DETECTION AND CHARACTERIZATION OF VIRULENT, HYPOVIRULENT, 
AND NONVIRULENT CLAVIBACTER MICHIGANENSIS SUBSP. MICHIGANENSIS

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Abstract

*Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), a seedborne quarantine pathogen, causes bacterial canker of tomato, which is a serious disease that can severely decrease yields in greenhouse and field production areas. Seed assays are used to prevent dissemination of *Cmm* through infested seed, but limitations in assay sensitivity and specificity allow canker outbreaks to continue. An assay was developed that detected *Cmm* when as few as 10 colony forming units (cfu) were present per 50 ml sample. The assay used a three-unit filtration system to capture bacterial cells, followed by a four-day membrane incubation and a colony blot immunoassay using the CMM1 monoclonal antibody (MAb). The filtration and immunoassay technique was more sensitive than a standard spread plating assay, and could potentially reduce current assay times by up to 3 weeks.

Assays done on nine seed lots yielded a high percentage (81%) of MAb CMM1-positive colonies that were hypovirulent or nonvirulent on tomato (*Lycopersicon esculentum* cv. Kewalo). All strains were confirmed as *Cmm* using the MicroLog™ identification system, rep-PCR, two PCR primer sets, and an endoglucanase assay. Of the assays tested, MicroLog™ and rep-PCR were the most consistent in identifying hypovirulent and nonvirulent MAb CMM1-positive strains as *Cmm*.

The CMM1 MAb was further used to quantify *in planta* movement and multiplication of a nonvirulent *Cmm* strain when coinoculated with a virulent *Cmm* strain. *In planta* coinoculation did not significantly alter growth or colonization habits of either strain. Thus, there is no evidence that nonvirulent *Cmm* strains play a role in
bacterial canker epidemiology. However, their continued isolation from diseased and infested tissues, and the importance of nonvirulent strains in other pathosystems, suggest significance and warrants further investigation.
# Table of Contents

Acknowledgements
Abstract
List of Tables
List of Figures
Chapter 1. Literature review
The disease: Bacterial canker of tomato
The pathogen: *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*)
Phytosanitary health and the international seed trade
Detection and identification of *Cmm* from seed
Filtration and filter immunoassays
Pathogenicity and virulence in *Cmm*
*Cmm* interactions *in planta*
Future outlook
Chapter 2. A filtration and colony blot immunoassay technique for detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seed
Abstract
Introduction
Materials and Methods
Results
Discussion
Chapter 3. Identification of *Clavibacter michiganensis* subsp. *michiganensis* strains isolated from infested tomato seed
Abstract
Introduction
Materials and Methods
Results
Discussion
Chapter 4. *In planta* interactions between coinoculated virulent and nonvirulent *Clavibacter michiganensis* subsp. *michiganensis*
Abstract
Introduction
Materials and Methods
Results
Discussion
Chapter 5. Conclusions
Literature cited
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Tomato seed lots used in this study</td>
<td>33</td>
</tr>
<tr>
<td>2.2</td>
<td>Seed lots tested for <em>Clavibacter michiganensis</em> subsp. <em>michiganensis</em> infestation for use in filtration assay validation studies</td>
<td>34</td>
</tr>
<tr>
<td>2.3</td>
<td>Comparison of a spread plating procedure and the filtration-colony blot immunoassay technique for detection of <em>Clavibacter michiganensis</em> subsp. <em>michiganensis</em> in infested tomato seeds</td>
<td>35</td>
</tr>
<tr>
<td>3.1</td>
<td><em>Clavibacter michiganensis</em> subsp. <em>michiganensis</em> reference strains</td>
<td>55</td>
</tr>
<tr>
<td>3.2</td>
<td>Characterization of <em>Clavibacter michiganensis</em> subsp. <em>michiganensis</em> reference strains</td>
<td>56</td>
</tr>
<tr>
<td>3.3</td>
<td>Characterization of 31 MAb CMM1-positive strains isolated from five infested seed lots</td>
<td>57</td>
</tr>
<tr>
<td>4.1</td>
<td>Strains of <em>Clavibacter michiganensis</em> subsp. <em>michiganensis</em> used in this study</td>
<td>73</td>
</tr>
<tr>
<td>4.2</td>
<td>Comparison of bacterial populations in plant tissues pre- and post-inoculation</td>
<td>73</td>
</tr>
</tbody>
</table>
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Three-unit filtration system for recovery of <em>Clavibacter michiganensis</em> subsp. <em>michiganensis</em> from seed extract.</td>
<td>36</td>
</tr>
<tr>
<td>2.2</td>
<td>Decrease in recoverable populations of <em>Clavibacter michiganensis</em> subsp. <em>michiganensis</em> from seed lot 19 over two years.</td>
<td>37</td>
</tr>
<tr>
<td>2.3</td>
<td>Photograph of a nitrocellulose membrane developed using the colony blot immunoassay procedure.</td>
<td>38</td>
</tr>
<tr>
<td>2.4</td>
<td>Recovery of <em>Clavibacter michiganensis</em> subsp. <em>michiganensis</em> from <em>Cmm</em>-only liquid suspensions using one- and three-unit filtration systems.</td>
<td>39</td>
</tr>
<tr>
<td>2.5</td>
<td><em>Clavibacter michiganensis</em> subsp. <em>michiganensis</em> recovery percentages from mixed liquid suspensions containing increasing concentrations of a single seed saprophyte strain.</td>
<td>40</td>
</tr>
<tr>
<td>3.1</td>
<td>Agarose gel electrophoresis of BOX-PCR products from <em>Clavibacter michiganensis</em> subsp. <em>michiganensis</em> reference strains and putative <em>Cmm</em> strains from seed.</td>
<td>59</td>
</tr>
<tr>
<td>3.2</td>
<td>Agarose gel electrophoresis of BOX-PCR products generated from seed saprophytes and putative <em>Clavibacter michiganensis</em> subsp. <em>michiganensis</em> seed strains.</td>
<td>60</td>
</tr>
<tr>
<td>3.3</td>
<td>Agarose gel electrophoresis of PCR amplification products from selected strains of <em>Clavibacter michiganensis</em> subsp. <em>michiganensis</em> using the CMM-5/CMM-6 primer set.</td>
<td>61</td>
</tr>
<tr>
<td>4.1</td>
<td>Enumeration of nonvirulent <em>Clavibacter michiganensis</em> subsp. <em>michiganensis</em> strain A4748 recovered from coinoculated plants.</td>
<td>74</td>
</tr>
<tr>
<td>4.2</td>
<td>Populations of nonvirulent (A4748) and virulent (A4758) <em>Clavibacter michiganensis</em> subsp. <em>michiganensis</em> recovered from single strain-inoculated and coinoculated tomato stems.</td>
<td>75</td>
</tr>
</tbody>
</table>
Chapter 1

