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GENETIC VARIATION IN STRAINS OF *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS* AND THE DEVELOPMENT OF BIRD'S EYE FRUIT LESIONS ON TOMATOES

By

Carmen M. Medina-Mora

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ABSTRACT

GENETIC VARIATION IN STRAINS OF *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS* AND THE DEVELOPMENT OF BIRD'S EYE FRUIT LESIONS ON TOMATOES

By

Carmen M. Medina-Mora

Bacterial canker of tomato is a major concern worldwide because disease occurrence is sporadic and may result in large yield reductions. Computer-assisted analysis of repetitive sequence-based Polymerase Chain Reaction (rep-PCR) genomic fingerprints generated with the universal primers BOX and $(GTG)_5$ suggested genomic variation in *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) is relatively limited. However, $(GTG)_5$-PCR fingerprint patterns do differentiate strains within the previously designated BOX-PCR types (A, B, C, D, and E). Intraspecific diversity using combined $(GTG)_5$ -PCR and BOX -PCR data is as follows; $C > A \geq B > D > E$. These data may provide useful genetic makers to trace *Cmm* strains used in epidemiological studies.

Fruit spotting resulting from *Cmm* infection is one of the most unpredictable tomato disease symptoms of bacterial canker in Michigan. Therefore, fruit spot formation was studied by spraying flowers with *Cmm* at various developmental stages. Maximum incidence of spotted fruit and maximum severity of fruit lesions resulted when inoculum was applied twice, a possible indication that a bacterial population threshold is required for the onset of fruit spots. Chemical control strategies could be formulated to reduce the level of unmarketable tomato fruit.

To my parents, husband, and
specially to my grandfather, Ramón I. Mora, on his memory...
With a lot of love and respect this is for you...

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Thanks to every past or present member of Dr. Fulbright's laboratory, Mario Mandujano, R. Scott Shaw, Virginia Rinkel, Steven Berkheimer, and Christa Chatfield who made me feel right at home, and shared professional and personal moments. My gratitude and deep respect to Dr. Julia Bell, for her support not only in research but also for her words of advice when I needed a kind of mom's talks. Also, for trusting me, and sharing with me the bacterial canker project. Special thanks for the rides home every now and then! Also, my special appreciation to Nicole Werner, for sharing with me the bacterial canker project as well as *Cmm* strains, and for the hours in front of GelCompar,

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and John Rogers, for your indescribable help with statistics, and for your special attention in regards to my cultural background.

I would like to give special thanks to my Puertorican friends, Lycely Sepúlveda, Hector Ayala, and Marcos Morales, who always made me remember where I came from, and to be proud of my culture. My deepest gratitude goes to all of you, for being near me in good and bad times, and for being my family at MSU.

Also, I would like to acknowledge my family, specially to my Mom and Dad, who taught me to fight for what I believe in and want from life, and who teach me the right way of doing whatever your hands and mind are on. Furthermore, thank you for showing me, through your own life, that in this life you have to give up some things to become a better person. And, thanks for many years of a wonderful childhood and, for giving me the pleasure and joy to be a sister, to be a teacher, and to be a friend of Anamaris.

And last but not least, I will like to give thanks to my left hand, my partner, my family, my friend, my love, and my husband, Vladimir Ferrer, for being always there on your own special way, day by day, providing me with strength when my faith was limited. For believing in me every time I have doubts about my professional career, and give me the liberty to grow as an individual. Furthermore, for just being YOU.

Thank you; I would not have reached this goal without all of you.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Heterotrophic bacteria associated with plants can be classified as parasites and pathogens, when they invade and live in plant tissues; epiphytes, when they live on the epidermis of the living plant tissue; or, saprophytes, when they degrade dead plant tissue. Various combinations also exist, for example pathogens may exist as epiphytes before, or as saprophytes after invading and killing tissues. Bacterial species may also demonstrate a specialization in terms of the host species, as well as the type of host tissue colonized. Epiphytic populations of bacteria can be found on leaves, stems, or buds, and may serve as the source of primary inoculum of disease in epidemics; but, the actual pathogenic phase may be limited to specific tissues on the plant. It would appear that the epiphytic stage serves the bacterium best, in terms of growth, in that populations are maintained on a living host absorbing nutrients without inciting disease and harming its host (Tsiantos, 1987; Goto, 1992). The epiphytic stage allows for population increases but it may not provide the needed protection for dissemination or overwintering (Gitaitis, 1989; Atlas & Bartha, 1998). Once a pathogenic stage is achieved and death of the host tissue follows, the bacterial species must shift to the saprophytic stage, where bacteria must compete against well adapted bacterial and fungal species specializing in colonization of dead tissue and soil survival (Goto, 1992). Phytopathogenic bacterial species, those bacterial species that cause plant diseases, frequently produce identifiable symptoms resulting from the infection process. Bacteria that cause leaf blighting and spotting generally infect through stomatal openings, during physiological conditions that promote high

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epiphytic populations due to physical action of wind driven rain or irrigation (Agrios, 1997). Bacterial species that inducing wilt can invade xylem tissue through natural openings, such as lenticels, or through wounds or insect bites. Still, other bacterial species may cause galls and hypertrophies of stem and root tissue after colonizing wounds, while other species specialize in post harvest decay of plant tubers and fruits (Atlas & Bartha, 1998). Few species of phytopathogenic bacteria produce more than one type of symptom while colonizing their host, and generally, have a particular host range, that is, they are restricted in the species or variety of plant they can infect (Tsiantos, 1987; Goto, 1992). Those pathogens that do produce symptoms on various tissues and can survive as epiphytes and saprophytes have received special attention due to their ability to cause severe crop losses.

Xanthomonas campestris pv. *campestris*, causal agent of black rot of crucifers, *Burkholderia* (*Pseudomonas*) *solanacearum,* causal agent of wilt of various tropical or subtropical plants, *Erwinia amylovora*, causal agent of fire blight of apple and pear and *Clavibacter michiganensis*, are only a few examples of pathogens that have the ability to cause large crop losses and show special adaptations in their pathogenic, epiphytic and survival stages. *Xanthomonas campestris* pv. *campestris*, is known to overwinter as an epiphyte on wild host species that live through the winter at the edges of fields (Schaad, 1981; Jones, 1991). The ability of this pathogen to disseminate from the crucifer weed to crucifer crop species is well known, but not well understood. Once infected, the host produces various symptoms including leaf necrosis, xylem colonization leading to systemic infection and ultimately a rot or disintegration of the host plant (Jones, 1991;

Agrios, 1997). Alternatively, the pathogen can initiate the infection process through seed dispersal and seedling infection.

Burkholderia solanacearum infects a large number of unrelated host species including banana, solanaceous species, and various ornamental species (Goto, 1992). This pathogen is not only unique because of its ability to infect many species, but also in its ability to survive in tropical and subtropical soils. Due to these characteristics, it can, for example, cause serious disease on newly planted banana plantations (Woods, 1984). *Erwinia amylovora* is different than *B. solanacearum* in that it is restricted in its host range to closely related species in the Rosaceae family, including apple, pear and pyrocantha (Agrios, 1997). It overwinters in flower buds and can cause leaf and flower blight and stem cankers. This pathogen can be found infecting trees of established orchards as well as on grafted nursery stock in nurseries (McManus & Jones, 1994).

Clavibacter michiganensis infects many different hosts and causes different types of symptoms on these hosts. Currently five subspecies are recognized, including *C. michiganensis* subpecies *michiganensis*, *nebraskensis*, *sepedonicus*, *tessellarius*, and *insidiosum*, based on the analysis of total protein profiles, immunological and metabolic differences such as pigment production and carbon source utilization patterns (Davis et al., 1984). The subspecies are restricted in their host range, for example, in other pathogenic species, the variants infecting different host are often called pathovars or pathogenic variants (Schaads, 1987; Agrios, 1997). This nomenclature was not adopted for *Clavibacter* because the characteristics of the subspecies are greater than just the pathogenic variation.

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The *Clavibacter* subspecies that infects tomato, *C. michiganensis* subsp. *michiganensis*, represents an example of a phytopathogenic bacterial species that shows unique abilities to survive and produce a wide range of symptoms on its host, *Lycopersicum esculentum*, the commercial tomato. This subspecies of *C. michiganensis* only known to infect tomato, although there have been a couple of reports of infection of pepper plants (reviewed by Strider, 1969a). This pathogen is unique due to its ability to survive in soil (Strider, 1967; Gaititis, 1989) and on seeds (Thyr, 1969; Dhanvantari, 1989a & 1989b; Gitaitis, 1989), as well as the production of an array of symptoms on its host including leaf blight, vascular infection, wilt, cankers and fruit spots (Gitaitis, 1993). This bacterium is capable of overwintering for several years in plant debris in the northern Midwest and tomato producers are warned to stay out of contaminated fields for at least 3 years (Stephens & Fulbright, 1986; Gitaitis, 1989). The relationship of *C. michiganensis* subspecies *michiganensis* to other subspecies of *C. michiganensis* is unknown and the evolutionary linkage of *C. michiganensis* subsp. *sepedonicus* infecting the solanaceous potato plant and *C. michiganesis* infecting tomato remains an intriguing mystery.

Tomato is one of the most important vegetable crops grown worldwide due to the demand for the product and high cost of production (Gould, 1992). Bacterial diseases of tomato are feared because of the ability of the bacterial diseases to rapidly reduce yield or make tomato fruits unmarketable (or unprocessable), and the producer's inability to manage them with pesticides (Bryan, 1930; Strider, 1969a; Ricker & Riedel, 1993). Tomato is host to at least six common species of phytopathogenic bacteria including *Pseudomonas syringae* pv. *syringae*, *P. syringae* pv. *tomato*, *P. corrugata*, *X. campestris*

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pv. *vesciatoria*, *B. solanacearum* and *C. michiganensis* subsp. *michiganensis*. Of these species and pathovars, *X. campestris* pv. *vesicatoria* and *C. michiganensis* subsp. *michiganensis* represent the greatest threat to Midwest tomato producers and processors since both species can cause devastating epidemics when not diagnosed early in the production cycle (Phoronezny & Volin, 1983; Gleason et al., 1993).

Bacterial Canker of Tomato

Clavibacter michiganensis subsp. *michiganensis* was one of the first bacterial pathogens discovered on tomato in the early part of this century. It was first called Grand Rapids disease and later changed to bacterial canker. It is considered a devastating disease of worldwide importance because of its sporadic and unpredictable nature and due to the severity of the disease in certain years (Stephens & Fulbright, 1986; Ricker & Riedel, 1993). Since its identification by Erwin F. Smith in 1909 (Smith, 1910), many studies have been conducted on *C. michiganensis* subsp. *michiganensis* to help understand various aspects of the disease, including the genetics of the pathogen, the host and the environmental and horticultural conditions that promote epidemics. Of these, the genetics of the pathogen has received the least attention due to its apparently homogeneous nature and its cell wall composition; Gram positive phytopathological species have received must less attention than Gram negative species due to classical work performed on *Escherichia coli*.

As destructive as this pathogen can be, and as well adapted to living on and causing disease on all plant parts of the plant, this pathogen does not cause economic losses each year (Emmatty & John, 1973; Hausbeck et al., 1999). Perhaps, the reason

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why more research has not been done on this serious disease is its sudden disappearance after a two or three year epidemic (Gleason et al., 1993). The determination of the origin of the primary inoculum has always been of interest in attempts to understand the epidemiological aspect of bacterial canker (Dhanvantari, 1989b). In trying to predict disease outbreaks, research has focused on seed dispersal, southern-state transplant infection, and soil and farm equipment overwintering and contamination (Strider, 1967; Basu, 1970; Dhanvantari, 1989a; Chang et al., 1991; Chang et al., 1992b; Gitatitis et al., 1992; Gleason et al., 1993; Carlton et al., 1994).

The disease affects plants in a wide range of geographical areas where processing and fresh market tomato varieties are grown (Gitaitis, 1991). After Smith's initial report, many countries confirmed the presence of bacterial canker in their greenhouses and fields (reviewed by Strider, 1969). Many outbreaks have been reported in the United States (Stephens & Fulbright, 1986; Gitatitis, 1991; Gleason et al., 1993) and Canada (Basu, 1966; Dhanvantari, 1989a; Speranzini, 1995) as well as in Europe (López et al., 1987; Vaerenbergh & Chauveau, 1987). Depending on when symptoms are first observed in a field, it can reduce yields by 50-80% due to smaller, weakened and killed plants (Strider, 1969a; Chang et al., 1992c; Gleason et al., 1993). In addition, profit can be reduced due to fruit spots that appear as a result of the infection of fruit (Bryan, 1930; Gleason et al., 1993).

Symptoms

Bacterial canker of tomato can be associated with diverse symptoms (Appendix A). The symptoms are defined as marginal necrosis, unilateral wilt, curling of leaves and

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stem canker caused by the systemic infection of *C. michiganensis* subsp. *michiganensis*. Although bacterial canker symptoms are often described for mature tomato plants, cotyledons and seedlings can show white blister and water-soaked lesions along the young petioles, stems, and leaves (Basu, 1966; Strider, 1969b). Under laboratory conditions, Chang et al. (1992a) were able to observed wilt and canker development on wounded two-week old seedlings.

In a fully-grown tomato plant, wilt is frequently caused by the lack of water and nutrient transport to the leaves due to the presence of high numbers of *C. michiganensis* subsp. *michiganensis* located in the vascular system. It is thought that the production of exopolysaccharides by the bacterium plays a role in the wilting symptom (Goto, 1992). Wilt was one of the first symptoms recognized by Smith (1910) during the description of this bacterium. He was able to demonstrate that the organism causing wilt to the tomato plants in Grand Rapids was not the same organism causing wilt to solanaceous crops in southern states, recognized as *Pseudomonas solanacearum*. When *C. michiganensis* subsp. *michiganensis* is the infectious agent, wilt development occurs slowly from the lower to the upper leaves while plants infected with *P. solanacearum* suffer a sudden death of the entire plant since the wilting of leaves occurs relatively fast (Goto, 1992). Necrosis of the foliage can be a confusing symptom in bacterial canker infection due to this symptoms's resemblance to symptoms caused by other bacteria and fungal infections (Gitaitis, 1991) as well as leaf injuries caused by fungicide applications (Thompson et al., 1989). Although, "leaf firing" or marginal necrosis of the leaves may be confusing to the inexperienced, it can be a distinct symptom for bacterial canker diagnosis (Basu, 1966).

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The brown sunken areas, called cankers, along the stems and petioles are not always the most common symptom associated with infection by *C. michiganensis* subsp. *michiganensis*. The name bacterial canker was used since bacterial wilt had already been used for *Pseudomonas solanacearum* on tomato. Cankers result due primarily to the presence of the pathogen in the vascular system. The pathogen is known to produce strong enzymes and these enzymes are involved in tissue destruction. Cankers can often result in areas where a wound has been produced after a branch, leaf, or flower bract has been removed due to natural or accidental injuries (Gitaitis, 1991; Carlton et al., 1994). It is thought that the severity and incidence of the disease is more intense in staked tomatoes than in tomatoes left growing on the ground due to the injuries obtained during the lifting and tying. Nevertheless, wounds are not required for the development of canker (Bryan, 1930).

C. michiganensis subsp. *michiganensis* may also produce spotted fruit showing symptoms that usually appear as small necrotic spots surrounded by white-chlorotic halos, known as bird's-eye lesions. During early development of the fruit lesions, bird's-eye spots appear as small superficial round white lesions on green fruits (Bryan, 1930). If bird's eye lesions are observed in the field, it is highly indicative that infection with *C. michiganensis* subsp. *michiganensis* has occurred due to the unique appearance of this fruit lesion. However, a few reports (Bryan, 1930; Weebb et al., 1967; Gould, 1992) have suggested the resemblance of the white bird's-eye lesions with early fruit infection with *X.campestris* pv. *vesicatoria* but both lesions show differences in their development. The main difference between these two types of fruit infection is observed as the disease progresses. Bacterial canker bird's-eye lesions remain small and superficial with

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necrotic lesions forming in the center of the white spot while fruit spots caused by *X. campestris* pv. *vesicatoria* develop into large deep necrotic lesions lacking the chlorotic-halo characteristic of bird's eye lesions (Bryan, 1930).

Infection avenues

The systemic invasion of the host by phytopathogenic bacteria may result from the ingress of bacterial cells through artificial or natural openings in the host tissues (Appendix C). It has been reported that the entrance of xylem-inhabitant bacteria, including *C. michiganensis* subsp. *michiganensis*, is solely dependent on the presence of wounds on target the host tissue (Pine et al., 1955) since no association with insects were known that could transfer bacterial cells directly to the host vascular system (Ark, 1944). In various laboratories, successful infection with *C. michiganensis* subsp. *michiganensis* was accomplished when tomato stems and leaves were mechanically damaged prior the application of bacteria (Bryan, 1930; Grogan & Kendrick, 1953; Thyr, 1968; Kontaxis, 1962; Layne, 1967; Farley, 1971). The theory that *C. michiganensis* subsp. *michiganensis* was a wound-dependent pathogen prevailed until the studies conducted by Kontaxis (1962) and Layne (1967). Independently, both studies provided evidence that trichomes on tomato leaves could serve as infection sites for *C. michiganensis* subsp. *michigenensis*.

Reports have confirmed the importance of natural openings such as stomata, hydathodes, and trichome-base holes on leaves and fruits for infection of phytopathogenic bacteria (Bashan et al., 1981; Getz et al 1983; Erhrig & Griesbach, 1985; Blanke 1986; Fankle et al., 1993; Carlton et al., 1998).

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Disease cycle—Source of inoculum

In North America, the appearance of the bacterial canker disease is primarily associated with the tomato production cycle (Appendix B). In general, the tomato production in the Midwest begins when a grower obtains seed from a seed-producing company or transplants from a greenhouse grower who had planted seeds. Transplants for field production are grown in a greenhouse for approximately two months. Generally during field production, bacterial canker symptoms are observed from the mid-growing season and later, however, symptoms may be observed in the greenhouse on transplants (Hausbeck et al., 1995b). At least three sources may be considered responsible for the origin of the primary inoculum in bacterial canker epidemics (Appendix C).

First, infected seed could be a source of the bacterial canker pathogen even after being screened by seed-certification programs for various bacterial pathogens, which includes *C. michiganensis* subsp. *michiganensis* (Grogan & Kendrick, 1953; Dhanvantari, 1989a; Chang et al., 1991; Parker et al., 1995). The acceptance *C. michiganensis* subsp. *michiganensis* as a seed-borne pathogen has been controversial for years because few studies have been conducted to establish the location on the seed where the pathogen survives (Bryan, 1930). Gitaitis et al. (1991) reported that one infected seed in 10,000 is capable of establishing 74-124 disease foci/hectare, resulting in a loss of yield and value. As Van Vaerenbergh & Chauveau (1987) stated "the production of tomato seed free from the pathogens is of great economic importance for both the growers and the seed industry." Although, *C. michiganensis* subsp. *michiganensis* is accepted as a seed-borne pathogen, seed-producing companies deny that they are responsible for the observed epidemics.

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A second possible source of primary inoculum is the transplants used to establish production fields. Transplants could become infected through contaminated seed or through contaminated greenhouse equipment and structures. Various studies have suggested that symptomless transplants could be the source of bacterial canker (Gitaitis et al., 1991; Chang et al., 1992a; Gleason et al., 1993; Hausbeck et al., 1995a & 1995b; Bell et al., 1996; Haubeck et al., 1999) introduction into the field environment. Hausbeck et al. (1999) suggested that symptomless transplants can harbor high population (10^6-10^7 colony forming units/gram of tissue) of *C. michiganensis* subsp. *michiganensis*, and serve as the foci for infection and diseased plants in the field. Further studies must be conducted to confirm this theory.

The third most probable way that bacterial canker epidemics are initiated is from tomato debris left from the previous years infected plants, since the pathogen has be found to overwinter in infested tomato debris for 2-3 years (Gorgan & Kendrick, 1953; Strider, 1967; Basu, 1970; Gleason et al., 1991; Chang et al., 1992b). Many growers recognize this as a potential source of infection, and rotate accordingly.

Regardless of the primary source of inoculum, various aspects of the bacterial canker disease cycle concern tomato growers such as dissemination and spread of the pathogen (Appendix C) and management strategies to control bacterial canker (Appendix D).

Dissemination of *C. michiganensis* subsp. *michiganensis*

Use of certified seed and transplants from certified seed, as well as the removal of tomato debris has not stopped the dissemination in the Midwest of *C. michiganensis* subsp. *michiganensis.* Regardless of the source of primary inoculum seed, infected

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transplant or tomato debris, the pathogen most likely spreads as an epiphyte, slowing building its population until a threshold is reached (Gleason et al., 1991; Chang et al., 1992b). *C. michiganensis* subsp. *michiganensis* can spread from diseased to healthy plants in the same field as a result of natural events such as rainsplash or overhead irrigation (Ark, 1944; Strider, 1967; Strider, 1969a; Tsiantos, 1986; Gleason et al., 1993). Water droplets can carry a large number of bacterial cells that can survive as an epiphytic population on the host tissue. As favorable environmental conditions emerge, the cells of the epiphytic population can multiply, and if infection avenues are available in the host, invasion of susceptible sites could result in systemic infection (Stephens & Fulbright, 1986; Carlton et al., 1998).

Cultural practices such as pruning, staking, fumigation, and or any other type of human contact with diseased and healthy plants during any stage of tomato production may also serve as a mechanism for spread (Strider, 1967; Chang et al., 1991; Chang et al., 1992b; Carlton et al., 1994). Gitaitis et al. (1991) provided evidence that commercial cultural practices such as clipping the top of systemically infected transplants to control plant height could result in the dissemination of the pathogen throughout healthy transplant fields.

The dissemination of *C. michiganensis* subsp. *michiganensis* from one year to another year could also occur through the epiphytic population of various weed species such as nightshade, horsenettle, and jimsonweed (reviewed by Strider, 1969a; Ricker, 1989; Chang et al., 1992b). It has been reported that these weed species serve as alternative hosts of high population numbers of the pathogen.

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Management Strategies

Clearly, numerous sources of primary inoculum and as well as opportunities for the dissemination of the inoculum exists in the tomato production cycle, thus various management strategies have been integrated to reduce the spread of the pathogen. These strategies can be accomplished through certification programs such as pathogen-free seed and pathogen-free transplants. Other practices as well can be instituted dependent on grower cultural practices and sanitation.

