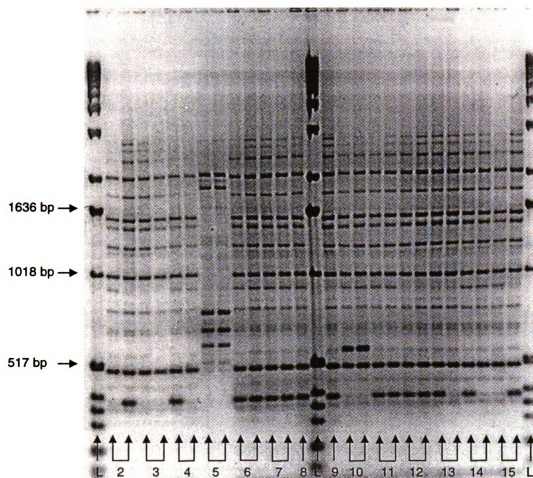


Figure 42. Rep-PCR using the REP primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- ATCC-29227 and Illinois-control (II-Ps 1). Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (II-Ps 1), respectively. 3-Ohio (DC 283); 4-Ohio (DC 160); 5-Ohio (DC 130); 6-Iowa (SW 87-8); 7-Iowa (SW-87-7); 10-Iowa (SW-87-6); 11-Indiana (SW 14); 12-Tennessee (SW 36); 13-North Carolina (SW-39); 14-Illinois (SW 45).

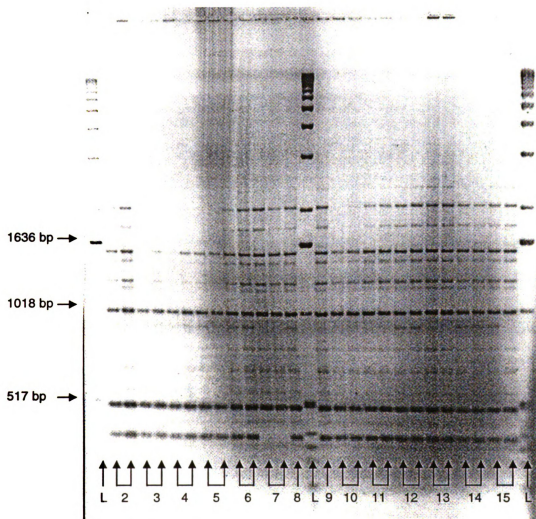


Figure 43. Rep-PCR using the REP primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- ATCC-29227 and Illinois-control (II-Ps 1). Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (II-Ps 1), respectively. 3-Michigan (II-B-CP-1); 4-Iowa (SW-87-4); 5-Iowa (SW-87-5); 6-Iowa (SW 87-18); 7-Iowa (SW-87-17); 10-Iowa (SW-87-3); 11-Iowa (SW 87-2); 12-Michigan (II-B-CP-7); 13-Iowa (SW-87-1); 14-Michigan (II-B-CP-5).

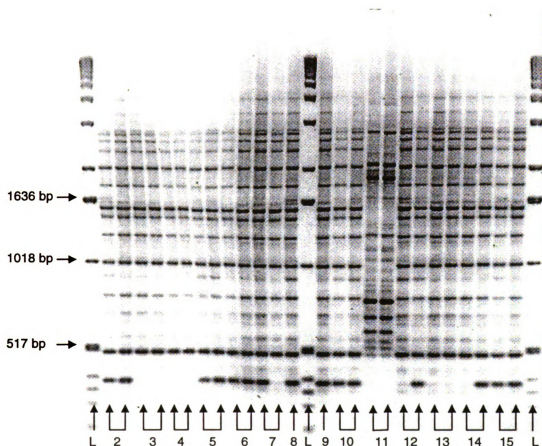


Figure 44. Rep-PCR using the REP primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- ATCC-29227 and Illinois-control (II-Ps 1). Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (II-Ps 1), respectively. 3-Iowa (Es 9246); 4-Iowa (Es 9212); 5-Iowa (Es 9210); 6-Iowa (Es 9208); 7-Iowa (Es 9207); 10-Iowa (Es 9206); 11-Ohio (SS 104); 12-Iowa (Es 9264); 13-Ohio (DC 283); 14-Ohio (MU 51).