Literature review

The disease: Bacterial canker of tomato

Bacterial canker of tomato (Lycopersicon esculentum Mill.), caused by the gram positive pathogen, Clavibacter michiganensis subsp. michiganensis (Smith) Davis et al. (1984), is an important disease affecting both greenhouse and field tomato crops. First thoroughly described in Michigan by E. F. Smith (1910), incidence of bacterial canker was later reported from most tomato-growing regions of the world, including all major tomato seed-producing areas (Strider, 1969). In Hawaii, canker outbreaks have occurred infrequently, but have caused significant damage to the local tomato industry when they have appeared (Chun, 1982).

Disease symptoms differ depending upon whether the infection is systemic or localized (Gleason et al., 1993). These symptoms may also vary with differences in virulence of the infecting strain, environmental conditions, cultivar susceptibility and age at infection, and the site of infection (Strider, 1970; Forster and Echandi, 1973; Chang et al., 1992). For vascular infections, unilateral wilting of the leaves, often starting with the lower leaves, normally occurs first. Wilting is followed by canker development on the plant stems and petioles. Symptoms from a localized infection may appear as leaf spotting or blight, or marginal necrosis (“firing” of the foliage). Under certain circumstances, the pathogen may enter the vasculature of the plant from these localized lesions and cause symptoms of systemic infection as well (Strider, 1969). Heavily

1
infected plants may not set fruit. Fruit that develop from infected plants may be stunted or malformed, and may exhibit yellowing or browning of the vascular strands. Fruits may also produce raised pustules surrounded by white halos (bird’s-eye spots) resulting from surface infection (Medina-Mora et al., 2001). Economic losses from bacterial canker outbreaks reflect decreased tomato production due to plant death, but can also be caused by declines in fruit production exhibited by infected plants, or reduced fruit quality for fresh-market sale.