The primary control strategy for seed-borne pathogens like *C. michiganensis* subsp. *michiganensis* is based on the use of pathogen-free, certified seed. The production of non-infected seeds is dependent on the use of fruit from uninfected plants, and treatment of seed with disinfectants to eliminate any bacterial cells present. Seed treatments have included soaking the seeds in disinfectants including bleach, alcohol, and chemicals such as and hydrochloric or acetic acids which have been shown to efficiently eradicate the bacteria from the seed coat and seed-hairs (Bryan, 1930; Ark, 1944; reviewed by Strider, 1969a; Weebb et al., 1967; Dhanvantari, 1989; Gleason et al., 1995; Ricker, 1995). Another seed treatment process frequently used is the fermentation of the tomato pulp before seed extraction, although this is not as efficient as the chemical treatments since it reduces the number of bacterial cells but does not eradicate the pathogen.

The use of certified transplants and cuttings, is another method to prevent the onset of an epidemic in a disease-free location (Strider, 1969a) primarily if symptomless transplants and cuttings are sampled for the presence of C. *michiganensis* subsp. *michiganensis* prior to distribution (Gitaitis et al, 1991). Both of these strategies are

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based on the sensitivity of detection and identification protocols. Fortunately, highly sensitive techniques are now available to detect low number of phytopathogenic bacteria.

Since the bacterial canker pathogen can persist in soil where tomato debris is present, crop and field rotations of 2-5 years have been routinely recommended (Bryan, 1930; Strider, 1969a; Gleason et al., 1993). Also, reducing the potential of alternate hosts like nightshade and jimsonweed from both the greenhouse and fields will decrease the potential of weeds as the source of inoculum (Ricker, 1989; Chan et al., 1992b). Other suggestions have been reported such as the use of fumigation or steam sterilization of greenhouse soils and fall plowing of fields (Weebb et al., 1967). Each of these techniques has questionable environmental aspects as well as added costs.

A thorough sanitation program consisting of cleaning equipment used during tomato production such as transplant trays, pruning tools, and stakes has been strongly recommended as a management strategy to reduce bacterial canker outbreaks. Sanitation can be achieved with the use of disinfectants such as chloride, bromide, or formalin (Gitaitis et al., 1992). Since the pathogen can be disseminated through splashing water, uncontrolled and excessive periods of overhead irrigation should be minimized.

Perhaps the biggest breakthrough in management schemes is the preventive application of copper-based bactericides to transplants while in the greenhouse. The control provided by bactericides is accomplished by reducing the number of bacterial cells which will delay the onset of bacterial canker, thus an increase in yield and high quality fruits can be achieved (Hausbeck et al., 1997; Hausbeck et al., 1999). The application of streptomycin provides an effective control to *C. michiganensis* subsp. *michiganensis*, unfortunately, the application of compounds with this antibiotic agent is

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prohibited in the greenhouse and in the field in some regions of the country (Hausbeck et al., 1995a).

Another mechanism of control is the use of varieties resistant to *C. michiganensis* subsp. *michiganensis*. Some companies have reported the production of breeding lines and varieties with tolerance or moderate resistance to bacterial canker (Ricker et al., 1997). The resistance obtained by some of the tomato lines is based on characters such as the delay of onset of symptoms, low incidence of bacterial canker symptoms, and a decrease in severity of specific bacterial canker symptoms. Since resistance is based on the genetic composition of the pathogen and the host, studies involving the characterization of various genetic aspects will provide knowledge to improve the development of fully resistant tomato lines.

The use of biological agents to control bacterial canker has not been reported and however, earlier descriptions and characterization of naturally avirulent strains and their ability to produce bacteriocins still seems promising (Echandi, 1976). The biological agents could provide control through various mechanisms such that the population of virulent strains is reduced to a safe level, or completely inhibited.

Characterization and Identification

The identification of phytopathogenic bacteria is based on Koch's postulates which include the isolation of a pure culture of the presumptive pathogenic organism, the induction and evaluation of symptoms caused by this organism, and the reisolation of the same organism from infected tissue (Goto, 1992; Agrios, 1997). If the isolation and growth on culture media of the organism is feasible, and if pathogenicity tests and the

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expression of symptoms can be easily obtained under laboratory conditions, the reisolation of the pathogenic bacteria could be a challenge. For example, distinguishing *C. michiganensis* subsp. *michiganensis* from saprophytic bacteria found on diseased tissue can be problematic if only colony morphology is used due to the similarity in color and texture of the saprophytes and the pathogen (Gitaitis & Beaver, 1990). The use of amended culture media with nalidixic acid has been a successful approach because it inhibits the growth of a significant amount of Gram-negative bacteria which enhances the screening process (Fatmi & Schaad, 1983). Fatmi & Schaad (1983) developed a semi-selective culture media (SCM) which has facilitated the isolation and recovery of *C. michiganensis* subsp. *michiganensis* from seed extracts even when low number of bacterial cells are present. SCM has presented an advantage over general media because *C. michiganensis* subsp. *michiganensis* colonies develop a specific coloration and texture on culture plates.

The identification of *C. michiganensis* subsp. *michiganensis* and diagnosis of bacterial canker can be a problem when small populations of bacteria are living epiphytically and only invading the host from time to time (Dreier et al., 1995). The early recognition and confirmation of the presence of the pathogen are critical in preventing and slowing bacterial canker epidemics. Therefore, there is a need for fast, accurate and reliable detection techniques especially during the tomato seedling stage where symptoms are rarely present (Gleason et al., 1993). Symptomless seedlings may harbor large population numbers of *C. michiganensis* subsp. *michiganensis*, which can cause significant yield reduction once the plants have been transferred to the field (Hausbeck et al., 1995b).

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As the number of samples increase due to the sampling and screening techniques, the development of detection techniques based on the production of antibodies against specific pathogenic bacteria have been employed to enhance the rate and accuracy of identification and enumeration (de Boer & Wieczorek, 1984; van Vuurde, 1987; Baer & Gudmestad, 1993; Drennan et al., 1993). The success and efficiency of immunology-based techniques are often firmly established on the use of highly specific antibodies targeted to unique bacterial cell wall components. The production of monoclonal antibodies has an advantage over polyclonal antibodies established by the reduction of false-positive results (de Boer & Wieczorek, 1984). As of today, a large number of specific monoclonal antibodies have been produced for the detection of phytopathogenic bacteria that retain a zero tolerance status. For instance, various studies (de Boer & Wieczorek, 1984; Gudmestad et al., 1991; de Boer & Gudmestad, 1993; de Boer et al., 1994; de Boer & Hall, 1996) have successfully achieved the production of highly specific monoclonal antibodies against the potato pathogen, *C. michiganensis* subsp. *sepedonicus*, a pathogen typically given "zero tolerance" status where any plants observed with this pathogen are destroyed.

Although *C. michiganensis* subsp. *michiganensis* is not considered a pathogen with "zero tolerance," antibodies have been produced to accurately detect the pathogen in early stages of tomato production. Screening early could possibly prevent the onset of a bacterial canker epidemic. Immunology-based techniques such as enzyme linked immunology assay (ELISA), and immunoisolation are commonly used for detection of *C. michiganensis* subsp. *michiganensis*. Stephens et al. (1988) evaluated the detection level of ELISA wells pre-coated with antiserum against *C. michiganensis* subsp.

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michiganensis. They showed that the lower level of detection was $1x10^3$colony forming units/ELISA well. Gitatits et al. (1991) analyzed seedlings for the bacterial canker pathogen using ELISA plates consisting of antiserum against *C. michiganensis* subsp. *michiganensis* and they were able to successfully detect *C. michiganensis* subsp. *michiganensis* in symptomless seedlings. However, from this assay bacterial cultures cannot be obtained to confirm the presence of the pathogen or its pathogenic nature. Gharbi & Nameth (1992) developed a monoclonal antibody specific for the detection of *C. michiganensis* subsp. *michiganensis*. They were able to detect the bacterial canker pathogen from symptomless tomato plants through ELISA and dot immuno-blots.

Immunoisolation protocols can overcome the limitations of ELISA because the technique combines the use of specific antibodies with the culture of presumptive pathogenic cells in solid culture media. Franken et al. (1993) evaluated the potential of immunoflourescence (IF) and subsequent plating on semiselective media to detect *C. michiganensis* subsp. *michiganensis* from tomato seeds. They accurately identify the bacterial canker pathogen after pathogenicity assays were conducted for the bacterial colonies obtained from positive seed lots. The limitation of this technique is the relatively long time necessary to conduct pathogenicity tests for the confirmation of false positive results. Although the efficiency and specificity between detection methods is routinely under evaluation, immunology-based techniques have the potential to detect naturally avirulent *C. michiganensis* subsp. *michiganensis* isolates (van Vaerenburgh & Chauveau, 1987).

As the number of samples to be tested increases, various techniques have been developed to reduce the time necessary for characterization and detection of *C.*

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michiganensis subsp. *michiganensis*. For example, fatty acid methyl ester (FAME) analysis and microplates (e.g. Biolog) based on the cell wall constituents and biochemical characteristics, respectively, have accelerated the detection process but have limited potential for characterization at the subspecies level. FAME uses fatty-acid composition, as determined by gas chromatography to help differentiate unknown isolates of bacteria. Gitaitis & Beaver (1990) constructed a library of FAME profiles of *C. michiganensis* subsp. *michiganensis*. They found that the ratio between specific fatty acids was distinct for species that showed similar colony morphologies which were recovered from various host tissues. In comparison to semiseletive media and ELISA, FAME analysis was less sensitive and more difficult to use for detecting latent, symptomless infections of C. *michiganensis* subsp. *michiganensis* in tomato seedlings (Gitaitis et al., 1991).

Biolog is an identification system based on a database containing the results of biochemical reactions obtained for each species tested with the system. The identification process is dependent on microplates where its wells are coated with reagents that will simultaneously perform a number of microbiological tests. Using Biolog GN microplates designed for Gram-negative species, Jones et al. (1993) were able to successfully identify strains of the Gram-positive genus *Clavibacter* from Gram-negative phytopathogenic genera, *Agrobacterium, Erwinia, Pseudomonas*, and *Xanthomonas*. The introduction of plates designed specifically for Gram-positive bacteria, including *C. michiganensis* subspecies has improved the accuracy of this assay for the pathogenic coryneform species, although certain carbon sources must be included in the system to improve the identification at the subspecies level (Harris-Baldwin &

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Gudmestad, 1996). Clearly even more sensitive and specific assays for identification and characterization are needed.

Since the acceptance of molecular biology, DNA hybridization and polymerase chain reaction (PCR) techniques have revolutionized cell detection techniques making it more reliable, specific and less-time consuming (Vandamme et al., 1996). PCR based techniques are ten times more sensitive than microplate identification and immunology-based procedures (Hu et al., 1995). Many reports on the use of DNA probes and PCR primers have demonstrated the efficiency and success in the detection of important phytopathogenic species such as *Erwinia caratovora* (de Boer & Ward, 1995), *E. amylovora* (McManus & Jones, 1995) *C. michiganensis* subsp. *sepedonicus* (Verrault et al., 1988; Johansen et al., 1989; Drennan et al., 1993; Rademaker & Janse, 1994), *C. michiganensis* subsp. *michiganensis* (Thompson et al., 1989; Rademaker & Janse, 1994), and *C. xyli* subsp. *xyli* (Pan et al., 1998).

For example, Verrault et al. (1988) were able to detect up to 1 nanogram of the DNA of *C. michiganensis* subsp. *sepedonicus* after the development of specific probes. Later, Thompson et al. (1989) constructed a chromosomal library from stains of *C. michiganensis* subsp. *michiganensis*. After the digestion of clones with endonucleases, they obtained a 5 kilobase pair (kbp) fragment unique to this pathogenic subspecies. Using the 5 kb fragment as a probe, they were able to successfully detect virulent and avirulent strains of the bacterial canker pathogen. Rademarker & Janse (1994) used commercially available probes, MIC 1 and Diagen, to detect *C. michiganensis* subsp. *michiganensis* and *C. michiganensis* subsp. *sepedonicus*, respectively. To confirm the specificity of the two probes, they derived PCR primers to amplify chromosomal

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fragment of the two *C. michiganensis* subspecies. After treating the PCR products with endonuclesases, the restriction analysis of the amplified fragments supported the specificity of the probes developed since other *C. michiganensis* subspecies were not detected with this method. Recently, Sousa-Santos et al. (1997) developed two PCR primers, CM3 and CM4, that confirmed the presence of *C. michiganensis* subsp. *michiganensis* isolated from tomato seed extracts. A minimum of 40 cells/ ml were detected with CM3 and CM4 probes, and no positive results were observed with the saprophytic bacteria tested.

The use of other than entire chromosomal sequences such as intergenic spacer sequences between ribosomal genes, and specific sequences on the 16S ribosomal gene (Mirza et al., 1993; Mills et al., 1997; Daffonchio et al., 1998) have provided a higher level of specificity to the identification process. Besides the advantages offered by new identification protocols for identifying subspecies of C. *michiganensis*, some of these techniques have increased the amount of information to conduct epidemiological studies (Mirza et al., 1993; Rademaker & Janse; 1994; Rivera et al., 1995; Liu et al., 1995; Colombo et al., 1997) and formulate novel management strategies (Pan et al., 1998).

The use of DNA-hybridization in combination with PCR protocols has also increased our knowledge of pathogenicity characters. For instance, Dreier et al. (1995) were able to distinguish pathogenicity genes on *C. michiganensis* subsp. *michiganensis*. They described a pathogenicity gene (*pat1*) with unknown function and an endocellulase gene (*celA*). Both genes are found in *C. michiganensis* subsp. *michiganensis* isolates on two plasmids, pCM1 and pCM2, respectively. After digestion of total DNA from *C. michiganensis* subspecies and subsequent treatment with endonucleases, the DNA was

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probed with celA and an RFLP pattern was obtained for *C. michiganensis* subsp. *michiganensis*. A 3.2 kbp fragment was obtained specific to *C. michiganensis* subsp. *michiganensis* since the fragment was lacking in other *C. michiganensis* subspecies tested. The probe patlwas shown to be specific for virulent strains of *C. michiganensis* subsp. *michiganensis* as none of the laboratory-generated avirulent strains tested showed a hybridization signal for this fragment. They also developed a set of PCR primers, CMM-5 and CMM-6, derived from the patl gene to test their ability to identify virulent bacteria from infected plant extracts and seeds harvested from infected tomato plants. They were able to detect virulent isolates from 50 seeds containing 1,000 bacterial cells. They obtained negative results from healthy plants and plants infected with an avirulent strain. The detection limit of the suggested PCR protocol was of 200 bacterial cells/ml of plant extract without prior isolation and enrichment of the pathogen. The only limitation observed in this technique was the use of plants infected artificially in the laboratory with a known concentration of the pathogen and not from naturally infected plants that may contain a higher population of saprophytes that serve as PCR inhibitors decreasing the detection limit. Once more, this study proved the potential of PCR-based assays to provide speed, sensitivity, and specificity that will reinforce the results from standard detection methods.

The development of DNA probes require previous knowledge of DNA sequence, a task that can be time-consuming as well as less attainable due to its high cost. An alternative approach to avoid the extra time and costs is the use of universal PCR primers to generate DNA fragments unique to bacterial species. DNA-fingerprinting, as this type of gene amplification protocol is generally termed, has been intensively applied in studies

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dealing with topics such as genomic diversity and variability (Rivera et al., 1995; Rodríguez-Barradas et at., 1995; Balerias Couto et al., 1996; van Belkum et al., 1996; Appuhamy et al., 1997; Murry et al., 1997; Sechi et al., 1998; Vásquez-Arroyo et al., 1998; Jersek et al., 1999), and taxonomy (Balkwill et al., 1997; Vinuesa et al., 1998). DNA-fingerprinting techniques have been applied to a broad number of genera of microorganisms from fungi to bacteria (Weising et al., 1991; de Bruijn, 1992; Weising et al., 1995; Louws et al., 1996; Thanos et al., 1996), and plant pathogenic bacteria have not been an exception (McManus & Jones, 1995, Louws et al., 1994; Louws et al., 1995; Smith et al., 1995; Opgenorth et al., 1996; Pooler et al., 1996; Vera Cruz et al., 1996; Weingart & Völksch, 1997; Louws et al., 1998; Rademaker & de Bruijn, 1997; Rademaker et al., 1997; Jaunet & Wang, 1999; Rademaker et al., 1999a).

DNA fingerprinting

The repetitive sequence-based PCR (rep-PCR) is one of the two DNA fingerprinting techniques used to define genetic characters and polymorphic regions of prokaryotic and eukaryotic genomes (Louws et al., 1996; Schneider & de Bruijn, 1996). The fingerprints generated from rep-PCR technology originated from naturally occurring repetitive sequences that are randomly arranged in high copy number on the circular bacterial genomes (de Bruijn, 1992; Schneider & de Bruijn, 1996; Vera Cruz et al., 1996; Versalovic et al., 1997). Three groups of repetitive sequences have been studied in detail and have been used to generate primers for the amplification of DNA through PCR in various bacterial species (Versalovic et al., 1991). The three primers used for the production of rep-PCR genomic fingerprints consist of 18-22 base pair oligonucleotide

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sequences recognized via DNA hybridization studies (Versalovic et al., 1994). They include the repetitive extragenic palindromic (REP) sequence, the enterobacterial repetitive intergenic consensus (ERIC) sequence, and the BOX element (Louws et al., 1996; Schneider & de Bruijn, 1996; Versalovic et al., 1997). These primers were developed after their characterization in the Gram-negative enteric bacteria, *Escherichia coli* and *Salmonella typhimurium* for the REP and ERIC primers, respectively. The combination of boxA, boxB, and boxC elements was first characterized in the Gram-positive bacterium, *Streptococcus pneumonia* and an unrelated Gram-negative species used in characterizing the BOX primer (Versalovic et al., 1994; Koueth et al., 1995).

A different group of primers can be generated from the minisatellite sequences, which consist of 10-60 base pairs arranged in a head to tail fashion with two to several thousand motifs, most likely containing non-coding DNA (revised by Weising et al., 1995). The polytrinucleotides, $(GTG)_5$ and $(GCC)_5$ are examples of primers complementary to minisatellites (Versalovic et al., 1994). The precise function of the repetitive and minisatellite sequences is unknown, but it has been postulated to be important in chromosomal organization, DNA replication and prevention of DNA degradation, as well as in regulation of gene expression (de Bruijn, 1992; Weising et al., 1995). Meyer et al. (1993) demonstrated the usefulness of $(GTG)_5$ as rep-PCR primers for the differentiation of eukaryotic species. Some of the first reports where $(GTG)_5$ was used to primer rep-PCR were realized with human pathogenic species such as the fungus *Cryptococcus neoformans* (Meyer et al., 1993; Meyer & Mitchell., 1995). Various scientists showed that minisatellite sequences are randomly spread through the entire fungal and prokaryotic genomes (Meyer et al., 1993; Meyer & Mitchell, 1995; Weising et

al., 1995; Warren et al., 1996). These studies were important in generating information related to origin and geographic distribution of various strains; data necessary for epidemiological studies. A recent report has demonstrated the usefulness of $(GTG)_5$-PCR genomic fingerprinting for assaying genetic variability in various fungal species (Balerias Couto et al., 1996).

As in medical pathology, PCR-based techniques have revolutionized the detection and differentiation of pathogens in plant pathology (Roberts et al., 1996; Sadowsky et al., 1996; Lee et al., 1997a & 1997b; Rademaker & de Bruijn, 1997; Sousa Santos et al., 1997; Rademarker et al., 1999a & 199b). Specifically, rep-PCR genomic fingerprinting has provided useful information for the reevaluation of taxonomic names and evolutionary relationships within phytopathogenic bacterial species. For example, Louws et al. (1994 & 1995) were able to redefine phylogenetic groups and effectively differentiate phytopathogenic strains within the pseudomonad and xanthomonad species that can cause symptoms on a wide variety of hosts. More recently, Louws et al. (1998) were able to rapidly and effectively differentiate five *Clavibacter michiganensis* subspecies using rep-PCR. The *C. michiganensis* subspecies analyzed are among characterized by their virulence on different hosts. They also reported a higher level of genetic resolution at the subspecies level when a wide collection of strains of *C. michiganensis* subsp. *michiganensis* was analyzed with BOX-PCR. They defined four BOX-PCR (A, B, C, and D) types based on polymorphic DNA bands after agarose-based electrophoresis. During the completion of Louws et al. (1998) manuscript a new BOX-PCR genotype was described as type E (Bell et al., 1997). Although the biological significance of the BOX-PCR types was not established, genetically typing the strains

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provided useful groupings. For example, almost all of the *C. michiganensis* subsp. *michiganensis* strains that are asymptomatic on tomato (avirulent strains) belong to BOX-PCR type A, while highly virulent strains belong to BOX-PCR type C.

Taxonomy

The assignment of genus and species to bacterial isolates has been primarily based on morphological, physiological and biochemical characteristics. The taxonomy of the bacterial canker pathogen has been controversial since its discovery (Smith, 1910) due to limited physiological and biochemical tests. The bacterial canker pathogen was first named *Bacterium michiganense* due to the similarities in colony morphology with *Bacterium campestre* and *B. solanacearum*. *Bacterium michiganense* colonies were described as pale yellow with smooth round surface and edges, and with a wet shinny appearance. Bacterial cells were described as short rods (0.35-0.4 x 0.8-1.0 microns in diameter). This pathogen was recognized as a Gram-positive bacterium, non-spore forming, and slow growing on solid media even at optimum temperatures (25°C) Biochemical tests such as reduction of nitrogen source (nitrate and nitrite), and starch and gelatin degradation were also analyzed. Later, *B. michiganense* was named *Aplanobacter michiganense* due to the absence of flagella (non-motile) and morphological similarities with the potato pathogen, *Aplanobacter rathayi*. Thereafter, additional attempts at renaming the tomato pathogen included *Pseudomonas michiganensis*, *Phytomonas michiganensis*, *Erwinia michiganensis*, *Mycobacterium michiganense*, and *Corynebacterium michiganense* (as indicated by Strider, 1969a). The latter nomenclature remained until recent molecular-based analyses were included as taxonomic criteria.