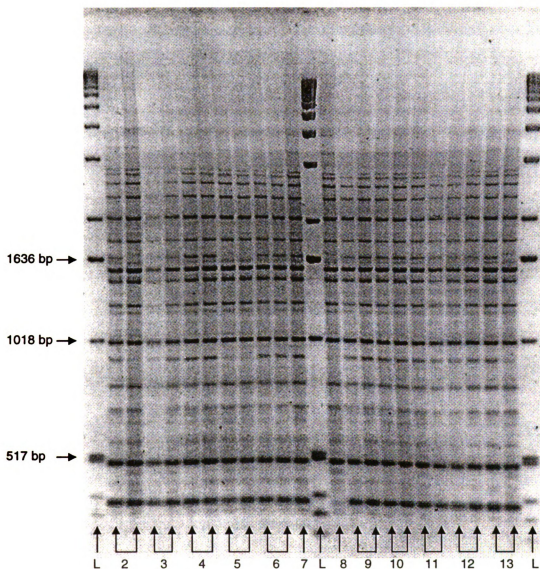


Figure 45. Rep-PCR using the REP primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- ATCC-29227 and Illinois-control (IL-Ps 1). Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (IL-Ps 1), respectively. 3-Illinois (VI-A-CP-1-1); 4-(SW 45); 5- (SW 39); 6-Illinois (V-A-CP-3-2); 9-Illinois (VIII-A-CP-3-2); 10-Illinois (V-A-CP-4-2); 11-Illinois (VI-A-CP-2-1); 12-Illinois (VI-A-CP-4-1).

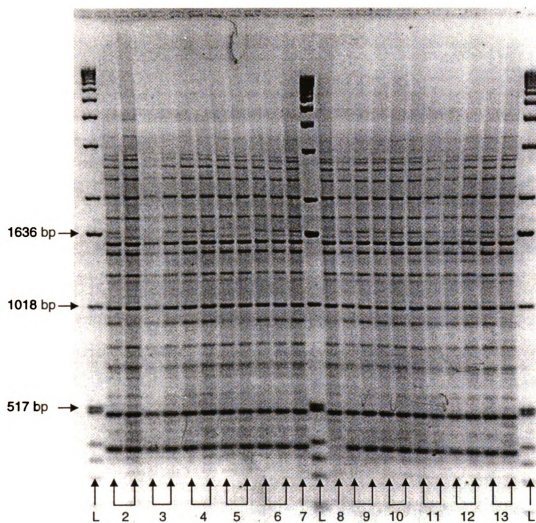


Figure 46. Rep-PCR using the REP primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- ATCC-29227 and Illinois-control (II-Ps 1). Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (II-Ps 1), respectively. 3-Illinois (VI-A-CP-2-3); 4-Illinois (V-A-CP-1-2); 5- Illinois (V-A-CP-3-1); 6-Illinois (V-A-CP-1-1); 9-Illinois (V-A-CP-4-1); 10-Illinois (VI-A-CP-1-3); 11-Illinois (VI-A CP-1-2); 12-Illinois (VIII-A-CP-3-1).

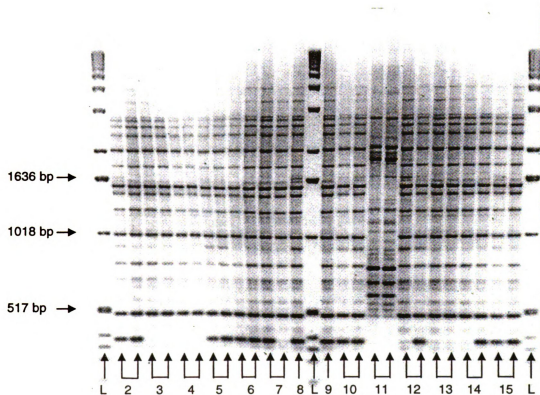


Figure 47. Rep-PCR using the REP primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- ATCC-29227 and Illinois-control (II-Ps 1). Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (II-Ps 1), respectively. 3-Ohio (GAL E8); 4-Ohio (MU 51); 5-Illinois (SW 45); 6-North Carolina (SW 39); 7-Tennessee (SW-36); 10-Indiana (SW 14); 11-Ohio (DC 130); 12-Ohio (DC 160); 13-Ohio (DC 283); 14-Ohio (SW 1).