The pathogen: *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*)

*Cmm* is one of five separate bacterial populations that make up the *C. michiganensis* species. The five subspecies (*insidiosus*, *michiganensis*, *nebraskensis*, *sepedonicus*, and *tessellarius*) were first combined under the name *Corynebacterium michiganense* based on cellular protein similarities (Carlson and Vidaver, 1982), and were later moved to the newly-created genus *Clavibacter* based on the presence of 2,4-diaminobutyric acid in their cell wall peptidoglycans (Davis et al., 1984). Although they share high DNA homology, all are readily distinguishable from each other by their distinct host ranges.

*Cmm* can infect a number of solanaceous hosts (Thyr et al., 1975), but is only economically important on tomato and, under some conditions, on pepper (Volcani et al., 1970; Lai, 1976; Lewis Ivey and Miller, 2000). Infection of tomato can occur through stomata (Smith, 1914), hydathodes (Carlton et al., 1998), and through injured or uninjured trichomes (Kontaxis, 1962). When introduced directly into the vascular
system, as few as 5 to 9 cells are needed to infect two to four week old seedlings (Thyr, 1968; Strider, 1970).

Outbreaks of bacterial canker initiated by *Cmm* populations harbored in infested tomato debris or soil (Grogan and Kendrick, 1953; Strider, 1967), or by epiphytic populations of *Cmm* (Gleason et al., 1991; Chang et al., 1992), have been found to play a role in disease epidemiology under favorable conditions. However, the primary source of inoculum implicated in canker epidemics worldwide is that carried into a production area by infested seed (Strider, 1969) or through latently infected tomato transplants (Chang et al., 1991; Gitaitis et al., 1991). Smith (1920) first hypothesized that *Cmm* could infest tomato seeds after observing “yellow slime” close to the seeds in immature fruit. Bryan (1930) first provided direct evidence for seed transmission with her findings of the bacterium in the fleshy outer seed coat. Seed transmission is highly variable, and has been reported to range from less than 1% to 100% (Fenner, 1931, as cited in Strider, 1969; Grogan and Kendrick, 1953; Strider, 1969; Fatmi and Schaad, 1988). It has been estimated that even one infested seed in 10,000 that grows into an infected plant could potentially infect 74-124 other plants/ha during normal production procedures (Gitaitis et al., 1991). When used for production of tomato transplants, rates of seed transmission as low as 0.01% to 0.05% may result in field epidemics (Chang et al., 1991). The importance of preventing seed lots with even low *Cmm* infestation from commercial distribution, therefore, cannot be overemphasized.
Phytosanitary health and the international seed trade

The estimated value of the world seed market is between $40-60 billion dollars (International Seed Federation [http://www.worldseed.org/statistics.html]; Condon, 1997). The United States commercial seed market comprises a tenth of this value (Condon, 1997). Because a large portion of this trade depends upon the international movement of seeds, developing and implementing accurate and reliable seed health testing methods is critical in preventing worldwide dissemination of potentially devastating diseases (Hutchins, 1997). At present, national and international seed testing methods lack uniformity that sometimes results in seed lots of questionable quality being released for sale from certain testing sites (Meijerink, 1997; Tylkowska, 1997).

Compounding this quality control problem is the inherent difficulty of extracting certain types of pathogens from infested seed. Unlike fungal pathogens that usually can be detected by low-power microscopic examination of individual seeds for spores, bacterial pathogens typically exist in low populations on and/or within the seed and, many times, are greatly outnumbered by faster growing, non-target saprophytic organisms. This makes it difficult to test for the slower-growing pathogens using single seed plating methods since the saprophytes often overpopulate a plate before the pathogen has time to establish itself and, in some cases, may also compete with or antagonize the target organism (Alvarez et al., 1997; van Vuurde, 1997; Schaad et al., 1999).

Numerous assay procedures have been developed to detect plant pathogenic bacteria from seed, either by direct methods (growing-on tests), indirect methods (bioassays, selective media plating of seed extract) or by nonviable methods to detect the pathogen directly from extract (antibody-based assays, DNA probes, phage testing).
Schaad, 1982, 1989a). Suggested methods to determine optimal extraction procedures and assay development parameters have also been published (Schaad, 1982; Roth, 1989). At present, polyphasic schemes involving one or more of the above methods dominates testing protocol. Seed extract plating to semiselective or selective media, in particular, is widely used because isolated colonies are then available for further identity confirmation tests using differential media, bioassays, or, more frequently now, immunodiagnostic or DNA-based assays (Schaad, 1989a).