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Based on classical taxonomy, the genus *Corynebacterium* consisted of coryneform bacteria regardless of their host specificity. Morphological, biochemical, or physiological characters used to discriminate between plant pathogenic and animal/human pathogenic bacteria were lacking until Yomada & Kogamata (1972a & 1972b) conducted a numerical analysis. In the numerical analysis, they used the type of cell division, bacterial cell wall composition, and guanine plus cytosine (G+C) DNA content as the primary characters for the differentiation of coryneform bacteria. They analyzed 112 strains from the designated genera *Corynebacterium, Microbacterium, Cellulomonas, Arthrobacter,* and *Brevibacterium.* They were able to differentiate the strains into seven groups where the main distinction was the presence or absence of diaminopimelic acid (DAP). As a result, strains without DAP were assigned to a single group. This single group consisted of two plant pathogenic species, *C. michiganense* and *C. insidiosum.* These species contained diaminobutyric acid (DAB) as a principal cell wall amino acid, a bending type cell division, and a GC DNA content range from 69-78%. As a result plant pathogenic species were recognized to be different from the animal/human pathogenic species.

After recognizing the differences between plant and animal pathogenic coryneform bacteria, the next challenge to be accomplished was the differentiation among phytopathogenic species. Starr et al. (1975) used DNA-DNA homology and GC content to differentiate seven *Corynebacterium* species. Since the pathology of the species was highly specific, they suspected that DNA will provide useful information to genetically group the seven species. Indeed, they were able to established two groups; one, consisting of *Corynebacterium michiganense*, *C. insidiosum*, and *C. sepedonicum*

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strains, and the second of *C. poinsettiae, C. betae, C. flaccumfaciens*, and *C.ilicis*. They observed a low level of DNA-homology (15%) between the species in each group, and low genetic variability between species in the same group. Later, Döpfer et al (1982) confirmed the homology between *C. michiganense, C. insidiosum*, and *C. sepedonicum* by the determination of the type of peptidoglycan layer and DNA homology. Their results indicated a close relatedness *between C. michiganensis* and *C. insidiosum*, and a degree of similarity between *C. sepedonicum, C.michiganense* and the corn pathogen *C. nebraskense*. However, they suggested that the presence of type B2γ peptidoglycan layer is strong evidence to consider the strains as a single species. Therefore, they suggested that these strains should not be considered subspecies.

As the controversy continued, techniques other than DNA homology were used to differentiate closely related *Corynebacterium* species. Carlson & Vidaver (1982) used polyacrylamide gel electrophoresis (PAGE) of cellular proteins of 13 *Corynebaterium* species including *C. michiganense, C. insidiosum, C. nebraskensis*, and a new wheat pathogen named as *C. tessellarius*. They concluded that small differences in PAGE-protein profile dissimilarity observed between the species showed these strains to be subspecies of *C. michiganense*. Thus, they proposed the recognition of the strains as *C. michiganense* subspecies *michiganense*, subspecies *insidiosum*, subspecies *nebraskense*, and subspecies *tessellarius*. Later, Davis et al. (1984) combined morphological, biochemical, cell wall amino acid and sugar characteristics, GC-DNA content, and PAGE-protein profile results to corroborate subspecies subscription on strains of *C. michiganense*. Recently before Davis et al (1984) study concluded, a xylem inhabitant fastidious organism with similar morphological, biochemical and physiological

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characteristics was isolated and described (Davis et al., 1980). The bacterium isolated from stunted sugarcane plants was included in the genus *Corynebacterium*, and named as *C. xyli* due to the morphological, biochemical, and physiological similarities with the *Corynebacterium* phytopathogenic bacteria. Davis et al. (1984) proposed the reclassification of all the *Corynebacterium michiganense* subspecies to be included in the genus *Clavibacter*, and *C. xyli* and bermudagrass pathogenic coryneform bacteria as a subspecies of *Clavibacter xyli*.

Although the genus *Clavibacter* has been redefined, various studies have suggested the use of the subspecies level within the species *C. michiganensis* (Henningson & Gumestad, 1991; de Bryne et al., 1992; Kampfer et al., 1993). For example, Hennington & Gumestad (1991) subdivided phytopathogenic coryneform strains representing the genera *Arthrobacter, Clavibacter, Curtobacterium*, and *Rhodococcus* based on the analysis of fatty acids by gas chromatography. The analysis of the FAME profiles suggested the division of four genera without the taxonomic classification at the subspecies level. Kampfer et al. (1993) conducted a numerical classification study based on 280 physiological characters. They observed a high similarity between the genera *Clavibacter* and *Curtobacterium*, and a close relationship of these genera to *Microbacterium*. Individually, these studies suggested that *C. michiganensis* should be returned to a full species status.

As of today, *Clavibacter michiganensis* subspecies status prevails since the studies suggesting the full species status lacked the analysis of more than one data source which helps on the resolution of the small differences between the subspecies. The pathovar designation for *Clavibacter michiganensis* has been avoided since genetic

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differences have been reported besides their host specificity. Therefore, *Clavibacter michiganensis* subspecies has been circumscribed to plant pathogenic xylem-inhabitant bacteria with the following distinctive characters, pleomorphic gram-positive rods which are often arranged in V or L formation, obligately aerobic, DAP as the major component in their cell wall, MK-9 as the main respiratory molecule, with anteiso- and iso-methyl branched fatty acids, and about 70 % CG DNA content (Collins & Bradbury, 1991). Evidently, *Clavibacter* is still the most clearly taxonomically defined genus of the plant pathogenic bacteria.

Objectives

In spite of the fact that many studies have provided answers to the differentiation and characterization of the bacterial canker pathogen, *C. michiganensis* subsp. *michiganensis,* many questions regarding the epidemiological basis of the pathogen still exists. Defining genetic variability should help us understand more about the role of the strains in the host/pathogen relation and may provide us with powerful tools in examining the epidemiological picture of this disease. To this end, I have focused on two objectives:

1) To determine if another rep-PCR universal primer, $(GTG)_5$, supports the subdivision of *C. michiganensis* subsp. *michiganensis* into the five genotypes established with BOX-PCR; and, if so,

2) Determine if the measurable diversity allows for a better understanding of the disease and the origin of the strains within the epidemic.

Although the application of copper-base chemicals in the greenhouse have provided management strategies to reduce yield loss caused by bacterial canker pathogen

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infection, these control strategies do not appear to provide control for the occurrence of fruit spotting in the field. It is reasonable to suggest that in order to formulate novel management strategies to control bacterial canker at the fruit spot level, a better understanding of *C. michiganensis* subsp. *michiganensis* infection and bird's-eye lesions development is required. Therefore, two objectives were set to help me determine the role of the pathogen in causing fruit spots:

1) To determine if bird's eye lesions are the result of external flower infection; and,

2) If it is, determine the flower stage which provides the maximum number of fruit spots.

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CHAPTER 2

GENETIC VARIATION IN STRAINS OF *CLAVIBACTER MICHIGANENSIS* SUBSPECIES *MICHIGANENSIS*

The genetic characterization of the bacterium *Clavibacter michiganensis* subspecies *michiganensis*, causal agent of bacterial canker on tomatoes, is limited and until recently (Louws et al., 1998) no consistent genetic variability has been noted. Identification and detection of the pathogen at various times of tomato production is critical for development of control strategies and similarly assessment of the genetic diversity of the pathogen in the field is necessary for epidemiological studies as well as host/pathogen studies. Identification and assessment of diversity is the cornerstone of taxonomy. Yet, over the years classification of *Clavibacter michiganensis* subspecies *michiganensis* has been accomplished through physiological, chemical, serological, and pathogenic analyses, which have proved controversial due to the high degree of genetic similarity between the *Clavibacter michiganensis* subspecies (Yamada & Komagata, 1972a & 1972b; Carlson & Vidaver, 1982; Davis et al., 1984; Lee et al., 1997a & 1997b) and the lack of diversity among strains of *C. michiganensis* subsp. *michiganensis*. The use of protein measurable and DNA-based techniques such as protein profiles, restriction fragment length polymorphism (RFLP), and polymerase chain reaction (PCR) technology have provided needed insight and understanding of the genetic character of this phytopathogenic species (Davis et al., 1984; Mogen et al., 1990; Rademaker & Janse, 1994; Drier et al., 1995; Louws et al., 1998). Since the discovery and identification of DNA repetitive sequences found in prokaryotic and eukaryotic genomes, a wide variety of protocols to amplify genes with PCR using these primers has been developed for the

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detection, characterization, and differentiation of microbes, including bacteria and fungi of ecological, medical, and agricultural importance. Each PCR based technique designed provides different levels of taxonomic resolution, ranging from genus to subspecies and strain discrimination (Louws et al., 1996; Vandamme et al., 1996; Rademaker & de Bruijn; 1997). However, the repetitive-sequence based polymerase chain reaction (rep-PCR) has provided several advantages over other DNA techniques. For example, with this technology it is possible to generate a genomic fingerprint without previous knowledge of DNA sequences at a resolution that identifies species, subspecies, and strains of various species (Louws et al., 1996; Rademaker & de Bruijn; 1997). The most appealing attributes of the rep-PCR technique are its reproducibility, reliability, speed, and cost when compare against other DNA fingerprinting techniques (Versalovic et al., 1994; Weising et al., 1995; Schneider & de Bruijn, 1996; Versalovic et al., 1997).

The rep-PCR protocol is one of the two DNA fingerprinting techniques used to define genetic characters and polymorphic regions of prokaryotic and eukaryotic genomes (Louws et al., 1996; Schneider & de Bruijn, 1996). The fingerprints generated from rep-PCR technology originated from naturally occurring repetitive sequences that are randomly arranged in high copy number on the circular bacterial genomes (Versalovic et al., 1991; de Bruijn, 1992; Koueth et al., 1995; Schneider & de Bruijn, 1996; Vera Cruz et al., 1996). Three groups of repetitive sequences have been studied in detail have been used to generate primers for the amplification of DNA through PCR in other bacterial species. The three primers used for the production of rep-PCR genomic fingerprints consist of 18-22 oligonucleotides sequences recognized through DNA hybridization studies (Versalovic et al., 1991; Versalovic et al., 1994). They include the

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repetitive extragenic palindromic (REP) sequence, the enterobacterial repetitive intergenic consensus (ERIC) sequence, and the BOX element (Louws et al., 1996; Schneider & de Bruijn, 1996). These primers were developed after their characterization in the Gram-negative enteric bacteria, *Escherichia coli* and *Salmonella typhimurium* for the REP and ERIC primers, respectively. The combination of boxA, boxB, and boxC elements were first characterized in Gram-positive bacterium, *Streptococcus pneumonia* (Koeuth et al., 1995) and unrelated Gram-negative species were used in characterizing the BOX primer (Versalovic et al., 1994; Versalovic et al., 1997).

A different group of primers can be generated from minisatellite sequences, which consist of 10-60 base pairs arranged in a head to tail fashion with two to several thousand motifs, most likely containing non-coding DNA. The polytrinucleotides, $(GTG)_5$ and $(GCC)_5$ are examples of primers complementary to minisatellites (Versalovic et al., 1994). The precise function of the repetitive and minisatellite sequences is unknown, but it has been postulated to be important in chromosomal organization, DNA replication and prevention of DNA degradation, as well as in regulation of gene expression (de Bruijn, 1992; Weising et al., 1995). Meyer et al. (1993) demonstrated the usefulness of $(GTG)_5$ as rep-PCR primers for the differentiation of eukaryotic species. Some of the first reports where $(GTG)_5$ was used to prime rep-PCR were realized with human pathogenic species such as the fungus *Cryptococcus neoformans* (Meyer et al., 1993; Meyer & Mitchell., 1995). Various scientists showed that minisatellite sequences are randomly spread through the entire fungal and prokaryotic genomes (Weising et al., 1991; Meyer et al., 1993; Meyer & Mitchell., 1995; Weising et al., 1995; Warren et al., 1996). These studies were important in generating information related to origin and geographic distribution of

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various strains; data necessary for epidemiological studies. A recent report has demonstrated the usefulness of $(GTG)_5$-PCR genomic fingerprinting for assaying genetic variability in various fungal species (Balerias Couto et al., 1996).

As in medical pathology, PCR-based techniques have revolutionized the detection and differentiation of pathogens in plant pathology (Rademaker & Janse, 1994; Roberts et al., 1996; Sadowsky et al., 1996; Lee et al., 1997a & 1997b; Rademaker & de Bruijn, 1997; Sousa Santos et al., 1997). Specifically, the use of rep-PCR genomic fingerprinting has provided useful information for the reevaluation of taxonomic names and evolutionary relationships within phytopathogenic bacterial species that are of serious economical and agricultural concern. For example, Louws et al. (1994 & 1995) were able to redefine phylogenetic groups and effectively differentiate phytopathogenic strains within the pseudomonad and xanthomonad species that can cause symptoms on a wide variety hosts. More recently, Louws et al. (1998) were able to rapidly and effectively differentiate five *Clavibacter michiganensis* subspecies using rep-PCR. The *C. michiganensis* subspecies analyzed are among characterized by their virulence on different hosts. They also reported a higher level of genetic resolution at the subspecies level when a wide collection of strains of *C. michiganensis* subsp. *michiganensis* was analyzed with BOX-PCR. They defined four BOX-PCR (A, B, C, and D) types based on polymorphic DNA bands after agarose-based electrophoresis. During the completion of Louws et al. (1998) manuscript a new BOX-PCR type was described as the E-type (Bell et al., 1997). The number of E-type strains is limited and these have been recovered only from two geographical locations. Although the biological significance of the BOX-PCR types was not established, genetically typing the strains provided useful groupings. For

example, almost all of the *C. michiganensis* subsp. *michiganensis* strains that are asymptomatic on tomato (avirulent strains) belong to BOX-PCR type A, while highly virulent strains belong to BOX-PCR type C.

The creation and analysis of dendrograms based on more than one DNA fingerprint can help support the subdivision of strains into genotypes based on genetic diversity (Schneider & de Bruijn, 1996; Louws et al., 1996; Rademaker et al., 1997; Rademaker et al., 1998; Vinuesa et al., 1998; Jaunet & Wang, 1999; Rademaker et al., 1999a; Rademaker et al., 1999b). That *C. michiganensis* subsp. *michiganensis* can be divided into five genotypes based on BOX-PCR protocol suggests that this subspecies is genetically narrow. Defining genetic variability should help us understand more about the role of the strains in the host/pathogen relation and may provide us with powerful tools in examining the epidemiological picture of this disease. To this end, I have focused on two objectives; 1) to determine if other rep-PCR universal primers support the subdivision of *C. michiganensis* subsp. *michiganensis* into the five genotypes established with BOX-PCR; and if so 2) determine if the measurable diversity allows for a better understanding of the bacterial canker disease cycle and the relationship of the strains.

MATERIALS AND METHODS

The 6 *Clavibacter michiganensis* strains representing different subspecies of C. *michiganensis* and the 175 representing *C. michiganensis* subsp. *michiganensis* strains used in this study were obtained from a culture collection and are described in Table 1. *Clavibacter michiganensis* subsp. *michiganensis* strains listed in Table 1 are categorized by their previous BOX-PCR type as described by Louws et al. (1998). Subdivision of

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Table 1. Strains of subspecies of *Clavibacter michiganensis* used in this study.

Subspecies	PCR type[a]	Origin[b]	Variety[c]	Tissue[d]	Virulence[e]	Strain(s)[f]
insidiosum						CDA2(ATTCC3314)
sepedonicus						CIC4 (NCPPB2139)
nebraskensis						CIC13, CIC17
tessellarius						CIC21, CIC22
michiganensis	A	MI	P	seedling	V	139, 210, 211, 297, 300, 301
					I	129
					AV	132, 133, 134, 136, 138, 207, 302
				foliage	V	T3, T4, T5, T66, T67, 936
				fruit	V	T1, T8, T12, T13, T17, T18, T19, T20, T23, T24, T27, T35, T41, T42, T43, T45, T56, T57, T63, T65, T70, T80, 294
					AV	T2
				nk	V	229, 292, 294, 672, 676, T73
					I	671
					AV	209, 673, 674, 675
					ND	622, 624, 625, 899, 900, 933, 934, 935, 950
			FM	fruit	V	299
				nk	V	127
					ND	357, 360
		OH	P	fruit	V	353, 355, 356
					ND	363
			nk	nk	AV	9, 29
	B	MI	P	fruit	V	30
			FM	fruit	V	12
				foliage	V	35
			nk	nk	ND	619, 620, 621, 623, 668
		OH	FM	foliage	V	69
				fruit	V	68, 77
			nk	nk	V	226
		CA	FM	foliage	ND	CA-01, CA-10R, CA-Y
				fruit	ND	CA-5, CA-5R
		ON	nk	nk	V	R28

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Table 1. (cont'd)

Subspecies	PCR type[a]	Origin[b]	Variety[c]	Tissue[d]	Virulence[e]	Strain(s)[f]
michiganensis	C	MI	P	foliage	V	955, 958, 961, 962, 963
					I	15, 33
			FM	foliage	V	18, 954, 956
					I	24
					ND	NW89, NW90, NW99, NW100, NW107, NW108, NW110
				fruit	V	8, 44, 53, 236
				fruit	I	56
			nk	foliage	V	237
			nk	fruit	V	234
					I	218
			nk	nk	V	13
					I	216
					ND	S1, S3, S4, S6, S12
			FM	foliage	V	122
				nk	V	117, 230
			nk	nk	V	27, R19
					I	26
		NC	nk	foliage	V	21
				fruit	V	3
				nk	V	54, R4
					ND	14
		CA	P	foliage	V	GH-197
				seed	V	GH-7902
		Chile	P	foliage	V	GH-213
				seed	V	GH-202A, GH-71033
					I	GH-202B
		China	P	seed	V	GH-211, GH-212, GH-71127
			FM	seed	V	GH-7168, GH-71182, GH-71196, GH-71235, GH-71290, GH-71335, GH-71341,GH-71412, GH-17427, GH-71428, GH-71430, GH-71431, GH-71472, GH-71564, GH-71474, GH-713350
					I	GH-71176

Table 1. (cont'd)

Subspecies	PCR type[a]	Origin[b]	Variety[c]	Tissue[d]	Virulence[e]	Strain(s)[f]
michiganensis	C	nk	nk	nk	V	R13
		Morroco	FM	seed	ND	GH-7904
	D	MI	FM	foliage	ND	Bet1-5-1, Bet1-5-2, Bet3-5-1, Bet3-5-2, FM1-4-1, MF1-5-1, MF1-5-2, MF3-5-1
				fruit	I	286, 288
		India	FM	seed	ND	GH-7137P
		Morroco	FM	seed	ND	GH-7219, GH-7219P, GH-7388, GH-7388C
	E	MI	nk	foliage	I	2S, 10S, 14S, 30S, 31S
					ND	48S
				fruit	I	1F, 9F, 10F
	E	OH	nk	foliage	AV	118

[a] PCR= polymerase chain reaction designated type using BOX primer (Louws et al., 1998).

[b] Original source of *C. michiganensis* subspecies other than subspecies *michiganensis* were reported on Louws et al., 1998. Michigan=MI, Ohio= OH, North Carolina=NC, California=CA, Nebraska=NE, and Ontario=ON.

[c] Processing variety=P, Fresh-Market variety=FM, and not known=nk.

[d] not known=nk.

[e] Virulence= producing canker and wilt; I=intermediate, producing canker only; AV=avirulent,producing no symptoms; and ND= not determined (Louws et al., 1998).

[f] Strain source: T=F.J.Louws collection, CA and GH= California, CDA= California Department of Agriculture, CIC= Carol Ishimaru collection, R= Mark Ricker, and Bet,FM,MF,& NW= Nicole Werner; Michigan State University, Dept. of Botany & Plant Pathology.

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each PCR-type (A, B, C, D, and E) is based on the geographical location, plant variety, plant source, and virulence. Virulence of each strain used was previously determine by our laboratory using the pathogenicity and hypersensitive response (HR) tests as reported by Louws et al (1998). All bacteria were stored in glycerol at -20° C or -70° C. Bacterial strains were grown on nutrient both yeast extract (NBY; Fatmi & Schaad, 1988) agar (no glucose) at 27° C for 4 to 10 days depending of the subspecies.

The $(GTG)_5$-PCR and BOX-PCR protocols were carried out as described by Versalovic et al. (1994) with the following modifications. For the amplification with $(GTG)_5$ primer, the annealing time was lowered to 30 sec, and mineral oil was not used to overlay each PCR reaction mixture as the thermal cycler (Genemate-Techne, Princeton, NJ, USA) used consisted of a heated lid which prevented evaporation of the product during the amplification procedure.

The $(GTG)_5$ and BOX primers were synthesized by the Macromolecular Structure, Sequence and Synthesis Facility at Michigan State University as the agarose gel electrophoresis and gel photographic conditions were similar to those reported by Louws et al. (1998). Ninety five percent of the strains included in Table 1 were amplified with each primer at least two times during separate $(GTG)_5$-PCR and BOX-PCR reactions. Genomic DNA from Cmm 936 used as positive control in every PCR reaction was prepared as described by Louws et al. (1998).

Computer-image analysis of photographs of the ethidium bromide-stained agarose gels was performed using the GelCompar gel analysis system program (Applied Math, Kortrijk, Belgium) following the recommendations of the manufacturer and those reported by Vauterin & Vauterin (1992), and Rademaker & de Bruijn (1997).

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Photographs were scanned using a scanner (Hewlett Packard, Scan Jet 3C), and raw images were stored. Each fingerprint pattern was standardized to the 1 kilobase pair (1 Kb) DNA maker (Gibco, USA) on three lanes in each gel. Normalized gel tracts for each fingerprint pattern was digitized and stored for further analysis. An area of the densitometric curves for each gel tract was selected before a similarity matrix was generated; for $(GTG)_5$-PCR and BOX-PCR the area corresponding from the 2.2 to 0.5 kb and from the 2.0 to 0.4 kb range of fingerprint bands were selected, respectively. Similarity matrices and dendrograms were generated using product-moment correlation and unweighted pair group with arithmetic averages (UPGMA) clustering, as previously described by Louws et al. (1994) and Rademaker et al. (1999b). Dendrograms from single fingerprint patterns (either $(GTG)_5$-PCR or BOX-PCR) and from combined $(GTG)_5$-PCR and BOX-PCR fingerprint patterns were generated following the recommendations of the manufacturer.