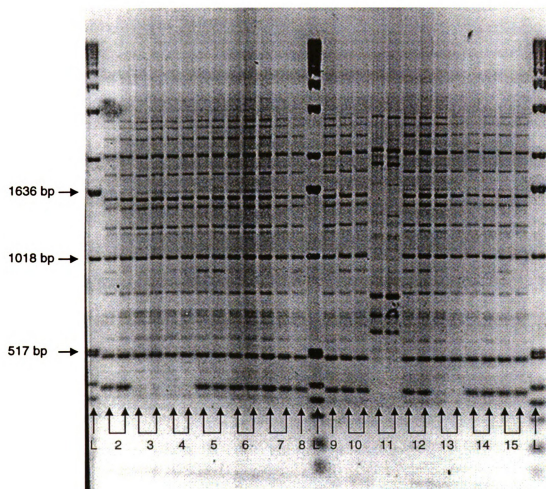


Figure 48. Rep-PCR using the REP primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- ATCC-29227 and Illinois-control (II-Ps 1). Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (II-Ps 1), respectively. 3-Ohio (GAL E8); 4-Ohio (MU 51); 5-Illinois (SW 45); 6- North Carolina (SW 39); 7-Tennessee (SW-36); 10-Indiana (SW 14); 11-Ohio (DC 130); 12-Ohio (DC 160); 13-Ohio (DC 283); 14-Ohio (SW 1).

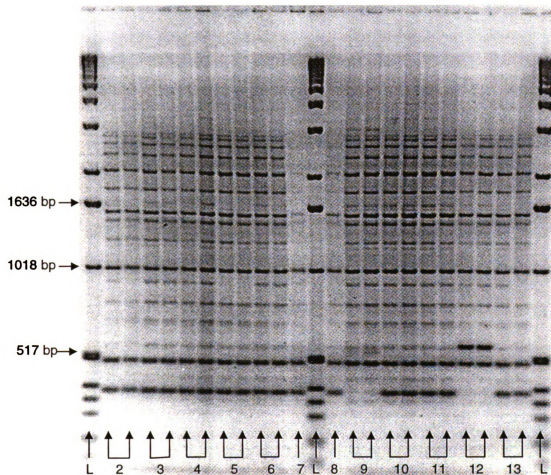


Figure 49. Rep-PCR using the REP primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- ATCC-29227 and Illinois-control (II-Ps 1). Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (II-Ps 1), respectively. 3-Iowa (87-13); 4-Iowa (87-15); 5-Iowa (87-16); 6-Iowa (87-20); 9-Iowa (87-19); 10-Iowa (87-22); 11-Iowa (87-21); 12-Iowa (87-6).

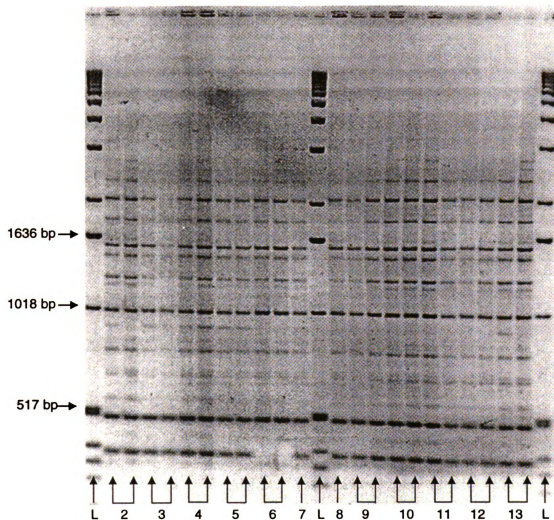


Figure 50. Rep-PCR using the REP primer generated genomic fingerprint, on a 1.5% agarose gel, of *Pantoea stewartii* strains. The numbered brackets below indicate the strain of *Pantoea stewartii* that was analyzed. Each strain is repeated in adjacent lanes and represented by the same number. The three lanes labeled with an 'L' contain a 1 Kb DNA Ladder. Lanes 2 and 15 contain the positive controls- ATCC-29227 and Illinois-control (II-Ps 1). Lane 8 and 9 also contain strain ATCC-29227 and Illinois-control (II-Ps 1), respectively. 3-Iowa (87-7); 4-Iowa (87-8); 5-Iowa (87-18); 6-Iowa (87-17); 9-Iowa (87-9); 10-Iowa (87-10); 11-Iowa (87-11); 12-Iowa (87-12).



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