Immunodiagnostic techniques such as immunofluorescence (IF) and indirect and direct ELISA are commonly used to identify pathogens isolated from seed (Sheppard et al., 1989; Schaad, 1989b; van Vuurde and van den Bovenkamp, 1989; Gleason et al., 1993). IF and the related technique, immunofluorescent colony staining (IFC; van Vuurde, 1997), are also used to detect target pathogens directly from seed extract. IFC, in particular, can provide 10,000-fold greater sensitivity than direct ELISA. With the IFC technique, sample size may be increased and smaller numbers of cells (1 to 10 cfu/sample) can be detected (Alvarez et al., 1997). One major drawback cited of immunodiagnostics—lack of adequate specificity—has been nearly overcome by replacing polyclonal antibodies with more specific monoclonal antibodies. Commercial ELISA kits are now available for a wide range of pathogens and are being used routinely for pathogen confirmation (Alvarez, 2001).

DNA-based techniques using species-specific oligonucleotide primers with the polymerase chain reaction (PCR) have gained rapid popularity for confirmation purposes due to their specificity and speed. In addition, methods using nested primers (Schaad et al., 1995), and primers that amplify conserved regions of the bacterial genome in order to
generate a strain “fingerprint” (Versalovic et al., 1994) have been developed that improve upon specificity or sensitivity of the standard technique.

Detection assay sensitivities vary with individual protocols, and depend in part upon the amount of analyte (in most cases, seed or plant extracts) they are designed to test as well as the host-pathogen system being tested. Schaad et al. (1999) found direct PCR and other DNA-based methods to be more sensitive than those based on ELISA when used to detect C. michiganensis subsp. sepedonicus (Cms). Slack et al. (1996), on the other hand, reported comparable sensitivity with direct PCR and indirect ELISA, but decreased sensitivity using DNA hybridization when detecting Cms in infected potato tubers. In another study with Xanthomonas oryzae pv. oryzae, DNA-based assays performed directly on subsamples of seed extract were less sensitive than assays using enrichment followed by an immunodiagnostic procedure. In addition to increasing initial pathogen concentrations through an enrichment step, the latter assay was also capable of handling sample sizes that were 20 to 50 times larger than the DNA-based tests (Alvarez et al., 1997).

Detection and identification of Cmm from seed

The use of sensitive and reliable seed testing methods becomes especially important with internal pathogens that are difficult to eradicate with standard seed treatments (Fatmi et al., 1991), as is the case with Cmm. Unfortunately, accurate diagnoses from infected or infested seed are sometimes difficult due to the uneven distribution and apparently low levels of pathogen in even “highly infested” seed lots.
Several methodologies for detecting *Cmm* in seed and plant material have been described. Early seed assays for *Cmm* included direct plating of seeds onto agar plates (Bryan, 1930), grow-out tests (Ark, 1944), or tomato bioassays with ground seed extract (Thyr, 1969). Numerous semiselective media have been developed for direct plating of seed extract (Chun, 1982; Fatmi and Schaad, 1988, Kritzman, 1991; Waters and Bolk, 1992; Alvarez and Kaneshiro, 1999). Presently, the most common methods of detection employ either semiselective plating or IF using polyclonal or monoclonal antibodies, or a combination of the two (Fatmi and Schaad, 1989; OEPP/EPPO, 1998; ISHI, 2001). More recently, a nondestructive PCR assay was developed that shows potential commercial use (Zriba et al., 1999).

Isolated *Cmm* colonies from seed extract can be identified with ELISA using monoclonal antibodies (Alvarez et al., 1993), by *Cmm*-specific PCR primer sets (Dreier et al., 1995; Sousa Santos et al., 1997), or by plant bioassays (Fatmi and Schaad, 1989; Gitaitis, 1990; OEPP/EPPO, 1998; ISHI, 2001). Repetitive sequence-based (rep)-PCR fingerprinting is also used to generate strain fingerprints that can confirm the identities of putative *Cmm* (Fulbright et al., 1998; Louws et al., 1998; Medina-Mora et al., 1999; Werner, 2001).