Cluster designation for each dendrogram was accomplished after the definition of an arbitrary baseline. To define the baseline for the computer-generated similarity index for each dendrogram, two reproducibility experiments were conducted. Ten independent PCR master-mixes for each primer ($(GTG)_5$ and BOX) were prepared on one day with the same stock-aliquot for each PCR reagent. One *Clavibacter michiganensis* subsp. *michiganensis* strain of each BOX-PCR type (Table 1) was used as DNA template; strains Cmm 936 (type A), Cmm 68 (type B), Cmm 236 (type C), Cmm 286 (type D), and Cmm 10F (type E), and genomic DNA was used as positive control for strain Cmm 936. From the same strain cultured on NBY agar, six colonies for each *C. michiganensis* subsp. *michiganensis* strain was used as DNA templates for each PCR reaction. Five of

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the colonies were used as template in five independent PCR reactions and one of the colonies was repetitively used for the remaining five PCR reactions. PCR amplification protocols and thermal cycler conditions were adjusted as previously indicated depending on the primer used; therefore, $(GTG)_5$-PCR and BOX-PCR amplifications were conducted on consecutive days. Six microliters of each PCR product from the same *C. michiganensis* subsp. *michiganensis* strain were resolved at the same time in a single agarose gel (1.75% in 0.5 X TAE) under the same electrophoretic conditions (83 volts at 4°C). Each agarose gel was stained, photographed, and computer analyzed as mentioned, above. Two experimental variability baselines were established; one for dendrograms of fingerprint patterns from a single primer, and another for dendrograms of combined fingerprint patterns.

RESULTS

$(GTG)_5$-PCR differentiates subspecies of *Clavibacter michiganensis*. To determine if the genetic diversity of *Clavibacter michiganensis* subsp. *michiganensis* resolved by BOX-PCR into genotypes A, B. C, D, and E, can be further resolved using another primer, known as $(GTG)_5$, a collection of 175 *C. michiganensis* subsp. *michiganensis* strains was studied. The genomic fingerprint obtained with $(GTG)_5$-PCR consisted of series of amplified bands with a more complex pattern when compared to genomic fingerprints generated with BOX-, ERIC- and REP-PCR. The number of strongly amplified bands obtained with BOX-PCR was approximately 15, whereas 11 were obtained when using $(GTG)_5$-PCR; however, less strongly amplified bands were generated with the $(GTG)_5$ primer than the BOX primer.

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To demonstrate that *Clavibacter michiganensis* subsp. *michiganensis* could be differentiated from other subspecies of *Clavibacter michiganensis* using $(GTG)_5$-PCR, 12 strains from a collection of 175 strains were selected for $(GTG)_5$-PCR amplification and characterization (Table 1). Of the 12 strains, two each represented the type A, B, D, and E groups as distinguished by BOX-PCR, and four represented the larger type C type group. The other subspecies of *C. michiganensis* represented strains previously compared to *C. michiganensis* subsp. *michiganensis* using BOX-PCR (Louws et al., 1998) and are listed in Table 1. Approximately twenty-five visible bands between the 2.2 to 0.5 kb region were included in the cluster analysis of the $(GTG)_5$-PCR genomic fingerprints (Figure 1). One half of the visible bands from the *C. michiganensis* subsp. *michiganensis* fingerprints co-migrated with bands from the four *C. michiganensis* subspecies (Figure 1). Based on the four *C. michiganensis* subspecies used in this study, the cluster analysis indicated that all of the *C. michiganensis* subspecies could be easily differentiated from each other based solely on the fingerprint pattern obtained. Therefore, $(GTG)_5$ was capable of distinguishing *C. michiganensis* subsp. *michiganensis* from other subspecies, however, it is more intrinsically difficult to use and interpret than BOX-PCR due to the large number of weakly amplified bands.

Reproducibility of $(GTG)_5$-PCR fingerprints and definition of clusters. If the fingerprint patterns obtained from the $(GTG)_5$ primer are to be useful in differentiating strains of *C. michiganensis* subsp. *michiganensis* the amount of variability due to artifacts of the PCR reaction must be gauged. Two experiments were performed to test this experimental variability. In the first experiment, the reaction mixtures were held

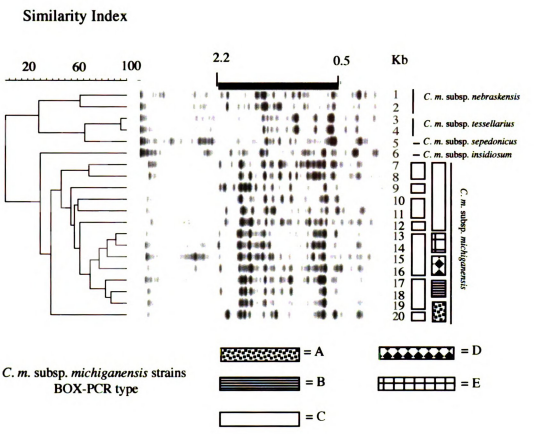

Figure 1. Cluster analysis (UPGMA) of $(GTG)_5$-PCR-generated genomic fingerprints of strains of *Clavibacter michiganensis* subspecies. The gray bar above the fingerprint patterns ranging from 2.2 to 0.5 kilobase pairs represents the area of the banding pattern used to generate the dendrogram shown on the left side of the figure. The columns of non-patterned and patterned boxes on the right side of the figure represent the results from the $(GTG)_5$-PCR obtained from this study and the BOX-PCR genotypes previously designated by Louws et al. (1998), respectively. Lanes 1 to 6, $(GTG)_5$- PCR generated fingerprint patterns for *C. michiganensis* subspecies including subsp. *nebraskensis* strains CIC 12 (lane 1) and CIC 16 (lane 2); subsp. *tessellarius* strains CIC 21 (lane 3) and CIC 22 (lane 4); subsp. *sepedonicus* strain CIC 4 (lane 5); and subsp. *insidiosum* strain CDA 4 (lane 6). Lanes 7 to 20, $(GTG)_5$- PCR generated fingerprint patterns for *C. michiganensis* subsp. *michiganensis* including representatives of the designated as type C strains on the basis of BOX-PCR (GH-7904 (lane 7), GH-71033 (lane 8), Cmm56 (lane 9), Cmm122 (lane 10), GH-71182 (lane 11), and Cmm14 (lane 12)), type E strains (Cmm118 (lane13) and Cmm14S (lane14)), type D strains (Cmm288 (lane 15), and GH-7137P (lane 16)), type B strains (CA-5 (lane 17) and Cmm30 (lane 18)), and type A strains (Cmm936 (lane 19), and CmmT13 (lane 20)).

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constant and five individual colonies of one strain were amplified with BOX and $(GTG)_5$ primers. In the second experiment five different reactions with BOX and $(GTG)_5$ primers were obtained from the same colony of the strain assayed in the first experiment. These experiments were performed on a strain representing BOX-PCR types A, B, C, D and E.

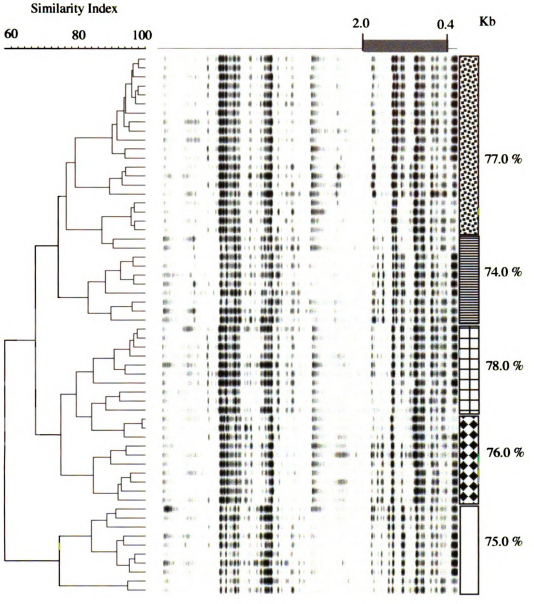
The overall amount of variability observed in these reactions indicated that each clonal strain showed a rather large amount of experimental variability based solely on the PCR reaction protocol, and not due to the genetics of the strain (Figures 2 and 3). When the cluster analysis (UPGMA) was made for single primers, the experimental protocol alone could account for up to 25% of the observed variability and up to 20% for the variability observed in combined primers (Figure 4). This experimental variability was taken into consideration when strains were compared for genetic diversity.

Genetic variability measured by $(GTG)_5$-PCR fingerprint analysis in strains of *C. michiganensis* subspecies *michiganensis* designated as BOX-PCR Type A.

In a preliminary study, 23 type A strains with greatest overall diversity, including BOX-PCR fingerprint type, origin of recovery, *etc.* were selected to represent the 73 strains listed as type A strains in Table 1. These 23 strains were amplified with BOX and $(GTG)_5$ primers and the resulting banding patterns were analyzed for differences through cluster analysis (UPGMA; Figure 5).

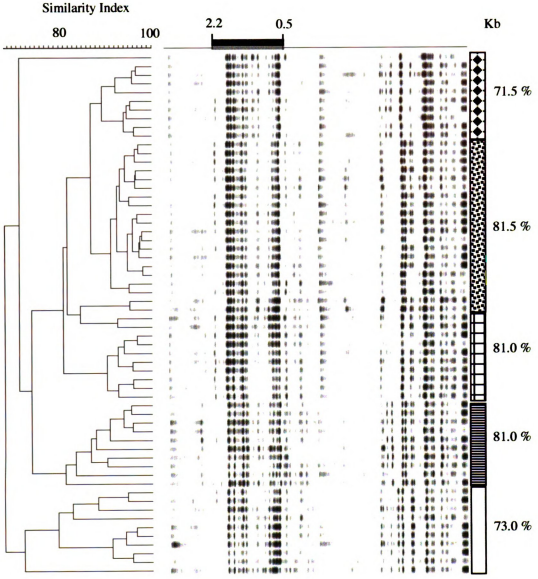
As expected with the BOX primer, all BOX-PCR type A strains clustered as one large group when the experimental variability baseline was subtracted (25%). Because the strain appeared so similar, an outgroup consisting of 6 type C strains was included in the analysis to provide a similarity index less than 75% (Figure 5A).

Figure 2. Cluster analysis (UPGMA) using product moment correlation of BOX-PCR-generated genomic fingerprints of *Clavibacter michiganensis* subsp. *michiganensis* strains used to define the experimental variability baseline. The gray bar above the fingerprint patterns ranging from 2.0 to 0.4 kilobase pairs represents the area of the banding pattern used to generate the dendrogram shown on the left side of the figure. The percent value next to each BOX-PCR type (patterned boxes) corresponds to the similarity value observed for the BOX-PCR-generated fingerprints using the same culture as the DNA template. The average of the similarity values observed among the strains used was considered to be the experimental variability baseline (approximately 75.0%) for the analysis of the following dendrograms.



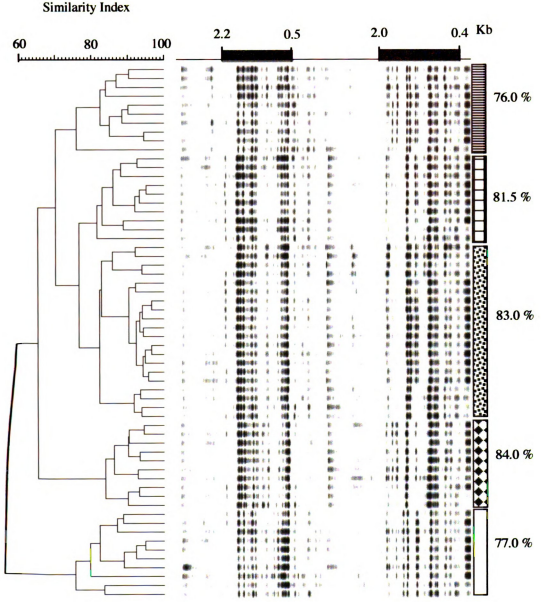
C. michiganensis subsp. *michiganensis* BOX-PCR types

Figure 3. Cluster analysis (UPGMA) using product moment correlation of $(GTG)_5$-PCR- generated genomic fingerprints of *Clavibacter michiganensis* subsp. *michiganensis* strains used to define the experimental variability baseline. The gray bar above the fingerprint patterns ranging from 2.2 to 0.5 kilobase pairs represents the area of the banding pattern used to generate the dendrogram shown on the left side of the figure. The percent value next to each BOX-PCR type (patterned boxes) corresponds to the similarity value observed for the $(GTG)_5$-PCR-generated fingerprints using the same culture as the DNA template. The average of the similarity values observed among the strains used was considered to be the experimental variability baseline (approximately 75.0%) for the analysis of the following dendrograms.



C. michiganensis subsp. *michiganensis* BOX-PCR types

Figure 4. Cluster analysis (UPGMA) using product moment correlation of two linearly combined $(GTG)_5$-PCR and BOX-PCR-generated genomic fingerprints of *Clavibacter michiganensis* subsp. *michiganensis* strains used to define the experimental variability baseline. The gray bars above the fingerprint patterns ranging from 2.2 to 0.5 and 2.0 to 0.4 kilobase pairs represent the area of the banding patterns used to generate the dendrogram shown on the left side of the figure. The percent value next to each BOX-PCR type (patterned boxes) corresponds to the similarity value observed for the linearly combined $(GTG)_5$-PCR and BOX-PCR-generated fingerprints using the same culture as the DNA template. The average of the similarity values observed among the strains used was considered to be the experimental variability baseline (approximately 80.0%) for the analysis of the following dendrograms.



C. michiganensis subsp. *michiganensis* BOX-PCR types

With $(GTG)_5$, the BOX-PCR type A strains clustered into three major group after the experimental variability (25%) was taken into consideration (Figure 5B). The same type C strains were used as an outgroup to define the branch of a cluster. When both primers were analyzed simultaneously, four groups were differentiated after the experimental variability (20%) was taken into consideration and each subgroup was designated as types A-1, A-2, A-3, and A-4 (Figure 5C).

Looking at these four groups in more detail offers some interesting insights into the genetic diversity of A type *C. michiganensis* subsp. *michiganensis* strains (Figure 6). The A-1 group was composed of strains primarily from Michigan isolated from processing tomato plants in the 1990's. The A-2 group was composed of 2 strains (Cmm355 and Cmm360). In the BOX-PCR reaction, these two strains were found to be similar to each other, and were the most distinct strains (lanes 18 and 19) in that single large group. While both of these strains were found in the same group when $(GTG)_5$ reactions were analyzed, other than year of isolation from diseased tomato plants, no other obvious pathological or epidemiological characteristic united these two strains or set them apart from other strains. Based on $(GTG)_5$ and BOX-PCR fingerprint patterns, these two strains would be seen as genetically similar, yet, each was recovered from completely distinct types of tomato, one a fresh market variety and the other from a processing variety, from fields 220 km apart.

The A-3 group is composed of a single representative strain, Cmm302, which was also part of $(GTG)_5$ group 2. Looking at the BOX-PCR analysis, this strain grouped with the single large group but was placed in that group with strains isolated in 1993 from processing varieties recovered from the southwestern part of Michigan. Cmm302 was

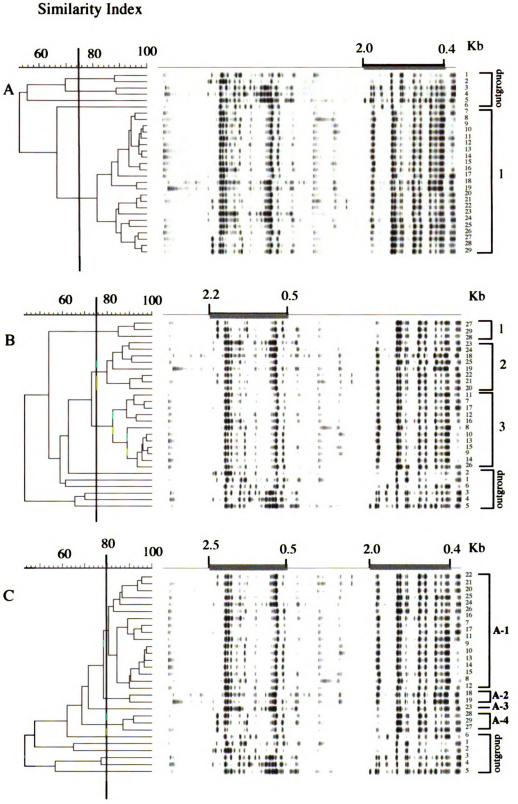
isolated in 1994 from a diseased processing tomato plant recovered from southeast Michigan. Another representative strain, Cmm134, isolated from plants recovered from that location in the same year grouped in the large A-1 group in the combined PCR analysis.

The A-4 group consisted of 3 representative strains that were considered $(GTG)_5$ PCR group 1. These strains were also contained in the single large group in BOX-PCR analysis and grouped together with another isolate CmmT56. CmmT56 had an identical BOX-PCR pattern with the A-4 isolates, but when $(GTG)_5$-PCR fingerprints alone were analyzed, CmmT56 was not grouped with the A-4 group. It is easy to see why they were not grouped together (Figure 5). A prominent large band approximately 2 kb in size was present in the A-4 isolates and lacking in the CmmT56 isolate. Therefore, where BOX-PCR failed to pick up a distinction, the $(GTG)_5$-PCR reaction was able to demonstrate a strong difference in genotype. When taking into consideration the epidemiological differences, both the strains making up the A-4 group and CmmT56 were all isolated from in 1993 from southwest Michigan farms planted to processing tomato varieties. CmmT56 was isolated from a different farm than the other strains.

Genetic variability measured by $(GTG)_5$-PCR fingerprint analysis in strains of C. michiganensis subspecies michiganensis designated as BOX-PCR Type B.

Seventeen type B *C. michiganensis* subsp. *michiganensis* strains were amplified with BOX and $(GTG)_5$ resulting in banding patterns analyzed for differences through cluster analysis (UPGMA; Figure 7). The results of the B type strains were different than those with A type strains as BOX-PCR resolved the B type strains into at least three subgroups. With $(GTG)_5$, alone, all BOX-PCR type B strains clustered into one large

Figure 5. Cluster analysis (UPGMA) using product moment correlation of two linearly combined $(GTG)_5$-PCR and BOX-PCR-generated genomic fingerprints of *Clavibacter michiganensis* subsp. *michiganensis* strains designated as type A. The gray bars above the fingerprint patterns ranging from 2.2 to 0.5 and 2.0 to 0.4 kilobase pairs for $(GTG)_5$-PCR and BOX-PCR, respectively, represent the area of the banding patterns used to generate the dendrograms shown on the left side of each panel. Panel A, analysis for BOX-PCR fingerprints; panel B, analysis for $(GTG)_5$-PCR fingerprints; and panel C, analysis for the two linearly combined $(GTG)_5$-PCR and BOX-PCR fingerprints. The vertical lines bisecting each dendrogram represent the experimental variability baseline used to define a cluster: 75.0 % for the dendrograms in panels A and B, and 80.0% for the dendrogram in panel C. The designation of groups for each analysis is represented on the right side of the panel


Similarity Index
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100
2.0
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0.5
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A-4

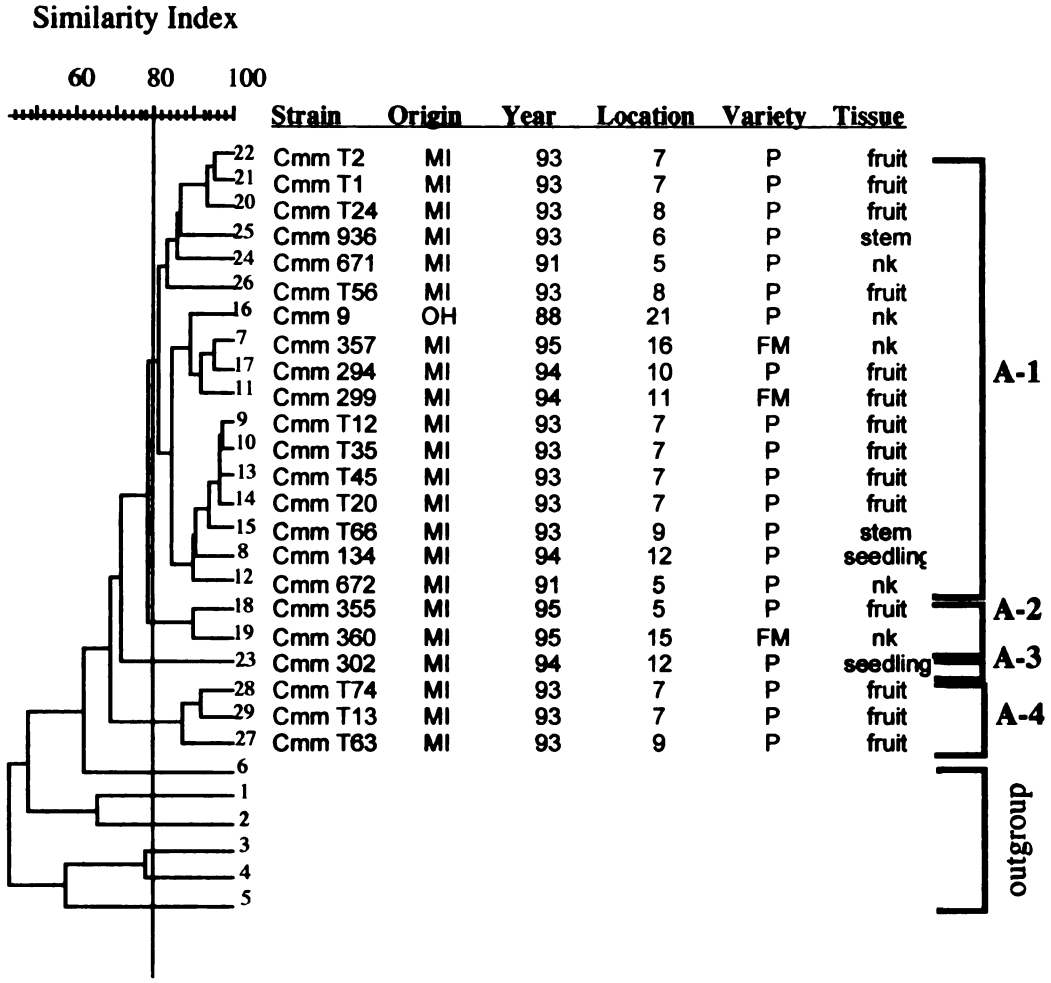


Figure 6. *Clavibacter michiganensis* subsp. *michiganensis* strains designated type A used for the cluster analysis (UPGMA) of the linearly combined $(GTG)_5$-PCR and BOX-PCR-generated genomic fingerprints in Figure 5C. The vertical line bisecting the dendrogram represents the experimental baseline (80%) used to define the groups. The designation of groups for the analysis of the linearly combined $(GTG)_5$-PCR and BOX-PCR-generated fingerprints, and six strains used toform an outgroup (Cmm12 (lane 6), GH-71182 (lane 1), Cmm56 (lane 2), GH-71033(lane 3), GH-7904 (lane 4), and Cmm14 (lane5)) are indicated on the right side of the figure.