**Filtration and filter immunoassays**

Filtration techniques for concentrating low numbers of bacteria in highly proteinaceous samples are commonly used in food microbiology, and are accepted detection methods used for a number of pathogens (Sharpe, 1994). In general, however, these methods tend to employ single filters, and membrane blockage by highly turbid
samples often limits the volume each system can handle efficiently. For detection of *Salmonella* in poultry, for example, between 200 µl to 10 ml of the initial sample can be efficiently passed through hydrophobic grid membrane filters for optical discrimination of target colonies (Farber et al., 1985; Sharpe, 1994; Hoszowski et al., 1996). As an alternative, a three-unit system consisting of seven filters and membranes was developed as a means to filter up to 400 ml of chicken carcass washings for *Salmonella* detection. This system enabled Hoszowski et al. (1996) to recover the pathogen from washes spiked with as few as 5 cfu of *Salmonella* per 400 ml sample. Multi-unit systems such as this, however, appear to be rare in the literature.

Detection of target pathogens recovered on a terminal filter is possible using immunodetection protocols. Colony blot immunoassays, specifically, have been developed for detection of a range of pathogens and probiotics in food microbiology (Hoszowski et al., 1996; Batina et al., 1997; Ingram et al., 1998; Carroll et al., 2000), clinical and veterinary microbiology (Roop et al., 1987; Gustafsson and Askelof, 1989; Magyar and Rimler, 1991; Duez et al., 2000), and in environmental monitoring (Bérubé et al., 1989; Szakál et al., 2001). Due to their relative simplicity and inexpensiveness, colony blot immunoassays are preferred over DNA-based identification methods, since they can be applied even in less developed geographic areas where workers have limited access to expensive equipment. They often also exhibit greater specificity and/or sensitivity, and are more rapid, than current protocols.
Pathogenicity and virulence in *Cmm*

Based on visual observations of infected plants (Pine et al., 1955; Patino-Mendez, 1966, as cited by Strider, 1969; Wallis, 1977) and on tomato cutting experiments using purified *Cmm* extracellular polysaccharides (EPS; Rai and Strobel, 1969), the typical wilting symptom of *Cmm*-infected plants was hypothesized to be caused either by water stress induced by a high titers of the bacterium in the vasculature, a phytotoxic EPS, or enzymatic degradation of the plant vessels by wilt-inducing enzymes. With the recent development of cloning vectors and transformation protocols for *Cmm* (Meletzus and Eichenlaub, 1991), the molecular basis of pathogenicity and virulence is now being studied (Jahr et al., 1999).

Recently, two large endogenous plasmids, pCM1 (27.5 kb) and pCM2 (72 kb) were isolated from a pathogenic strain of *Cmm* (Eichenlaub et al., 1991). Plasmid analysis of virulent *Cmm* strains had been done previously (Vidaver, 1982; Chamot and Fulbright, 1988; Finnen et al., 1990), but no correlation between pathogenicity and plasmid status was found. Curing of either plasmid pCM1 or pCM2 resulted in reduced and delayed wilting, whereas curing of both plasmids eliminated symptom expression altogether (Meletzus et al., 1993). Interestingly, cured strains were still capable of stem colonization and EPS production, both in culture and *in planta* (Eichenlaub et al., 1991; Meletzus et al., 1993). Insertion of the putative pathogenicity locus from pCM2 by electroporation of the plasmid vector into endophytic, plasmid-free *Cmm* converted nonpathogens into wilt inducers (Dreier et al., 1997).

It is now known that plasmids pCM1 and pCM2 carry the *celA* gene for endo-β-1,4-glucanase production, and the *pat-1* locus, of unknown function, respectively (Dreier...
et al., 1997; Jahr et al., 2000). Endo-β-1,4-glucanases, in particular, have been found important to disease development in both *Cmm* (Jahr et al., 2000) and in *Cms* (Laine et al., 1996). However, pathogenicity test results with *celA* and *pat-1* deletion clones (Dreier et al., 1997; Jahr et al., 2000) indicate that multiple genes encoded on these plasmids must be expressed for full virulence (Jahr et al., 1999).

**Cmm interactions in planta**

Previous work (Fulbright et al., 1998; Louws et al., 1998; Werner, 2001) has shown that both virulent and nonvirulent *Cmm* strains can be present in the same infected fields, and even coexist in mixed infections. The significance of these nonvirulent *Cmm* strains to the bacterial canker disease cycle has not been determined, but work with other plant pathogens may provide some initial clues.

Work with gram negative plant pathogens has revealed that at least two types of interactions can occur when nonvirulent strains are present *in planta*. These pathogens possess a suite of hypersensitive response and pathogenicity (*hrp*) genes that are necessary for pathogenicity on host plants (Lindgren, 1997). Virulent, wild type strains possessing functional *hrp* genes are also capable of producing a hypersensitive response (HR) on nonhost plants. Although *hrp* genes have not yet been found in gram positive organisms, *Cmm* and the closely related *Cms* are also capable of eliciting HR, and have been found to secrete HR-inducing proteins (Gitaitis, 1990; Nissinen et al., 1997; Alarcón et al., 1998). Studies with *hrp* mutants exhibiting attenuated *in planta* growth and virulent wild type strains of *Pseudomonas syringae* pv. *syringae* (Hirano et al., 1999) and *Xanthomonas campestris* pv. *campestris* (Kamoun and Kado, 1990) showed that
coinoculation allowed the mutant strains to multiply to near wild type levels. This phenomenon was hypothesized to occur due to exocellular components secreted by the wild type strains that complemented the defective gene in the mutant.

In other studies, two nonvirulent strains, each exhibiting only one of two phenotypic and/or genotypic characteristics normally expressed together in virulent wild type strains, could cause symptoms when coinoculated into a susceptible host (Bellemann and Geider, 1992; Hirano et al., 1999). The nonvirulent strains caused no symptom expression when inoculated individually, indicating that each strain contributed some factor(s) the other lacked to cooperatively cause disease. Interestingly, recent work with Cms indicated that two nonvirulent strains possessing certain phenotypic traits could complement each other in planta to elicit disease symptoms on eggplant (Nissinen et al., 2001).

As in the case of Cmm, plasmid-borne virulence factors exist in many plant pathogenic bacteria (Coplin, 1982; Vivian et al., 2001). Transfer of conjugative plasmids has been demonstrated in vitro (Coplin, 1982), but evidence of in planta plasmid transfer is lacking for most pathogen systems. The exception to this is Agrobacterium tumefaciens, which can mobilize its Ti plasmid into avirulent A. radiobacter and cause virulence (Kerr, 1969; Vicedo et al., 1996).

**Future outlook**

The Cmm-tomato system is currently being used as one model system for revealing the host-pathogen interactions of gram positive plant pathogens. Complete sequencing of the Cmm genome is presently underway (http://www.genetik.uni-
bielefeld.de/GenoMik/cluster2.html). In conjunction with a recently developed transposon mutagenesis protocol (Kirchner et al., 2001), this genome project should eventually reveal the chromosomal genes responsible for *Cmm* infection and colonization of the host plant. Additionally, planned studies on *in planta* interactions between virulent, hypovirulent, and nonvirulent *Cmm* strains should reveal the importance of each subpopulation to bacterial canker development, and the potential for gene transfer within strains of the subspecies.
Chapter 2

A filtration and colony blot immunoassay technique for detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seed

**Abstract**

A procedure consisting of a three-unit filtration system to capture bacteria from seed extract, followed by a membrane incubation period and a colony blot immunoassay was developed to test up to 50 ml seed extract volumes for *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) infestation. *Cmm* was recovered when as few as 10 colony forming units (cfu) were present per sample (0.2 cfu per ml). The CMM1 antibody used in the immunoassay differentiated *Cmm* colonies from bacterial saprophyte colonies in seed extract, even when *Cmm*-to-saprophyte ratios reached 1:149. When compared with a standard spread plate assay using two semiselective media, the filtration protocol identified 6 of 6 infested seed samples, while the spread plate assay identified only 4 of 6 samples. This protocol improves detection sensitivity over existing seed assays, allows accurate *Cmm* identification, and can reduce assay time by as much as 3 weeks.

**Introduction**

Current seed assays have limited ability to detect low concentrations of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), especially when contaminated seed lots also have high backgrounds of saprophytes with *Cmm*-like colony morphologies. Such assays are particularly limited when larger seed extract volumes are
needed to evaluate a representative seed lot. The purpose of this study is to develop a method that improves the sensitivity of existing seed assay protocols by condensing the target bacteria from a larger sample volume, selectively enriching viable cells, and detecting the resulting pathogen colonies by colony blot immunoassay.

A three-unit filtration system was first developed to concentrate Cmm cells on 0.45 μm terminal membranes. Filtration concentration is often done in food microbiology, where highly turbid samples are commonly processed (Sharpe, 1994). In one example, a three-unit system consisting of seven filters and membranes was developed as a means to filter up to 400 ml of chicken carcass washings. This system enabled Hoszowski et al. (1996) to recover Salmonella from washes spiked with as few as 5 cfu of Salmonella per 400 ml sample.