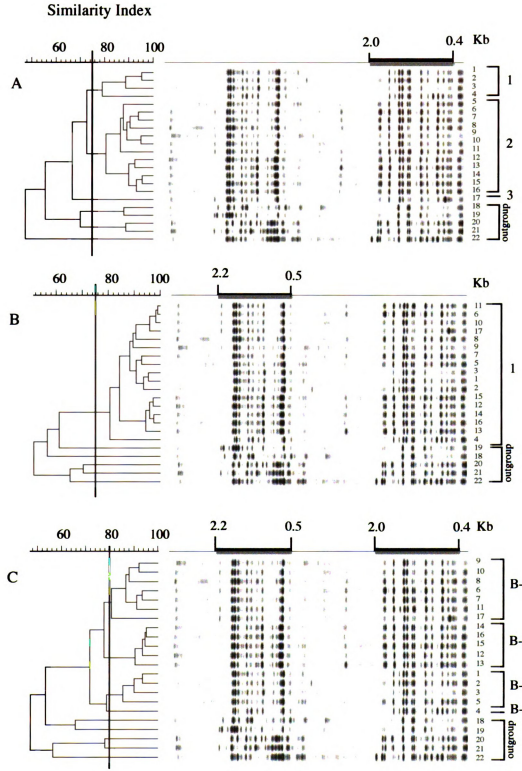
group. Because the relationship was so similar, the same 5 type C strains used in the type A strain analysis were used for the cluster analysis (Figure 7B).

When both primers and an 20% experimental variability baseline were used in the analysis, four subgroups with the B-type strains were resolved and are referred to as B-1, B-2, B-3, and B-4 (Figure 7C).

These subgroups, based on the combination of primers, appear to have some epidemiological merit in regard to the time and type of tomato plants grown (Figure 8). For example, two strains recovered from diseased fresh market tomato varieties on a farm in Michigan in 1986 were placed in the B-1 group along with three strains isolated from diseased fresh market tomato varieties growing in northwestern Ohio in 1987 and 1988. These strains were part of a large continuing epidemic (1984-88) that initiated the tomato bacterial canker workshop in 1984 (now referred to as the Tomato Disease Workshop). All 5 strains grouped as B-2 were isolated from California and sent for analysis indicating a geographic relationship. Similarly, 4 strains collected from southwest Michigan in 1991 were placed in group B-3 along with a fifth strain isolated from fresh market tomato in southwest Michigan earlier in 1987. The strain representing B-4 was isolated from the same location in the same year as the 3 strains in B-3 and the genetic distinction of this strain is in doubt as experimental variability should be taken into consideration due to the weak differences separating B-3 from B-4.

Many of these same groups can be seen in the BOX-PCR analysis if the experimental variability baseline is ignored. For example, the California isolates group together in the BOX-PCR analysis but do not fall out as a unique group if the experimental variability is taken in consideration. Interestingly, the 1991 strains isolated

Figure 7. Cluster analysis (UPGMA) using product moment correlation of two linearly combined $(GTG)_5$-PCR and BOX-PCR-generated genomic fingerprints of *Clavibacter michiganensis* subsp. *michiganensis* strains designated as type B. The gray bars above the fingerprint patterns ranging from 2.2 to 0.5 and 2.0 to 0.4 kilobase pairs for $(GTG)_5$-PCR and BOX-PCR, respectively, represent the area of the banding patterns used to generate the dendrograms shown on the left side of each panel. Panel A, analysis for BOX-PCR fingerprints; panel B, analysis for $(GTG)_5$-PCR fingerprints, and panel C, analysis for the two linearly combined $(GTG)_5$-PCR and BOX-PCR fingerprints. The vertical lines bisecting each dendrogram represent the experimental variability baseline used to define a cluster: 75.0 % for the dendrograms in panels A and B, and 80.0% for the dendrogram in panel C. The designation of groups for each analysis is represented on the right side of the panel.


Similarity Index
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B-4

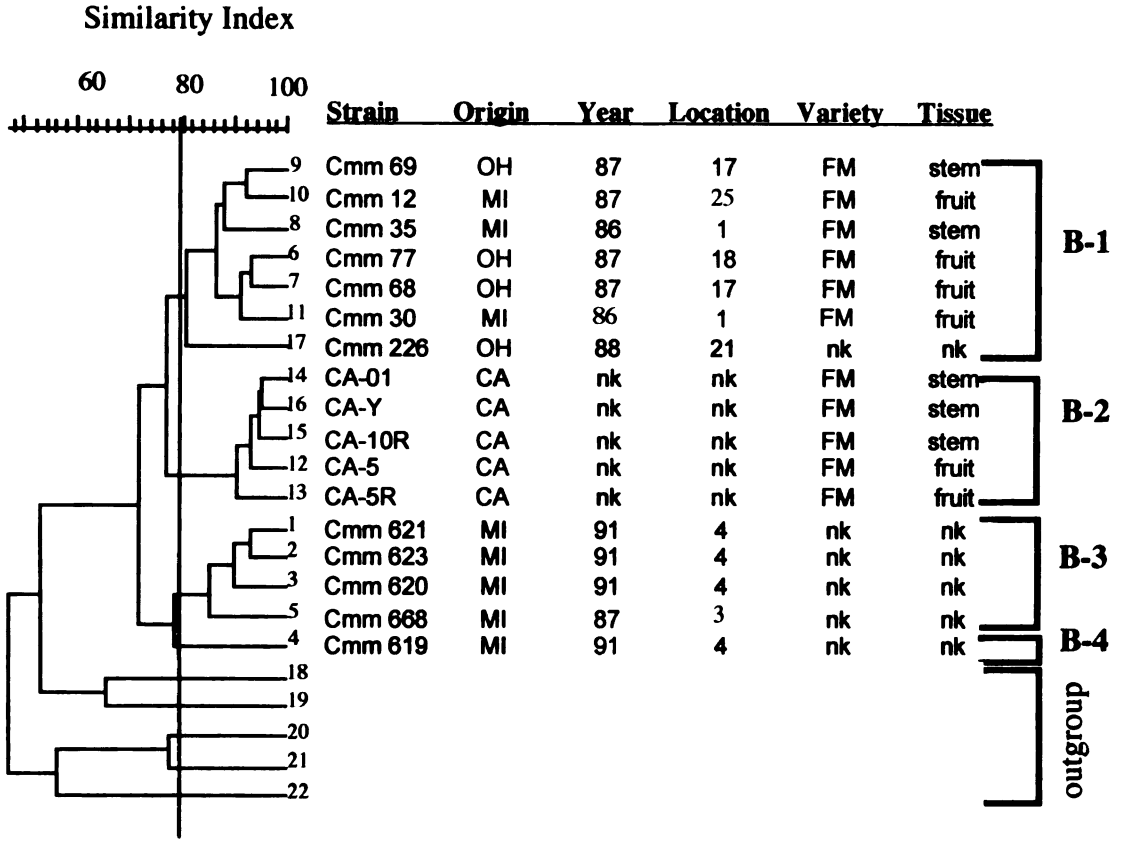


Figure 8. *Clavibacter michiganensis* subsp. *michiganensis* strains designated type B used for the cluster analysis (UPGMA) of the linearly combined $(GTG)_5$-PCR and BOX-PCR-generated genomic fingerprints in Figure 7C. The vertical line bisecting the dendrogram represents the experimental baseline (80%) used to define the groups. The designation of groups for the analysis of the linearly combined $(GTG)_5$-PCR and BOX-PCR-generated fingerprints, and five strains used toform an outgroup (GH-71182 (lane 18), Cmm56 (lane 19), GH-71033 (lane 20), GH-7904 (lane 21), and Cmm14 (lane22)) are indicated on the right side of the figure.

from Michigan cluster into BOX-PCR group 1 and the Michigan strain isolated in 1987, that the combination of primers grouped with the 1991 strains, was separated and placed with strains from Michigan recovered in 1986-87. This appears to be a more logical association, but it also indicates a potential hazard in interpreting similarities and differences among strains.

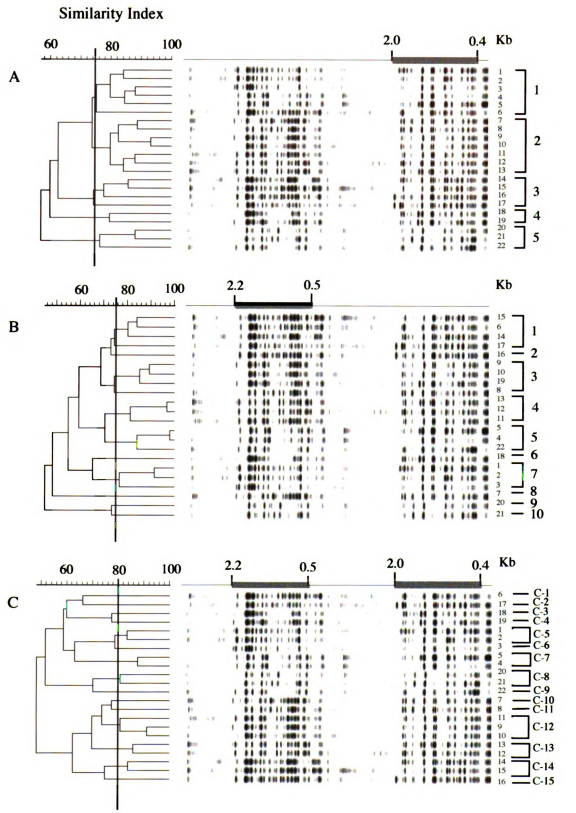
Genetic variability measured by $(GTG)_5$-PCR fingerprint analysis in strains of *C. michiganensis* subspecies *michiganensis* designated as BOX-PCR Type C.

In a preliminary study, 22 type C strains showing the greatest overall diversity, based on BOX-PCR fingerprint patterns, origin of isolation, *etc.* were selected to represent the 73 strains listed as type C in Table 1. These 22 strains were amplified with BOX and $(GTG)_5$ primers and the resulting bands were analyzed through cluster analysis (UPGMA; Figure 9).

When the experimental variability baseline was subtracted (25%) from the BOX-PCR banding pattern, five subgroups were obtained (Figure 9A). With $(GTG)_5$, the BOX-PCR type C strains cluster into 10 subgroups when 25% experimental variability baseline was taken into consideration (Figure 9B).

With both primers, 15 subgroups were observed with the type C strains using an 20% experimental variability baseline and each subgroup will be referred to as C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9, C-10, C-11, C-12, C-13, C-14, and C-15 (Figure 9C). The majority of the subgroups are represented by a single strain. From the subgroups that consist of more than one strain, these strains usually have some interesting relationships when characteristics other than PCR banding patterns are used to group the isolates. For

Figure 9. Cluster analysis (UPGMA) using product moment correlation of two linearly combined $(GTG)_5$-PCR and BOX-PCR-generated genomic fingerprints of *Clavibacter michiganensis* subsp. *michiganensis* strains designated as type C. The gray bars above the fingerprint patterns ranging from 2.2 to 0.5 and 2.0 to 0.4 kilobase pairs for $(GTG)_5$-PCR and BOX-PCR, respectively, represent the area of the banding patterns used to generate the dendrograms shown on the left side of each panel. Panel A, analysis for BOX-PCR fingerprints; panel B, analysis for $(GTG)_5$-PCR fingerprints; and panel C, analysis for the two linearly combined $(GTG)_5$-PCR and BOX-PCR fingerprints. The vertical lines bisecting each dendrogram represent the experimental variability baseline used to define a cluster: 75.0 % for the dendrograms in panels A and B, and 80.0% for the dendrogram in panel C. The designation of groups for each analysis is represented on the right side of the panel.


Similarity Index
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C-14
C-15

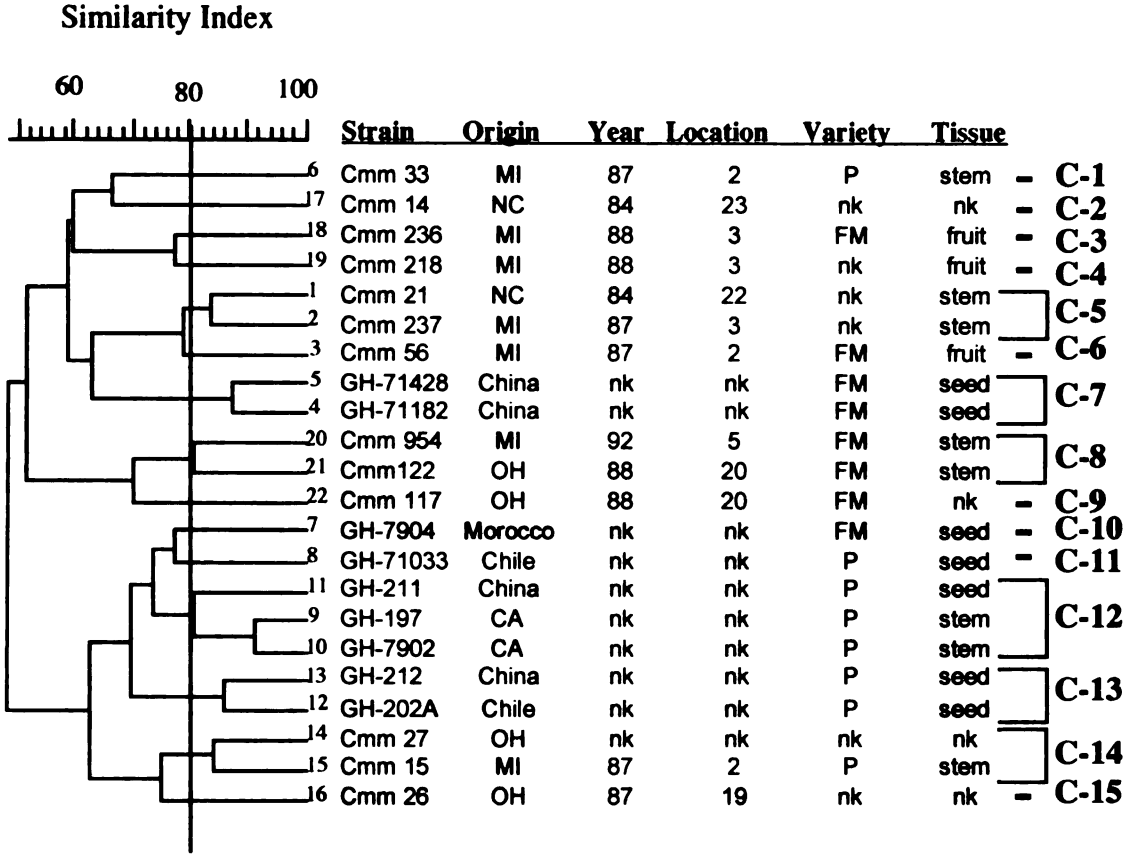


Figure 10. *Clavibacter michiganensis* subsp. *michiganensis* strains designated type C used for the cluster analysis (UPGMA) of the linearly combined $(GTG)_5$-PCR and BOX-PCR-generated genomic fingerprints in Figure 9C. The vertical line bisecting the dendrogram represents the experimental baseline (80%) used to define the groups. The designation of groups for the analysis of the linearly combined $(GTG)_5$-PCR and BOX-PCR-generated fingerprints are indicated on the right side of the figure.

example, two strains from California are grouped together and two strains from China are grouped together although another Chinese isolate is not grouped with the other two Chinese isolates (Figure 10). Not much is known about these strains however, the two grouped together are from fresh market varieties and the single isolate is from processing tomatoes. The strains from North Carolina were separated by fingerprints generated by all PCR primers, even though they were isolated in the same year, these strains were not from the same field as they came from different counties.

Genetic variability measured by $(GTG)_5$-PCR fingerprint analysis in strains of *C. michiganensis* subspecies *michiganensis* designated as BOX-PCR Type D.

Fifteen D type *C. michiganensis* subsp. *michiganensis* strains described in Table 1 were amplified with BOX and $(GTG)_5$ and resulting bands were analyzed through the cluster analysis (UPGMA; Figure 11). When the experimental variability baseline was subtracted (25%), the BOX-PCR fingerprints broke into 2 subgroups, where isolates were found to be clustered in correlation to their geographic origin. One group was from two farms in central Michigan and northeast Michigan and the other group was from foreign locations such as India and Morocco (Figure 11A). Within the large BOX-PCR group, isolates from Michigan were clustered into smaller subgroups. One of these subgroups contained D types from the two different farms mentioned above, both isolated in 1994. The other Michigan strains subgrouped away from the 1994 isolates were isolated in 1997 from the northeast farm.

With $(GTG)_5$, 3 groups were obtained after a 25% experimental variability baseline was established. Isolates from Michigan where represented in two of the three groups. The strains isolated in 1994 grouped together and with two other Michigan

strains isolated in 1997. The foreign strains split into two similar groups with one 1997 Michigan strain included in one of the two groups (Figure 11B).

With both primers taken into consideration and after the experimental variability was subtracted (20%), 3 subgroups were obtained and each subgroup will be referred to as D-1, D-2, and D-3 (Figure 11C).

The foreign strains fall into D-3 and no Michigan strains are positioned with them. The Michigan strains isolated in 1994 are found in D-2 alone. All the other Michigan isolates isolated in 1997 can be found in D-1. All the D type strains have only been found on fresh market tomatoes north and northeast of East (Figure 12).

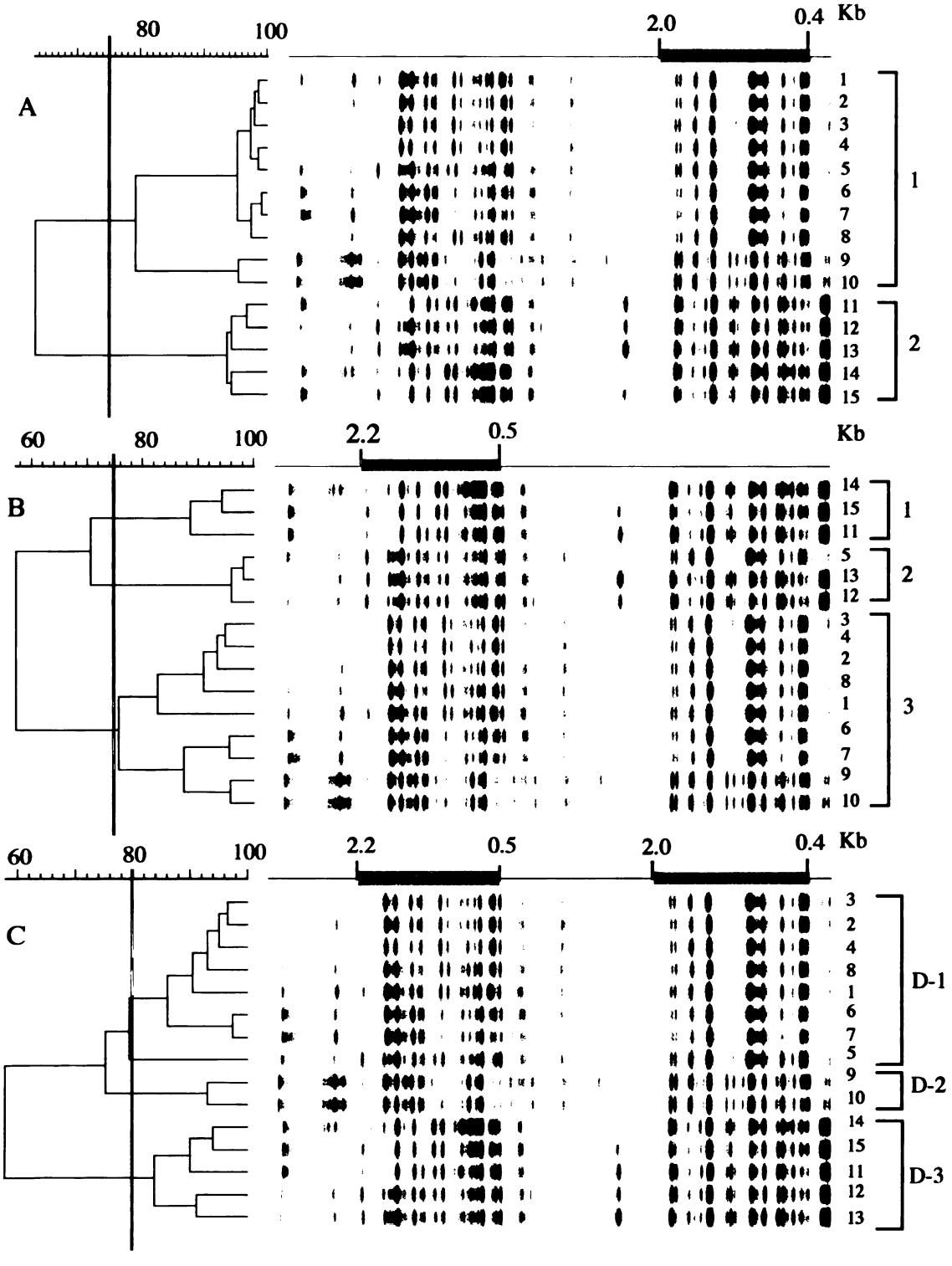
Genetic variability measured by $(GTG)_5$-PCR fingerprint analysis in strains of *C. michiganensis* subspecies *michiganensis* designated as BOX-PCR Type E.

Ten type E *C. michiganensis* subsp. *michiganensis* strains described in Table 1 were amplified with BOX and $(GTG)_5$, and resulted in bands that were analyzed through the cluster analysis (UPGMA; Figure 13).

When all primers were used and the experimental variability was subtracted (25%), all type E BOX-PCR strains formed one large group. Because the genetic similarity was so similar, an outgroup group consisting of 6 type C strains was added to the analysis to provide a similarity index of less than 75% (Figure 13).

At a higher similarity index (88%), two subgroups can be defined, although any relationships can be made because every isolate was collected at location #4 from Michigan with one exception. The second group contain two isolates from location #4 and the isolate recovered at location #20 from Ohio (Figure 14). This Ohio strain was isolated in 1988 and no other type E strains were recovered until 1996 in southwest

Figure 11. Cluster analysis (UPGMA) using product moment correlation of two linearly combined $(GTG)_5$-PCR and BOX-PCR-generated genomic fingerprints of *Clavibacter michiganensis* subsp. *michiganensis* strains designated as type D. The gray bars above the fingerprint patterns ranging from 2.2 to 0.5 and 2.0 to 0.4 kilobase pairs for $(GTG)_5$-PCR and BOX-PCR, respectively, represent the area of the banding patterns used to generate the dendrograms shown on the left side of each panel. Panel A, analysis for BOX-PCR fingerprints; panel B, analysis for $(GTG)_5$-PCR fingerprints; and panel C, analysis for the two linearly combined $(GTG)_5$-PCR and BOX-PCR fingerprints. The vertical lines bisecting each dendrogram represent the experimental variability baseline used to define a cluster: 75.0 % for the dendrograms in panels A and B, and 80.0% for the dendrogram in panel C. The designation of groups for each analysis is represented on the right side of the panel.


Similarity Index
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80
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Kb
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4
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8
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7
9
10
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2
3
60
80
100
2.2
0.5
2.0
0.4
Kb
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D-1
D-2
D-3

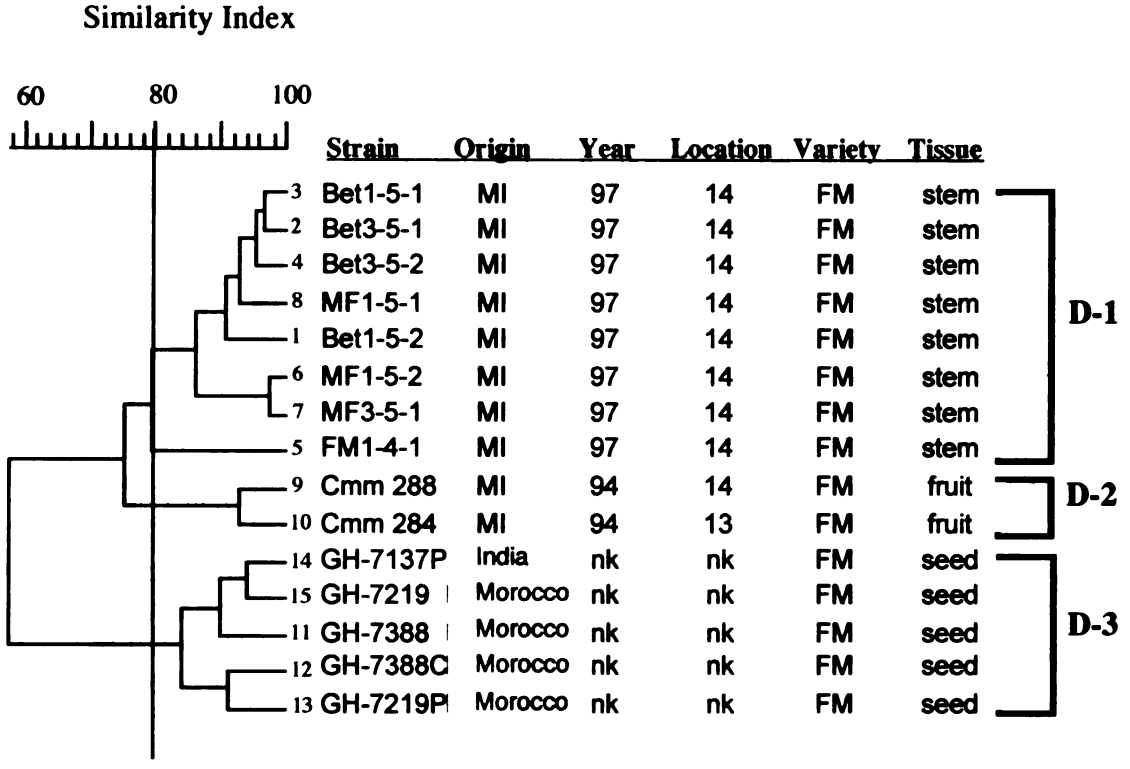
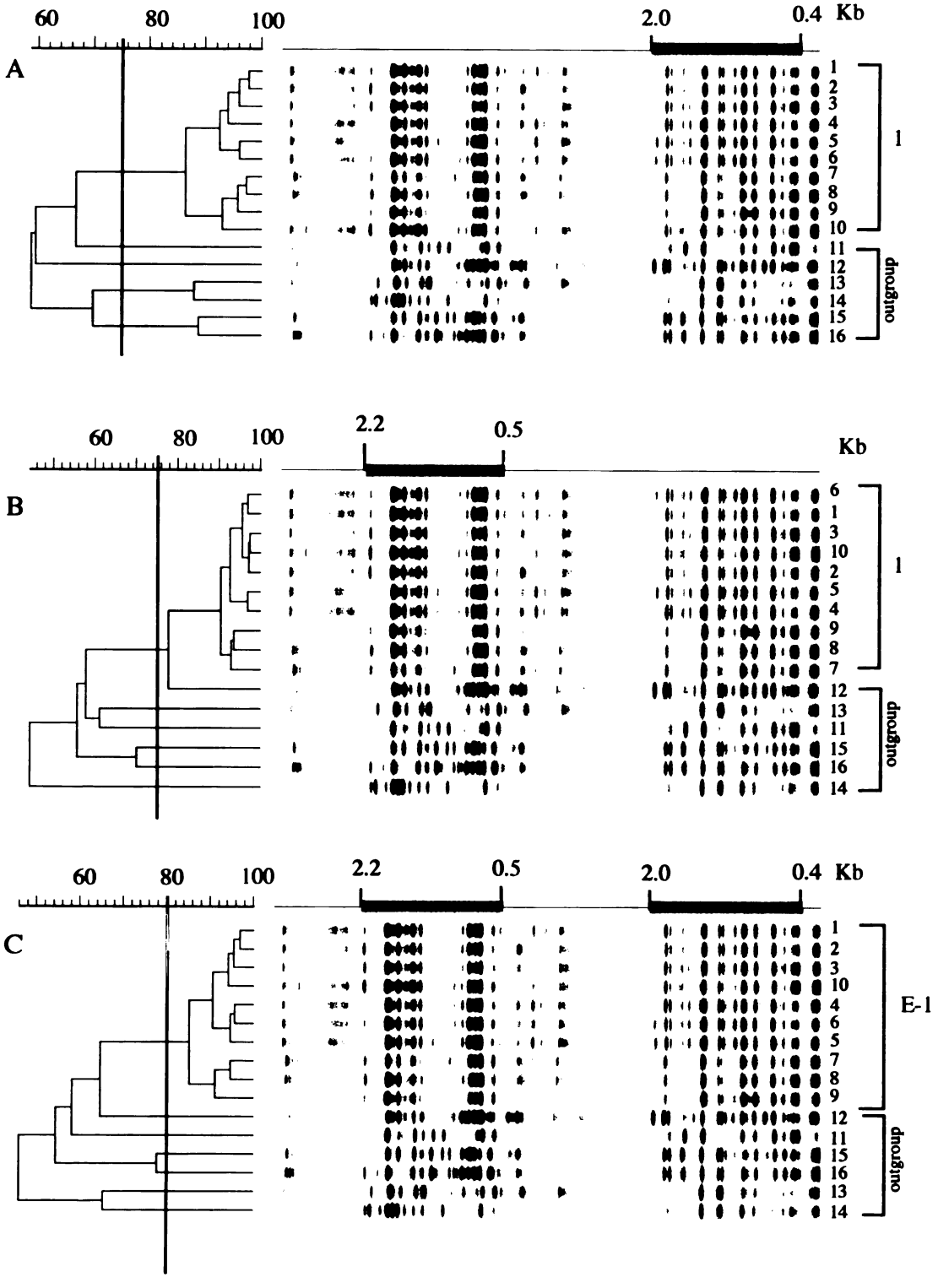


Figure 12. *Clavibacter michiganensis* subsp. *michiganensis* strains designated type D used for the cluster analysis (UPGMA) of the linearly combined $(GTG)_5$- PCR and BOX-PCR-generated genomic fingerprints in Figure 11C. The vertical line bisecting the dendrogram represents the experimental baseline (80%) used to define the groups. The designation of groups for the analysis of the linearly combined $(GTG)_5$-PCR and BOX-PCR-generated fingerprints are indicated on the right side of the figure.

Figure 13. Cluster analysis (UPGMA) using product moment correlation of two linearly combined $(GTG)_5$-PCR and BOX-PCR-generated genomic fingerprints of *Clavibacter michiganensis* subsp. *michiganensis* strains designated as type E. The gray bars above the fingerprint patterns ranging from 2.2 to 0.5 and 2.0 to 0.4 kilobase pairs for $(GTG)_5$-PCR and BOX-PCR, respectively, represent the area of the banding patterns used to generate the dendrograms shown on the left side of each panel. Panel A, analysis for BOX-PCR fingerprints; panel B, analysis for $(GTG)_5$-PCR fingerprints; and panel C, analysis for the two linearly combined $(GTG)_5$-PCR and BOX-PCR fingerprints. The vertical lines bisecting each dendrogram represent the experimental variability baseline used to define a cluster: 75.0 % for the dendrograms in panels A and B, and 80.0% for the dendrogram in panel C. The designation of groups for each analysis is represented on the right side of the panel

Similarity Index



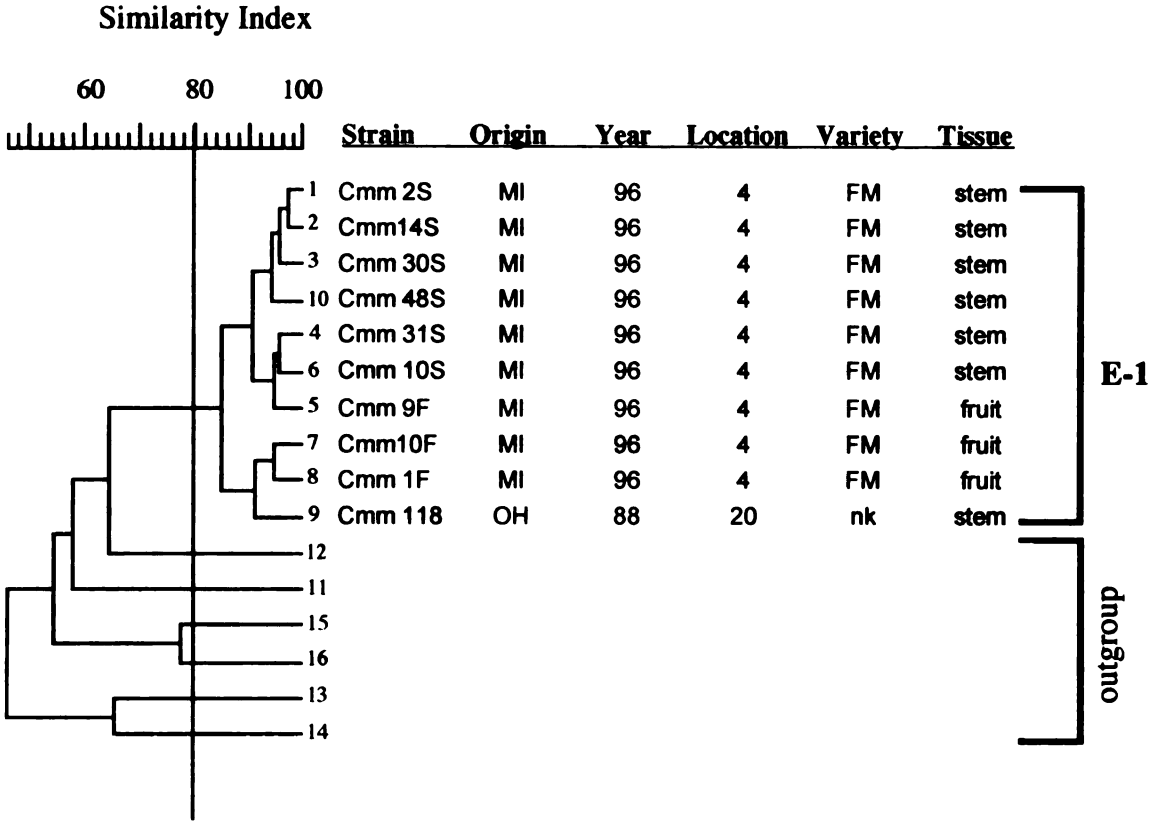


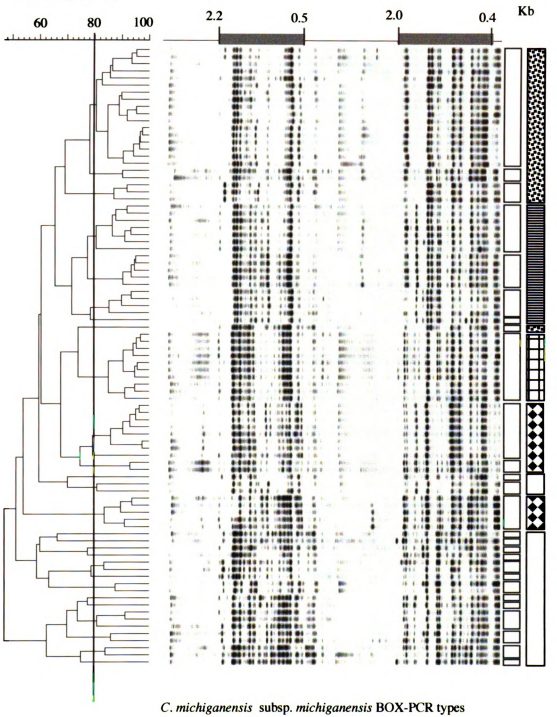
Figure 14. *Clavibacter michiganensis* subsp. *michiganensis* strains designated type E used for the cluster analysis (UPGMA) of the linearly combined $(GTG)_5$-PCR and BOX-PCR-generated genomic fingerprints in Figure 13C. The vertical line bisecting the dendrogram represents the experimental baseline (80%) used to define the groups. The designation of groups for the analysis of the linearly combined $(GTG)_5$-PCR and BOX-PCR-generated fingerprints, and six strains used to form an outgroup (Cmm14 (lane12), Cmm12 (lane 11), GH-71033 (lane 15), GH-7904 (lane 16), GH-71182 (lane 13), and Cmm56 (lane 14)) are indicated on the right side of the figure.

Michigan. This isolate always groups with two strains from Michigan, but these two isolates cannot be distinguished from other E type Michigan strains when the experimental variability baseline of 25% is used.

$(GTG)_5$-PCR fingerprints provides higher subdivision of *C. michiganensis* subspecies *michiganensis* strains.

Twenty-nine subgroups were resolved for the five BOX-PCR types defined for *C. michiganensis* subspecies *michiganensis* strains; five for type A, three for type B, twelve for type C, three for type D, and one for type E strains (Figure 15). The genetic diversity with $(GTG)_5$-PCR and BOX-PCR fingerprint patterns of the designated BOX-PCR types is as follows; $C > A \geq B > D > E$. Type C strains represent the most distinct group of the *C. michiganensis* subsp. *michiganensis* strains used in this study where these strains show 50% similarity to the other strains. Type D strains divided in two subgroups are 58% similar. The five subgroups of type A stains are 60% similar, while the type B strains are 62% similar to the other strains. Type E strains formed a single cluster, where the strains making up this group were 86% similar most likely because the majority of the strains were isolated from one location. The cluster analysis of $(GTG)_5$-PCR and BOX-PCR fingerprints indicate that type D, two subgroups of types C, B, and type E strains have closer similarity to the type A strains, while thirteen subgroups of the type C strains have more similar fingerprints among themselves. Overall, the cluster analysis of *C. michiganensis* subsp. *michiganensis* strains using both primers provided a more confident subdivision of the strains designated into the five BOX-PCR types probably because the combination of amplified bands consisted of information from more areas in the *C. michiganensis* subsp. *michiganensis* genome.

Figure 15. Cluster analysis (UPGMA) using product moment correlation of two linearly combined $(GTG)_5$-PCR and BOX-PCR-generated genomic fingerprints of eighty seven *Clavibacter michiganensis* subsp. *michiganensis* strains used in this study. The gray bars above the fingerprint patterns ranging from 2.2 to 0.5 and 2.0 to 0.4 kilobase pairs for $(GTG)_5$-PCR and BOX-PCR, respectively, represent the area of the banding patterns used to generate the dendrogram shown on the left side of the figure. The vertical line bisecting the dendrogram represents the experimental variability baseline (80.0%) used to designate the genotypes resulting from the linearly combined $(GTG)_5$-PCR and BOX-PCR-generated genomic fingerprints. The columns of non-patterned and patterned boxes on the right side of the figure represent the groups defined by the $(GTG)_5$-PCR and BOX-PCR-generated fingerprints in this study and the BOX-PCR genotypes previously designated by Louws et al. (1998), respectively.



C. michiganensis subsp. *michiganensis* BOX-PCR types

DISCUSSION

Most taxonomic studies of *Clavibacter michiganensis* have primarily focused on techniques that differentiated the various species of *Clavibacter* and the subspecies of *Clavibacter michiganensis* . With these studies and the recent reevaluation of the genus *Corynebacterium*, in which most of these species previously resided, *Clavibacter michiganenesis* is now arguably one of the best taxonomically resolved species of phytopathogenic bacteria (Davis et al., 1984). These prior studies, however, did not report on the diversity of strains making up the various subspecies, including *Clavibacter michiganensis* subsp. *michiganensis.* If diversity is present, recognizing this diversity could be useful in terms of understanding the dissemination and epidemiology of the pathogen around the world or in geographically localized regions.

Until this study was undertaken, our knowledge on the genetic diversity of *C. michiganensis* subsp. *michiganensis* strains was based on genomic fingerprints obtained with three rep-PCR primers; REP, ERIC, and BOX. With rep-PCR technology, Louws et al. (1998) grouped C. *michiganensis* subsp. *michiganensis* strains into four types based on a polymorphic region of the BOX-PCR fingerprint pattern and ignoring other polymorphic regions of the rep-PCR fingerprints. (Five types have since been recognized; Bell et al., 1997). They focused on the combination of bands obtained with BOX-PCR that migrated to the 1 kilobase pair (kbp) region. Strains described as type A fingerprints displayed three bands in this region, while type B and type E had only two bands each with different migration patterns. Louws et al. (1998) did not report on the newly recognized type E strains (Bell et al., 1997). Type C and D fingerprints also showed

banding pattern differences at the same 1 kbp region with both groups showing only one band but not at the same migration distance.

Using this narrow strain identification criterion, Louws et al. (1998) suggested that certain trends in strain recovery might prove useful in the understanding various aspects of bacterial canker epidemics that have occurred in Michigan and the Midwest since 1980. For example, they suggested type A strains were found mostly associated with processing tomatoes varieties; type B and C strains have been associated with both fresh market and processing tomatoes; and type D strains have, so far, only been associated with fresh market varieties. Although the biological significance of these trends is still unclear, a substantial number of strains (30%) in the type A group were avirulent in terms of wilt and canker symptom production and negative in the hypersensitive reaction assay. None of the strains in the other fingerprint groups were reported to be avirulent.

These studies were initiated to determine if a fourth primer, $(GTG)_5$ could provide more genotypic information or a greater resolution of the genomic fingerprint than had already been established using the BOX-PCR reaction. Our primary interest in this question stems from attempting to determine information about the primary source of infection in commercial Midwest tomato fields. Determining which strains may be found on certain farms in particular years may help determine if the inoculum is coming from previous infections on the farm or if the inoculum is newly introduced each year. For example, if the strains causing bacterial canker at a particular location were primarily BOX-PCR type C one year, and primarily of type A the next, it would be difficult to make any other conclusion than the inoculum was rapidly altered by a new source of

inoculum. But, if the fingerprint pattern remained the same year after year, then an argument could be made that the grower had an endemic source of inoculum on the farm or in the region that initiated the disease on the farm.

BOX-PCR provides opportunities to pursue these questions but it is important to determine if the genomic distinctions could be resolved to a greater level. For example, in work by Louws et al. (1998), they presented evidence that type C strains could be further resolved based on a few scattered polymorphic bands observed on the gels in areas other than the 1 kbp region. These polymorphisms were used in a study where strains were released in a greenhouse and several months later strains recovered from the field plot could be traced back to the greenhouse based on these bands and antibiotic resistance markers. These polymorphisms proved useful, and suggested that other, more useful and prevalent, fingerprint patterns still could be resolved.

My first attempt to answer questions involving phenotypic diversity with $(GTG)_5$-PCR was attempted on the BOX-PCR type A strains. Since many of these strains were recovered as avirulent as well as virulent, I wanted to determine if the resulting $(GTG)_5$-PCR banding patterns could be correlated to the virulence phenotype. The $(GTG)_5$-PCR reaction provided many more amplified bands, but the fingerprint patterns obtained with $(GTG)_5$ alone and in combination with BOX-PCR did not support any noticeable genomic differences based on virulence.

This was not too surprising as other research groups interested in the pathogenic response of *C. michiganensis* subsp. *michiganensis* to tomato have suggested that virulence genes are primarily harbored on two plasmids (Meletzus et al., 1993). Since it has been found that non-coding DNA regions are the main hybridization site for rep-PCR

primers and that plasmids are limited in the amount of non-coding sequences, it follows that the rep-PCR technique would not separate strains based solely on plasmid differences.

The question remained as to whether the computer-assisted analysis of BOX-PCR type strains (A-E) may be better delineated using genetic diversity of *C. michiganensis* subsp. *michiganensis* provided by $(GTG)_5$-PCR analysis. Other studies have demonstrated that the use of several primers provided more reliable information regarding the genetic diversity of a species because more areas in the genome are amplified resulting in more robust dendrograms (Louws et al., 1996; Schneider & de Bruijn, 1996; Rademaker & de Bruijn, 1997; Rademaker et al. 1999b). My studies using $(GTG)_5$-PCR and BOX-PCR fingerprint patterns agrees with previous studies in that clusters and strain identification were easily differentiated within each BOX-PCR genotype. Although *C. michiganensis* subsp. *michiganensis* strains are overall highly similar, more genetic variability usually resulted from the combined $(GTG)_5$-PCR and BOX-PCR fingerprint patterns than from using only one primer.

Perhaps the best example of this is with the BOX-PCR type A strains. Strains CmmT74, CmmT13, and CmmT63 were collectively grouped with all of the other type A strains when the BOX-PCR fingerprint was analyzed; however, when $(GTG)_5$-PCR fingerprint was analyzed these three type A strains were clearly differentiated based on a very prominent band; this band was not observed in any other A strain. Since there were other type A strains isolated from the same field in the same year that did not have the predominate large band, it can be clearly stated that tomato plants in these fields were infected by two different type A strains. Therefore, using just the BOX primer made the

infection of southwest Michigan's processing tomatoes appear like a clonal infection while $(GTG)_5$-PCR differentiated the strains involved in this infection providing a better genotypic resolution for multiple-year studies.