A colony blot immunoassay based on the tissue blot immunoassay of Hu et al. (1997) was then developed to detect the captured Cmm on the terminal membranes after a four-day enrichment. Colony blot immunoassays have been developed for detection of a range of pathogens and probiotics in food microbiology (Hoszowski et al., 1996; Batina et al., 1997; Ingram et al., 1998; Carroll et al., 2000), clinical and veterinary microbiology (Roop et al., 1987; Gustafsson and Askelof, 1989; Magyar and Rimler, 1991; Duez et al., 2000), and in environmental monitoring (Bérubé et al., 1989; Szakál et al., 2001).

It was predicted that together, the filtration protocol followed by the colony blot immunoassay would provide both sensitive and specific detection of Cmm from seed. This hypothesis was tested by comparative assays between the final developed system and a standard seed extract spread plating assay.
Materials and Methods

Identification of Cmm-infested seed lots. Samples from nine tomato seed lots with suspected Cmm contamination were assayed by seed extract spread plating (Table 2.1). For large samples (10 to 25 g seed), seed extract was generated using a modified version of the seed washing-semiselective medium procedure described by Fatmi and Schaad (1989). Seed samples were placed in mesh-lined plastic Stomacher laboratory blender bags. Extraction buffer (0.01M phosphate buffered saline [PBS, pH 7.4], containing 100 μg cycloheximide per ml and 0.02% Tween 20) was added to each bag at a 1:3 seed-to-buffer ratio. Samples were incubated at room temperature for 4 h, and then processed for 15 min using a Stomacher 400 laboratory blender (Seward, London, UK). Extract from small seed samples (one gram or less) was generated by macerating seed in extraction buffer using a sterile mortar and pestle. Seed extracts were spread plated in 100 μl aliquots to at least five petri plates containing Cmm1 medium (Alvarez and Kaneshiro, 1999; consisting of 15 g agar, 10 g sucrose, 1.2 g Tris base, 250 mg magnesium chloride·7 H2O, 5 g lithium chloride, 2 g yeast extract, 1 g ammonium chloride, 4 g casamino acids, 200 mg cycloheximide, 28 mg nalidixic acid, and 10 mg polymyxin B sulfate, per L). In some cases, a modified SCM semiselective medium (SCM-Y, modified from Fatmi and Schaad, 1988 by increasing yeast extract to 2 g per L) also was used for isolation. Plates were incubated for at least 7 days at 28°C before assessment.

Colonies exhibiting Cmm morphology on Cmm1 (yellow/orange, semi-fluidal, convex, entire margins) or SCM-Y media (yellow-gray, semi-fluidal, convex, entire
margins) were transferred to yeast sucrose calcium carbonate agar (YSC), modified from YDC (Wilson et al., 1967) by replacing dextrose with sucrose. Strains appearing typical (yellow/orange, semi-fluidal, convex, entire margins) were purified and tested with the CMM1 MAb (clone 103-142; Alvarez et al., 1993). Other saprophytes were also purified and tested with the antibody.

A "toothpick ELISA" procedure was used to rapidly screen colonies from YSC. A second, confirmatory ELISA using standardized antigen concentrations ($1 \times 10^8$ cfu/ml) was used to confirm initial results. For toothpick ELISA, cells were transferred with a toothpick to microtiter plate wells (Costar, Cambridge, MA) containing 100 µl carbonate bicarbonate buffer (CBC, pH 9.6; consisting of 1.59 g sodium carbonate and 2.93 g sodium bicarbonate, per L). For the confirmatory ELISA, cells were grown on YSC and then incubated in PBS-formalin (PBS containing 0.5% formalin). Fixed cells were washed three times with 0.85% sodium chloride, resuspended in CBC and adjusted to an absorbance value of $A_{600} = 0.1$ (approximately $10^8$ cfu/ml). One hundred microliter subsamples were pipetted to microtiter wells. Coated plates from both ELISA procedures were air dried in a circulating air incubator. Dried plates were wrapped in plastic and stored at 4°C until tested.