Strains placed in the A-1 group could be subdivided into two groups depending on where the experimental variability baseline (18 %) is placed. Placement of the experimental variability baseline was a concern I faced with all BOX-PCR types when analyzed with each primer alone or in combination. Type E strains remained the only exception as these strains consistently remained a single cluster. The experimental variability baseline for all reactions was calculated based on my finding that strains analyzed colony by colony and within the same colony showed up to a 75% variability when they, theoretically, should have been 100%. This variability could only be accounted for by experimental reaction error such as slightly different amounts of DNA, enzyme, or buffers added to each PCR reaction or by the placement of the reaction tubes in the heating block. These experimental reaction errors are only a concern if the strains are inconsistently placed within the different groups. If would be a major hindrance to this research effort if a known strain fell into group A-1 in one experiment and A-2 at another time. Therefore, until all strains have been analyzed several times for their position within each genotype, these dendrograms should be considered preliminary.

As stated above, my results suggested that type E strains represented a clonal population. Only if the experimental variability baseline is moved considerably, two strains, one from Michigan and the other from Ohio isolated several years earlier could be separated. However, there is no experimental support for doing this and the group, so

far, remains clonal. Recovering more type E strains and the use of other universal rep-PCR primers may prove useful in discovering diversity in the type E strains.

The type D strains required the use of a second primer to differentiate strains collected from the same location in different years. At first, the analysis of the strains recovered at the northeast Michigan farm was performed using only BOX-PCR and all strains appeared clonal to each other recovered from Michigan but distinct from some foreign strains sent to us from various locations throughout the world. When the isolates were analyzed with only $(GTG)_5$-PCR the strains showed genomic variability but nothing could account for the variability as Michigan isolates were mixed with the foreign isolates. After fingerprint patterns of the combined primers were analyzed, the type D strains collected from Michigan were separated from the foreign strains. Surprisingly, the type D strains isolated in 1994 were separated from the strains isolated in 1997. This indicated that the type D strains on the northeast Michigan farm in 1994 were not the same as those recovered on the farm in 1997 as each grouped into their own cluster. The differentiation observed between these strains provided a useful resolution that may contribute for a better understanding of bacterial canker epidemics because reproducible polymorphic DNA bands could be used as genetic marker to trace these strains to their primary source of inoculum.

Before too much emphasis is placed on the combined PCR genomic fingerprints in trying to explain the primary source of inoculum on a specific farm, in a specific region, during a given year or on a particular type of tomato, the nature of the PCR reaction itself should be analyzed and if possible confirmed using other molecular techniques such as restriction fragment length polymorphisms (RFLP) or monoclonal

immunological assays. The idea that one or two farms can harbor a specific pathogen, and that pathogen appears endemic in one PCR primer but appears genetically distinct when another PCR primer is used clouds the issue of genetic origin. How do these specific types come about? Is there some kind of genomic rearrangement within established strains? Could plamsids, by their presence, including integration or excision disrupt the genome such that genomic changes from year to year are observed in the population? Certainly this has not been observed readily in the subspecific grouping of pathovars of the xanthomonads, pseudomonads (Louws et al., 1995; Smith et al., 1995; Opgenorth et al., 1996; Pooler et al., 1996;and Weingart & Völksch, 1997) and erwinias (McManus & Jones, 1995). In those systems, clonality is commonly observed below the pathovar level. With *Clavibacter michiganensis* subsp. *michiganensis* diversity could be generated by small genomal rearrangements of the established strains. However, this argument is hard to explain in field environments, although an experiment conducted in closed environments have shown that over thousands of generations some changes of the genome can be observed using rep-PCR technology (Nakatsu et al., 1998)

Taking into considerations the cautions outlined above, it appears that we now have a powerful method to distinguish genomic variation among strains at a high level. Using both BOX-PCR and $(GTG)_5$-PCR reactions and GelCompar analysis, we should be able to determine if strains found on specific tomato farms change from year to year or season to season as would be expected with pathogen populations arriving by seed or seedling or whether they stay the same indicating clonal populations providing continuous reinfection on the farm. These studies will require long-term surveys of the farms and sampling procedures for adequate enumeration of genotypes.

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CHAPTER 3

DEVELOPMENT OF BIRD'S EYE FRUIT LESIONS AFTER THE APPLICATION OF *CLAVIBACTER MICHIGANENSIS* SUBSPECIES *MICHIGANENSIS* TO PROCESSING TOMATO FLOWERS

Bacterial canker of tomato, caused by *Clavibacter michiganensis* subspecies *michiganensis* (Smith, 1910) is a concern for tomato growers because the disease appearance is unpredictable and symptoms may have devastating consequences on tomato yield and quality (Bryan, 1930; Strider, 1969; Stephens & Fulbright, 1986; and Gitaitis, 1991). The systemic infection of tomato plants is primarily associated with marginal necrosis, wilt, curling of the leaves, and stem canker symptoms. Seedling infection may include white blister-like lesions on cotyledons and leaves (Basu, 1966). Infection of the fruit may result in the development of superficial small necrotic spots surrounded by white halos; these are called bird's eye lesions (Bryan, 1930; Strider, 1969). The presence of bacterial populations in the greenhouse as well as in the field may cause serious economic losses because infections can reduce the amount and the quality of the fruit produced from both processing and fresh market cultivars (Pohronezny & Volin, 1983; Chang et al., 1992b; Gleason et al., 1993; Ricker & Riedel, 1993). Populations of *C. m.* subspecies *michiganensis* may be introduced to a disease free area through the introduction of contaminated seeds (Bryan, 1930; Strider, 1969) and symptomless transplants (Gitaitis et al., 1992). Dhavantari (1989) reported that the use of 389 transplants from infected seeds could result in 74.9% disease incidence with a reduction of 53% in yield during a single year. Infected transplants may cause yield losses up to 80 percent for individual commercial growers (Gleason et al., 1993).

Cultural practices during tomato production may enhance secondary infection and dissemination in the field (Strider, 1969; Chang et al., 1991). The use of contaminated equipment such as pruning tools, wood stakes, transplanters, cultivators, and tillers may enhance the spread of the pathogen (Stephens & Fulbright, 1986; Carlton et al., 1994). In a more natural manner, splashing rain and overhead irrigation systems (Gitaitis et al., 1992) may also contribute to the dissemination of the epiphytic population harbored on the leaves, stems and fruits.

The production of tomatoes in a previously contaminated field may serve as an alternative source of inoculum since *C. m.* subsp. *michiganensis* can survive the winter season for at least one year in plant debris mixed within the soil (Strider, 1967; Basu, 1970). A study conducted in a California field demonstrated that more than 50% of the mature plants in the field showed canker symptoms when healthy transplants were sown on soil beds containing contaminated plant material from the previous year (Gorgan & Kendrick, 1953). Populations of *C. m.* subspecies *michiganensis* on plant debris may result in yield reduction on the following year's crop as shown in the studies conducted in Illinois and Iowa (Gleason et al., 1991; Chang et al., 1992a). Weeds within fields may also aid as a source of inoculum since nightshade, and jimsonweed species have been reported as epiphytic hosts (Weebb et al., 1967; Ricker, 1989; Chang et al., 1992a). Symptoms such as spotting, wilting, and vascular discoloration of cotyledons in alternative hosts such as pepper and eggplant have been reported (reviewed by Strider, 1969).

Prevention of the disease and control of the pathogen include the use of certified seed, healthy transplants, disinfected greenhouse materials and facilities, disinfected field

equipment, and fields rotated from tomatoes for at least two years (Bryan, 1930; Weebb et al., 1967; Dhanvantari, 1989; Gitaitis, 1991). Several reports conducted in Michigan have suggested the use of copper hydroxide at the greenhouse transplant stage to reduce the populations of *C. m.* subspecies *michiganensis* on transplants taken to the field. The suggested chemical regime benefits tomato production since there has been reported significant reductions in yield loss (Hausbeck et al., 1995; Bell et al., 1996; Hausbeck et al., 1999). Unfortunately, the application of these chemicals in the greenhouse do not appear to provide control for the occurrence of fruit spotting in the field (Hausbeck et al., 1995a; Hausbeck et al., 1999).

Some have concluded that the appearance and the development of fruit lesions are primarily dependent on systemic or superficial infections of injured fruits caused by abiotic events such as sandblasting (Pohronezny et al., 1992; Pohronezny et al., 1993). However, it has been well accepted that foliar infections do not require wounded tissue because tomato leaves contain trichomes which can harbor high populations of *C. m.* subspecies *michiganensis* (Kontaxis, 1962; Layne, 1967). Other reports (Bashan et al., 1981; Getz et al., 1983a; Ehrig & Griesbach, 1985) suggest that tomato fruit contain trichomes hairs similar to those found on leaves, suggesting that the trichomes may be involved in the fruit infection process. Blanke (1986) reported that the stomata present on the epidermis of tomato fruits may serve as portals of entrance during bacterial infection. Higgins (1922) suggested that there is only a short period of time early in the development of the pepper fruit when it is susceptible to infection with *Xanthomonas campestris pv. vesicatoria.* Getz et al. (1983a & 1983b) demonstrated that flower inoculation resulted in fruit infection especially during and after anthesis (pollen

maturity) as the ovary is already under development during these stages. Getz et al. (1983a) sprayed various flower developmental stages with *Pseudomonas syringae* pv. *tomato* with the resulting fruit developing speck lesions. Fankle et al. (1993) studied the ingress of watermelon fruit blotch after application of the bacterium *Acidovorax* (*Pseudomonas*) *avenae* subsp. *citrulli* to the surface of young fruit. They agree with the conclusion drawn by Getz et al. (1983a) in that young fruits are more susceptible to bacterial infection than older fruits. Such methods could be used to help understand *C. michiganensis.* subspecies *michiganensis* infection and bird's-eye lesions development. Therefore, the objectives of this work were to 1) determine if bird's eye lesions are the result of external flower infection and, 2) if it is, determine the flower stage which provides the maximum number of fruit spots.

MATERIALS AND METHODS

Strains used. The *Clavibacter michiganensis* subspecies *michiganensis* strains used in each experiment represent distinctive BOX-PCR fingerprint types designated as B type (Cmm 68) or A type (Cmm 292) (Louws et al., 1998). Cmm 68 and Cmm 292 were isolated from fruit lesions from Ohio (1987) and Michigan (1993) fields, respectively. Both strains were recovered from glycerol stocks (1 ml broth culture/0.5 ml 40% glycerol) kept at -80° C and streaked onto nutrient broth yeast extract agar without glucose (NBY; Fatmi & Schaad, 1988) plates. Pathogenicity tests on tomato plants and

the hypersensitive response (HR) (Gitaitis, 1990) on four-o'clock leaves (*Mirabilis jalapa*) were conducted with both strains to determine their virulence.

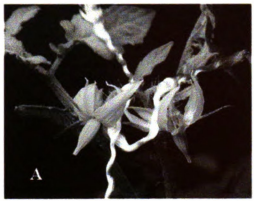
Inoculum was prepared by inoculating 5ml of NBY broth (no agar) with either bacterial strain and placing it on a shaker (New Brunswick Scientific NJ, USA) at 200 rpm at room temperature for 48 hours. A 100 ml of NBY broth was inoculated with the fresh broth culture and cultured for an additional 48 hours as described. A 10 ml dilution was prepared from each broth culture. Spectrophotometer (Baush and Laumb Spec 20) readings were performed at 600nm for each dilution to calculate the culture volume necessary to prepare a bacterial suspension of approximately 1 x 10^8 colony forming units (cfu)/ml (50% transmittance) with a final volume of 100 ml. The diluted culture was transferred to Oakridge tubes and centrifuged in a desktop clinical centrifuge (IEC Clinical Centrifuge MA, USA) for 15 min. at maximum speed. The pellet was resuspended in 20ml of sterile distilled water (sdW), recentrifuged, and the cells resuspended in 100ml of sdW. One milliliter of every bacterial suspension was stored in 0.5ml of glycerol (40%) in a micro-centrifuge tube (DOT Scientific MI, USA) at -20 C. One to three days after the inoculum preparation, a 10-fold serial dilution of each suspension was made by placing aliquots on NBY to enumerate the exact concentration of the inoculum used in each experiment. The final concentration of the inoculum used for all tomato flowers inoculations ranged from 3 x 10^7 - 3 x 10^8 cfu/ml. Prior to flower inoculation, HR of each bacterial suspension prepared was tested for its ability to induce an HR, a condition assumed to relate directly with the virulence of the pathogen.

Two tomato transplants of the susceptible processing variety (Heinz 8704), were sown in 12 inch clay pots amended with commercially prepared potting media (Bacto).

Tomato plants were grown in the greenhouse for approximately eight weeks under sodium lights with a photoperiod of 18 hours. Tomato plants were fertilized twice each week with a diluted solution (1/16) of Peter's fertilizer (20-20-20) at 20 ppm.

Tomato flowers were tagged at the petiole with colored yarn to differentiate various developmental stages. Inoculations were performed by applying approximately 0.7 ml of the washed *C. michiganensis* subspecies *michiganensis* suspension per flower using an air pressurized sprayer (Preval, Precision Valve Co. NY, USA); sterile distilled water (sdW) was used as control. A small circle, approximately 25 mm in diameter, was cut in the bottom of a clear plastic cup (2 ounces) large enough to cover a single flower with a wide open corolla. Individual flowers were covered with the plastic cup while applying the inoculum or sdW to direct the fine mist toward each flower and to limit the amount of aerosol landing on flowers and tissues of the immediate plants (Figure 16B).

Fruit spot development. To develop a repeatable method with which to obtain fruit spots similar to bird's-eye lesions, four treatments were initially evaluated. The first treatment consisted of a one-time spraying of 2-4 where the corollas were open and wide open (Figure 16A) per bract (1x Cmm). The second treatment consisted of a similar inoculation except three days following the initial spray, a second spray was made (2x Cmm). The third and fourth treatments were sprayed as in the treatments one and two above, and following each inoculation, individual bracts were covered with clear plastic bags (7in x 7in x 2.5in) for 16-18 hours (1x Cmm + bag and 2x Cmm + bag, respectively) (Figure 16C). For every combination mentioned above, sdW was substituted as a control inoculation (1x H_2O and 2x H_2O for one or two water applications, respectively). Each



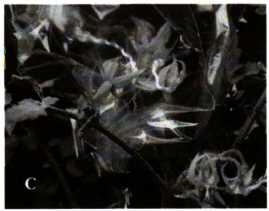


Figure 16. Spray inoculation method used to study fruit spot development on processing tomatoes. A) tomato flowers representing open corolla stages; B) use of plastic cup method to prevent spread of aerosol to other flowers and plants; and C) incubation of flowers for 16-18 hours with plastic bag used for third (1X Cmm + bag) and fourth (2X Cmm + bag) treatments.

treatment was conducted once in the spring and once in the fall. For the four treatments indicated above, an average of 66 and 30 flowers were used for each experiment in the spring and in fall, respectively. Three weeks after the second inoculation date for the second (2x Cmm) and fourth treatments (2x Cmm + bag), all of the tomato fruits were inspected for bird's eye-lesions; the number of fruit infected and the number of spots per fruit were recorded. All inoculations and incubations were performed during early evening when relatively cooler temperatures were recorded in the greenhouse. Although the two *C. michiganensis* subsp. *michiganensis* strains, Cmm68 and Cmm292, used in the spring and in fall experiments belong to different BOX-PCR genotypes, these experiments were considered as replicates because Cmm68 and Cmm292 shared phenotypic characters such as virulence, plant tissue from where these were isolated, and development of bird's eye fruit lesions with typical appearance.

To obtain a higher incidence of diseased fruit an alternative method of inoculation was attempted. Bacterial suspensions were prepared as described above and using a small artist paintbrush (camel's hair; #2) inoculum was directly applied to the surface of young green fruits 22 mm in diameter. Eight *C. michiganensis* subspecies *michiganensis* strains were selected based on their difference in BOX-PCR type and virulence rating (Louws et al., 1998). Strains Cmm 299 (A-type), Cmm 68R (B-type), and Cmm 285 (D-type) are classified as virulent; Cmm T33 (A-type), Cmm 236 (C-type), and Cmm 56 (C-type) represent strains with intermediate virulence; and Cmm 208 (A-type), and Cmm 133 (A-type) are classified as avirulent. Each bacterial suspension was applied to a total of five fruits; one fruit per plant, and tested for HR on four-o'clock leaves. Five fruits were inoculated with SDW to serve as the control. This experiment was conducted once

during spring. Four weeks after fruit inoculation, the number of infected and noninfected fruit and the number of spots per fruit were recorded.

The average number of spots per fruit for each treatment from both experiments were analyzed by using Kruskal-Wallis ANOVA by ranks ($P < 0.0001$) (SIGMASTAT). Differences between treatments were identified using the pairwise multiple comparison by Dunn's method.

Flower development stage and fruit spots. To determine the flower stage most susceptible to the development of fruit spots, the bacterial suspension was applied to five flower developmental stages by following the protocol for the third treatment (1x Cmm + bag) described above. The five flower developmental stages were defined using anthesis (pollen maturity) as the reference point. Bhadula and Sawhney (1987) established that calyces of *Lycopersicon esculentum* (Mill) showing 11.0 -12.0 mm in length with an open corolla corresponded to flowers at anthesis; therefore, the first stage of flower development, considered to be about five days prior to anthesis consisted of buds with closed calyces about 11-12 mm in length. The stages included in this experiment were five (-5) and three (-3) days pre-anthesis, anthesis (0), two (+2), and four (+4) days post-anthesis and each of these stages corresponded to the following flower appearance, closed calyx, open calyx, open corolla, wide open corolla, and half-closed corolla, respectively (Figure 17). For each flower developmental stage, sdW was used for control inoculations (H_2O). Three replicates (I, II, and III) were conducted in the spring, and an average of 57, 65, and 66 flowers were used for each replicate, respectively. When the youngest fruit reached 27 mm in diameter, the number of infected and noninfected fruit

Figure 17. Tomato flowers representing four of the five developmental stages used in the experiment to determine the susceptibility of flowers to infection with *C. michiganensis* subsp. *michiganensis*.

and the number of spots per fruit were recorded. All inoculations and incubations were preformed during early evening. The average number of spots per fruit for each replicate was statistically analyzed as mentioned above.

To confirm that the observed fruit spots were caused by the inoculum used for each experiment and for each replicate, randomly selected fruit spots were removed using a sterile scalpel. Each fruit spot was placed in a droplet of sdW for 5 seconds to let bacteria ooze from the plant material. Each droplet was streaked onto NBY plates and incubated for 96 hours at room temperature. After pure culture of single-colonies, suspected of being *C. michiganensis* subspecies *michiganensis* were subjected to rep-PCR fingerprinting protocol (Louws et al., 1998) to demonstrate that the BOX-PCR genotype was the same as the inoculum used in each experiment.

RESULTS

Fruit spot development. To determine the best technique with which to obtain fruit spots, flowers with open corollas were inoculated by spraying with 1 $X10^8$ *C. michiganensis* subsp. *michiganensis*. Both *C. michiganensis* subsp. *michiganensis* strains used in this study, Cmm68 and Cmm292, were considered virulent strains and produced strong hypersensitive reactions in the HR assay conducted in parallel with this study.

When they appeared, fruit spots could be found as typical or atypical spots. Typical fruit spots were observed as white lesions on small green fruit (10-12 mm in diameter), 6-8 days after inoculation. Three to five days after the white lesions first appeared, typical bird's-eye lesions developed, as the center of the lesion became necrotic

(Figure 18). Atypical fruit spots were necrotic spots similar in size to the bird's-eye lesions but lacking the characteristic white halo. The development of the necrotic area appeared about the same time after inoculation in both types of lesions. In spite of this difference, both the typical and atypical fruit spots were small, circular, and superficial with a raised blister-like appearance approximately 2 mm in diameter. Upon reisolation, both typical and atypical fruit spot lesions yielded the *C. michiganensis* subsp. *michiganensis* strains used as inoculum in each experiment, as determined by rep-PCR assays.

The number of inoculations and the treatment of the flower after inoculation were varied in these experiments. Fruit spots were observed in all treatments, but the number of spotted fruit obtained was affected primarily by the number of inoculations each flower received (Table 2). Fruit developing from flowers that were inoculated twice with either bacterial strain (2x Cmm treatments), showed the highest number of spotted fruit and the highest number of fruit spots per fruit. The maximum number of spotted fruit ranged from 78 to 80 %, and 53 to 70 % was observed on fruit that developed from flowers sprayed twice with Cmm68 and Cmm292, respectively. As with the results observed for the number of spotted fruit, the maximum number of spots per fruit was obtained from the flowers inoculated twice. The maximum number of spots per fruit ranged from 7.1 to 12.0, and 7.6 spots per fruit when Cmm68 and Cmm292, respectively, was applied to open flowers two times three days apart. The least amount of diseased fruit was obtained when the flowers were inoculated only once (1x Cmm) and these fruit averaged less than one spot per fruit. The minimum number of spotted fruit ranged from 14 to 32 %, and 9 to 28 % was observed on fruit that developed from flowers sprayed

Figure 18. Tomato fruit showing bird's eye lesions eight weeks after *C. michiganensis* subsp. *michiganensis* inoculum was sprayed on flowers with an open corolla.

Table 2. Fruit spotting development after spraying open corolla flowers with strains of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm68 and Cmm292).

Treatment	Total number of [1]		Incidence [2]	Average [3]
	flowers	fruit	(% diseased fruit)	# spots/ fruit
1x Cmm68	31	29	14	0.4 a [5]
2x Cmm68	31	30	80	12.0 b
1x Cmm68 + bag [4]	36	31	32	2.1 a
2x Cmm68 + bag	29	23	78	7.1 b
1x Cmm292	81	46	9	0.2 a
2x Cmm292	128	61	70	7.6 b
1x Cmm292 + bag	64	29	28	1.2 a
2x Cmm292 + bag	122	72	53	7.6 b

[1] Number of flowers (open corolla) sprayed with Cmm versus the number of fruit that developed from those flowers.

[2] Percent of fruit showing fruit spotting.

[3] Total number of spots observed per total number of fruit that developed per treatment.

[4] Incubation of Cmm on flowers after covering individual bracts with clear plastic bags for 16-18 hours after inoculation.

[5] Statistical analysis using Kruskal-Wallis ANOVA by ranks and pairwise multiple comparison by Dunn's method. (P= <0.0001)

once with Cmm68 and Cmm292, respectively. As with the results observed for the number of spotted fruit, the minimum number of spots per fruit was obtained from the flowers inoculated once. The minimum number of spots per fruit ranged from 0.4 to 2.1, and 0.2 to1.2 spots per fruit when Cmm68 and Cmm292, respectively, was applied to open flowers once regardless of the presence or absence of the bag. Bagging the flowers to hold in moisture after inoculation did not provide an obvious advantage as similar number of spots appeared on flowers inoculated twice regardless of the bagging regime used. Bagging may have increased the number of spots obtained with the single inoculation treatment, but the results were not statistically significant when compared to the flowers without bags. No fruit spots developed on uninoculated control flowers. The largest number of spotted fruit occurred when the surface of young fruit was inoculated using a paintbrush to apply the bacterial suspensions (Table 3). Strains determined to be virulent in previous studies and causing a hypersensitive reaction in the HR assay in this study resulted in 100% of the inoculated fruit developing fruit spots. Strains classified as intermediate in virulence and also causing positive hypersensitive reactions in HR assays in this study resulted in 67-100% of the inoculated fruit developing fruit spots. Surprisingly, strains determined to be avirulent in previous studies and not producing hypersensitive reactions in HR assays in this study resulted in 75-80% of the inoculated fruit developing fruit spots. The number of spots developing per fruit was not statistically related to the strains used, however, avirulent strains produced the lowest number of spots, and intermediate and virulent strains produced the highest number of spots with one exception. Strain Cmm236, previously determined to be intermediate in

Table 3. Fruit spot development after direct application of *Clavibacter michiganensis* subsp. *michiganensis* to the surface of young tomato fruit.

Virulence [1]	Strain(s) used	Incidence [2] (% diseased fruit)	Average [3] # spots/fruit
V	Cmm 299	100	60.8 a [4]
	Cmm 68R	100	66.8 a
I	Cmm T33	67	45.0 a
	Cmm 236	100	109.8 a
	Cmm 56	100	24.2 a
AV	Cmm 208	80	15.4 a
	Cmm 133	75	27.3 a

[1] Virulent= producing canker and wilt; I=intermediate, producing canker only; and AV= avirulent, producing no symptoms (Louws et al., 1998).

[2] Percent of fruit showing fruit spotting.

[3] Total number of spots observed per total number of fruit that developed per treatment.

[4] Statistical analysis using Kruskal-Wallis ANOVA by ranks and pairwise multiple comparison by Dunn's method. (P= 0.03)

virulence produced the largest number of spots per fruit than any other strain used in the study.

Flower development stage and fruit spot development. To determine the flower developmental stage most susceptible to infection, five flower developmental stages were established and flowers were inoculated with a strain of *C. michiganensis* subsp. *michiganensis* at each stage. The majority of the flowers inoculated post-anthesis (+2 and +4) developed into fruit with fruit spots (Table 4). The highest numbers of spots per fruit were also obtained from flowers inoculated during post-anthesis (+2 and +4). Those flowers inoculated prior to anthesis rarely developed fruit spots. No fruit spot lesions were observed when flowers were inoculated with water.

The number spots per fruit in this experiment was similar to those observed in the fruit spot development experiment described above where the bacteria were delivered by aerosol inoculation. For example, strain Cmm68 (Table 2) in the fruit spot development experiment produced approximately 2 fruit spots per fruit when inoculated once and bagged. In this experiment, strain Cmm68 was also inoculated once and bagged, and again, produced approximately 2 fruit spots per fruit. Although a higher incidence of fruit spots may have been achieved using two inoculations instead of one, in the flower development stage experiment, I decided to use only one inoculation since the flowers would have been at a different development stage when inoculated the second time, 3 days later.

Table 4. Fruit spotting after spraying flowers at various developmental stage wit *Clavibacter michiganensis* subsp. *michiganensis* strain Cmm68.

Flower Stage Anthesis (days)	Total number of fruits [1]			Incidence [2] (% diseased fruit)			Average [3] # spots/fruit		
	I [4]	II	III	I	II	III	I	II	III
-5	30/56	12/71	22/89	0	0	0	0.0 a [5]	0.0 a	0.0 a
-3	23/54	14/74	20/121	0	0	5	0.0 a	0.0 a	0.05 a
0	48/56	29/70	16/79	6	0	0	0.1 a	0.0 a	0.0 a
+2	54/55	47/72	57/102	30	15	42	1.7 b	0.8 a	2.8 b
+4	46/58	59/77	35/65	30	20	37	1.4 b	0.5 a	1.9 b

[1] Number of fruit that developed from flowers sprayed with Cmm68.

[2] Percent of fruit showing fruit spotting.

[3] Total number of spots observed per total number of fruit that developed for each treatment.

[4] I, II, and III = three independent replicates conducted during spring.

[5] Statistical analysis by Kruskal-Wallis ANOVA by ranks and pairwise multiple comparison by Dunn's method. (P= <0.0001)

DISCUSSION

Symptom expression in plants infected with bacteria is dependent on many factors including the genetics of the pathogen, age of the tissue infected, availability of infection sites, as well as environmental conditions (Strider, 1969). Previous studies with *C. michiganensis* subsp. *michiganensis* have shown that young leaves are more susceptible to infection by leaf blighting phytopathogenic bacteria than older leaves (Kontaxis, 1962; Layne, 1967). Leaf trichomes have been implicated in bacterial canker infection (Kontaxis, 1962) and Layne (1967), demonstrated that young tomato leaves contained higher numbers of trichomes per unit area than older leaves suggesting that the leaf trichomes of young leaves may provide an opportunity for growth and a more conducive environment for subsequent infection.

Fruit age also may influence the expression of the fruit spot symptoms in bacterial infection of plants (Gardner & Kendrick, 1923; Bryan, 1930; Getz et al., 1983; and Pohronezny et al., 1993). Getz et al. (1983b) reported that young fruit were highly susceptible to external infection with *Pseudomonas syringae* pv. *tomato* and showed the presence of trichomes on the surface of tomato ovaries soon after anthesis (Getz 1983a). They suggested that these fruit trichomes may provide an avenue for infection as natural openings occur through the process of trichome breakage. Scanning electron microscopy revealed the presence of *P. syringae* pv. *tomato* cells inside holes resulting from broken trichome-bases.

Similar processes with the bacterial canker bacterium may be involved with fruit infection. One confounding aspect of bacterial canker infection, that is not an issue with other tomato bacterial diseases that produce fruit spots, is that the disease is often

systemic, rapidly moving through the xylem. This has led some researchers to speculate that the fruit spots develop from internal systemic infection. All of my studies strongly suggest that flowers externally infected with *C. michiganensis* subsp. *michiganensis* early after anthesis would produce fruit with bird's eye lesions. It can be concluded that flowers at post anthesis are more susceptible to *C. michiganensis* subsp. *michiganensis* infection than flowers inoculated prior to anthesis. While the studies presented here do not rule out the possible role of internal infection causing fruit spots, they agree with the conclusions drawn by Getz et al. (1983) that spotted fruit can result from nonwounding, external infection of ovaries.

The large number of spots obtained when small green fruit were inoculated with bacterial suspensions may be a result of trichome injury, physical disruption of the epidermis and waxes positioned to prevent pathogen invasion or simply a more direct method of delivery of the pathogen to infection courts. This method of inoculation should allow a simple, more consistent and objective method to enumerate the effect of the pathogen on the host. It should be useful in selecting tolerant or resistant varieties judging efficacy of bactericides, or determining the virulence of *C. michiganensis* subsp. *michiganensis* strains.

Establishing the earliest developmental stage when flowers become susceptible to bacterial pathogens might be useful to formulate new, better targeted, control strategies. For example, the application of bactericide(s) at flowering or before flowering for the determinant tomato varieties may decrease the number of spotted fruit because populations of the pathogen may decrease and remain in low numbers during critical times for infection. Nontraditional control strategies such as biological control or

genetically engineered hosts could also benefit from knowledge of fruit spot development. Effective control agents could be selected that might specifically colonize flowers and ovaries out-competing or otherwise inhibiting the pathogen. Genetically engineered tomatoes could also deliver inhibiting compounds in targeted cells, such as trichomes on the ovary.

Surprisingly, few spotted fruit developed from flowers inoculated at anthesis or pre-anthesis stages after inoculation with high levels of inoculum. It seems that if a large population of the pathogen is in place a few days before the optimum infection point, those cells making up the population should still be in place when the appropriate time for infection arrives. Apparently, *C. michiganensis* subsp. *michiganensis* was not a competent epiphyte under conditions provided during this experiment. Several factors may be responsible for this observation. It is possible that bacterial multiplication may have been hampered due to the relatively low humidity found in the research greenhouses used in this study. Keeping the plants moist by placing a plastic bag around the flowers after inoculation did increase the number of spotted fruit, although not statistically significant (Table 2). The bagging treatment may have provided the moisture needed for continued colonization of the bacterial canker pathogen but the bacteria may not have been engaged in processes of active infection such as moving off of the petals to the trichomes and surface of the fruit. If bacterial multiplication continued and if the pathogen successfully colonized the early flower developmental stages, it could be possible that significant numbers of the bacterial population were lost during petal-fall, a common event during tomato fruit development. As a result, epiphytic cells were not efficiently delivered to the susceptible stage or tissue for fruit spot development. It is

also possible that the low number of fruit spots obtained when inoculating at anthesis or earlier is an artifact of the greenhouse inoculation protocol due to the lack of wind, rain and water splashing that would normally occur in the field. Pohronezny et al. (1992) reported that a high number of fruit spots resulted from infection with *Xanthomonas campestris* pv. *vesicatoria* if the pathogen is at high concentrations at natural openings. They suggested that, although the number of natural openings in pepper fruits is limited, the bacterial population is critical for the development of fruit spots in peppers. In their study, they showed that environmental factors, such as wind and rain splashing, could promote the production of natural openings on fruits.

It is also possible that certain *C. michiganensis* subsp. *michiganensis* strains are more genetically capable of dissemination than others and that the strains used in this study were not as capable of moving from petals to ovaries as others. I used at least two strains with different histories, as indicated in the Materials and Methods, in the fruit spot development experiments. More definitive answers on the effect of strains versus early flower inoculation could be obtained by doing similar experiments under controlled environments in growth chambers with other strains of the pathogen.

The most likely reason for delivering the inoculum prior to the most susceptible stage and not seeing significant infection is a combination of two events. The first event may be due to the bacterial population being delivered after it is already in the stationary phase of growth. This means that for active growth to occur on the new substrate (the plant), cells must go through a lag phase before growth can occur again. In the meantime, death phase has been reached in the older cells of the population while the flower is dramatically altering its tissues and shedding petals. Those flowers inoculated twice

probably had the population re-enforced during the most susceptible time for infection to occur.

The higher number of spotted fruit developing from flowers sprayed twice with or without a bag, supports the proposition that bacterial numbers are important for fruit infection. While obtaining large numbers of spotted fruit were convenient for purposes of these experiments, just a couple of fruit spots per tomato fruit can reduce the quality and acceptability of the fresh market tomato.

During this study, high levels of flower abscission occurred resulting in low number of fruit on several occasions. Getz et al. (1983b) and Pohronezny et al. (1993) also observed the abortion of a high number of non-mature tomato and pepper fruit during their studies. Various reasons may cause flower and young fruit abscission such as poor pollination, nutrient deficiency, inappropriate light intensity and soil media, as well as poor ventilation (personal communication with Dr. Ivin Widers- Michigan State University). The paintbrush method of applying the inoculum avoided the problem of flower abortion as only the fruit to survive flower abortion were inoculated. I observed no significant fruit abortion after inoculation of the fruit with the pathogen (data not shown).

A normal distribution of the number of spots per fruit was not observed in any of the experiments conducted in this study. The source of variability could have emerged from various factors with biological implications such as inconsistent environmental conditions, diverse localization of plants in the greenhouse as well as the localization of the flowers infected on each plant, and variability in the interaction between the host and the pathogen.

In conclusion, two protocols to obtain spotted fruit through external inoculation of tomato flowers and fruit under greenhouse conditions have been developed. Studies, such as this one, are necessary if we are to attempt further experiments involving the management of bacterial canker symptoms on fruit as well as increase our knowledge of the host-pathogen interaction of this important pathogen.

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REFERENCES

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CONCLUSIONS

Rapid and highly discriminatory strain identification has been accomplished by DNA-based fingerprinting techniques such as the repetitive sequence-based Polymerase Chain Reaction (rep-PCR). Rep-PCR protocols have been instrumental in differentiating and classifying strains of microorganisms of medical, agricultural, and economic importance. This technique has also provided new insights into the concepts of diversity and speciation. The use of universal rep-PCR primer such as REP, ERIC, and BOX has provided genetic information of microorganisms where genomic characteristics of the strains were limited or lacking. The accurate identification of genetic variants of pathogenic microbes is critical for understanding epidemics in regards to animal, human, or plant pathogens.

The study of genomic diversity in the phytopathogenic bacterium *Clavibacter michiganensis* subspecies *michiganensis* (*Cmm*), the causal agent of bacterial canker of tomato has proven to be an excellent example in which to demonstrate the discriminatory potential of rep-PCR. This is in part, due to the unique characteristics of the disease cycle in relationship with the tomato production in northern states. The presence of polymophic DNA fragments of *Cmm* strains below the subspecies level may provide useful tools for finding the primary source of inoculum. The results of this study have demonstrated the use of another rep-PCR primer, $(GTG)_5$, for the differentiation of *Cmm* strains. $(GTG)_5$- PCR can be used alone or in combination with BOX-PCR to provide even greater resolution of the genotypic diversity of *Cmm*. Various questions still remain unanswered in regards to the relationship between the sporadic appearance of the disease and the predominant genotype(s) responsible for the severity of the diverse symptom

expression in each field, in each region of Michigan and North America. *Cmm* strains of different pathogenic abilities, ie. avirulent and virulent, can be present in the same field during an epidemic. Unfortunately, the differentiation of these pathogenic variants was not possible using the combined data generated from the BOX-PCR and $(GTG)_5$-PCR fingerprinting patterns. The potential of other DNA fingerprinting techniques such as restriction fragment length polymorphism (RFLP) in conjunction with rep-PCR protocols to differentiate pathogenic types needs to be further explored.

Until this study was conducted, the origin of inoculum responsible for the fruit spots was unknown. In this study two protocols were developed to obtain spotted fruit. Using these techniques, I was able to conclude that epiphytic populations of *Cmm* was responsible for the primary source of epidermal fruit infection leading to the development of bird's eye lesions. The development of bird's eye lesions through application of *Cmm* through aerosols probably mimicked the natural mode of dissemination of the pathogen but it was time-consuming and tedious. The application of *Cmm* using an artist's paintbrush was more reliable and reproducible than aerosols but it was artificial in terms of inoculum application. Surprisingly, the paintbrush technique demonstrated that a strain recognized as avirulent in stem inoculation assays could still insight fruit spots, although at lower level than virulent strains. The paintbrush technique should prove valuable in enumerating studies involving virulence, resistance, chemical treatments, and biocontrol.

APPENDICES

APPENDIX A

BACTERIAL CANKER: SYMPTOMS

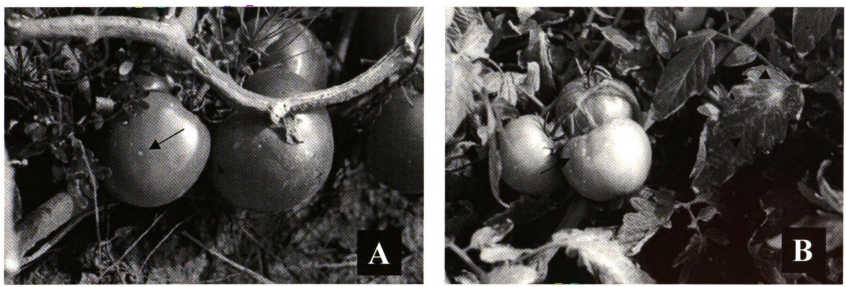

Figure 19. Bacterial canker symptoms on mature tomato plants and fruit. Panel A, canker-necrosis along the stem and fruit lesions (arrow); and panel B, marginal necrosis on leaves, known as "leaf firing" (arrow heads), and white lesions on fruit (arrow).

APPENDIX B

BACTERIAL CANKER: DISEASE CYCLE

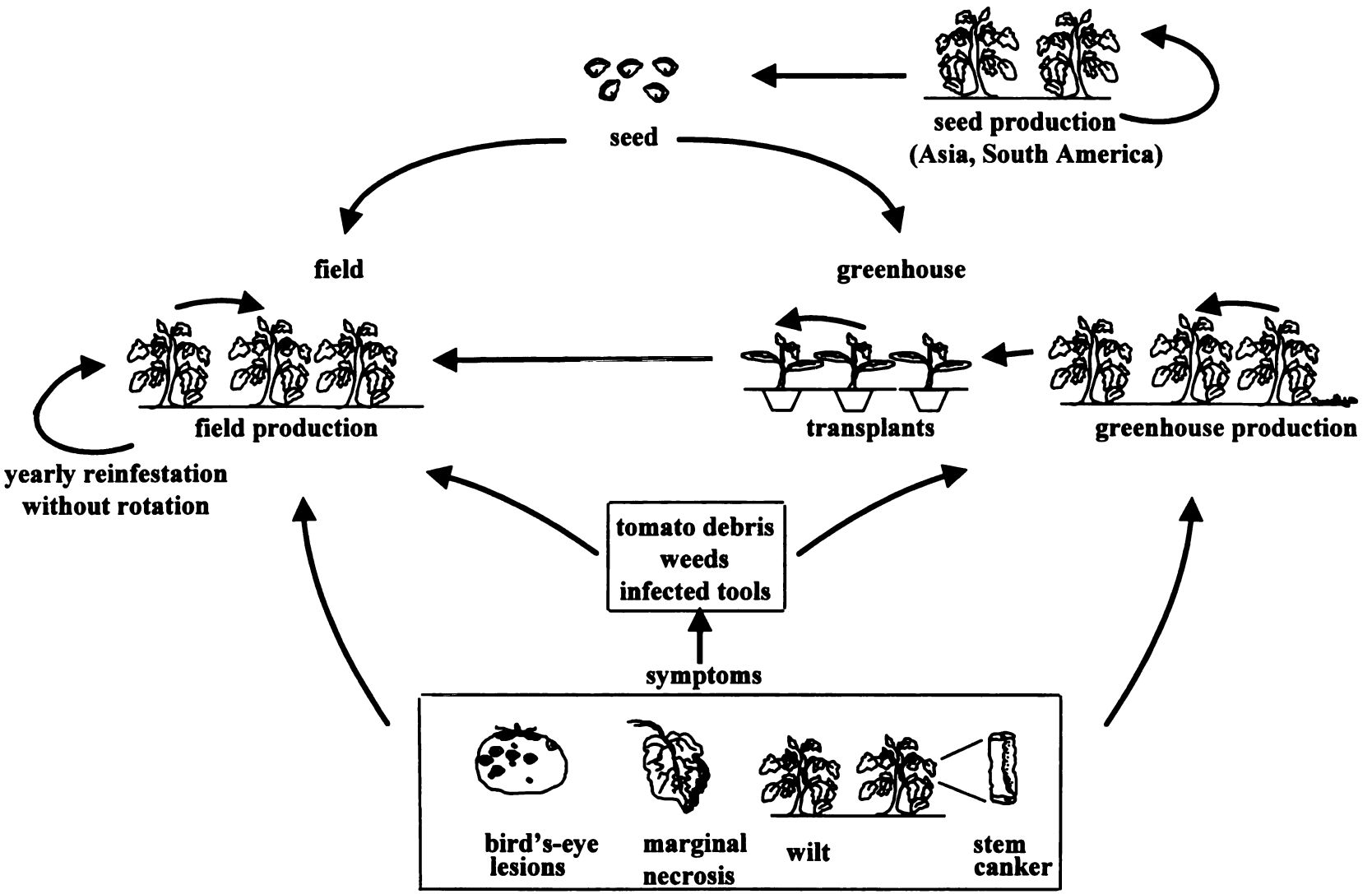

Figure 20. Disease cycle of the bacterial canker disease of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis*.

APPENDIX C

BACTERIAL CANKER: DISEASE CYCLE COMPONENTS

Table 5. Various components of the bacterial canker disease cycle caused by *Clavibacter michiganenesis* subsp. *michiganensis*.

Inoculum source	Infection avenues	Modes of dissemination
Seed **Tomato debris** **Alternative host** e.g. nightshade weed **Infected equipment**	**Natural openings** Trichomes Stomata Hydathodes **Tissue Injury** pruning/clipping/ tying wind- sandblasting	**Natural** seed, wind, rainsplash **Cultural practice** commercial seed trade overhead irrigation infected equipment e.g. pruning tools

APPENDIX D

BACTERIAL CANKER: CONTROL

Table 6. Management strategies for control of bacterial canker of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis*.

Management Strategies	
Cultural practices	**Copper-based bactericide**
certified seed and transplant	copper hydroxide (e.g.Kocide)
removal of tomato debris and alternative hosts	**Biological control**
field rotation	avirulent strains
disinfecting tools and equipment (e.g. mercury-based chemicals)	bacteriocin production
	Resistant cultivars
	possible, but not currently available

APPENDIX E

CHARACTERIZATION AND IDENTIFICATION

Table 7. Phenotypic and genotypic methods used for the characterization and identification of *Clavibacter michiganensis* subsp. *michiganensis* strains.

Phenotypic	**Genotypic**
Colony morphology • Nutrient Broth Yeast Extract (NBY) • Semiselective media for *C. michiganensis* (SCM)	DNA probes/ Restriction fragment Length Polymorphism (RFLP) • MIC 1
Serology (polyclonal and monoclonal antibodies) • ELISA • Immunology-based dot blot • Immunofluorescence (IF)	Polymerase Chain Reaction (PCR) primers • CM3 & CM4 • CMM-5 & CMM-6
Cellular fatty acid fingerprinting • Fatty Acid Methyl Ester Analysis (FAME)	Ribosomal DNA • ITS & 16S rDNA
Metabolism (Biochemical methods) • Biolog	**DNA fingerprinting** • Repetitive-sequence based PCR (rep-PCR); REP, ERIC & BOX primers • $(GTG)_5$-PCR