Both assays followed a horseradish peroxidase (enzyme) - 5-aminosalicylic acid (5-AS; substrate) procedure similar to that developed by Alvarez and Lou (1985). Unless otherwise noted, 100 µl of each reagent were added to wells and incubated at room temperature for 1 hour. Prior to blocking, plates were flooded with boric acid buffer (10.3 g boric acid, 7.85 g sodium chloride, 1.1 g sodium hydroxide, per L) and incubated at room temperature for 15 min. Plates were emptied, and rinsed twice with boric acid
buffer. Wells were blocked using 200 μl of a 5% (wt/vol) PBS-powdered skim milk solution, and incubated at room temperature for 30 min. Wells were then sequentially incubated with MAb CMM1 ascites (1:8000 dilution), rabbit anti-mouse ascites (1:1000 dilution), and Protein A-horseradish peroxidase conjugate solution (1:2000 dilution; Bio-Rad Laboratories, Richmond, CA). Solutions containing ascites or horseradish peroxidase conjugate were prepared in 1.25% (wt/vol) PBS-milk. Microtiter plates were washed three times with boric acid buffer after each incubation step. Peroxidase substrate was prepared by adding 20 mg 5-AS to 50 ml PB-EDTA buffer (consisting of 0.651 g monobasic monohydrate sodium phosphate, 0.737 g dibasic sodium phosphate, and 35 mg disodium EDTA, per L 0.01M phosphate buffer), heated to dissolve the 5-AS powder, and cooled to room temperature. Immediately before use, 100 μl of 3% hydrogen peroxide was added to the solution. Positive reactions were visible by a change in substrate color from clear to dark brown. Readings were taken after an hour incubation using a microtiter plate reader set at 450 nm.

Positive strains were characterized further (Chapter 3). Both MAb CMM1-positive and negative strains were stored in water vials at 4°C and at −80°C in a 1:1 solution of modified Luria-Bertani broth (consisting of 10 g tryptone, 5 g yeast extract, 2.5 g sodium chloride, per L) and glycerol. Seed lots exhibiting higher infestation rates were used in subsequent validation testing.

**Production of Cmm-infested seed lots.** Tomato plants (*Lycopersicon esculentum* cv. Kewalo) were grown to the fruiting stage in a glasshouse. Immature fruits (ca. 1 to 2 cm diameter) were inoculated with a virulent *Cmm* strain by stab inoculating the pedicel of each fruit using a sterile scalpel dipped in inoculum. Seeds were manually
extracted from the ripe fruit, rinsed with sterile tap water, and dried overnight in a circulating air incubator at room temperature. One-third of the seeds from each fruit were directly plated to Cmm1 medium, and incubated for up to 7 days at 28°C to detect Cmm contamination. Samples of the bacterial growth on and around the contaminated seeds were tested with toothpick ELISA using the CMM1 MAb. Cmm infestation percentages were estimated for each fruit. Seeds from fruit exhibiting 95% or higher infestation rates were pooled to produce seed lot 19. A second crop of tomato plants was inoculated when fruits were smaller (ca. 1 cm diameter) and processed as above to produce lot 20.

**Development of a filtration protocol.** Filter units and membranes were evaluated concurrently to determine the best three-unit combination for optimal recovery of Cmm from seed extract. Various positive- and negative-pressure filter units were assessed. Mixed cellulose ester membrane filters in a number of pore sizes were also evaluated. Prefilters were tested in a number of units to improve flow rates. Finally, insertion of mesh spacers to prevent “blinding” of downstream membranes was attempted to reduce the number of filter units required for the final system. All filter unit-membrane combinations were tested individually using Cmm suspensions in PBS buffer. Cmm recovery percentages and filter retention rates were then determined for each combination. Selected units and membranes were further tested in three-unit configurations using pure Cmm suspensions, mixed suspensions of Cmm and a seed saprophyte strain, and seed extract spiked with Cmm. Recovery percentages and retention rates were again evaluated.
**Final filtration protocol.** The final protocol was designed to filter seed extract volumes of 5 ml or more, and consisted of three separate negative pressure filter units (Fig 2.1). Unit 1 consisted of a 90 mm diameter Buchner funnel, containing one Whatman 1 filter paper (Whatman Inc., Clifton, NJ). Units 2 and 3 were 47 mm diameter fritted glass microanalysis filter holders, containing one 42.5 mm diameter G8 glass fiber prefilter (Fisher Scientific, Pittsburg, PA) and one 47 mm diameter 3.0 µm mixed cellulose ester membrane filter (Millipore, Bedford, MA), or one 47 mm diameter 0.45 µm mixed cellulose ester membrane filter (Pall Corporation, Ann Arbor, MI), respectively. Twenty-five milliliters of PBS were passed through the assembled units and then discarded. In most cases, 50 ml of seed extract was then filtered successively through units 1 and 2. A post-filtration rinse (25 ml PBS) was passed through the two units, and the filtrate collected. Filtrate volumes exceeding 50 ml were separated into at least three equal aliquots for individual filtration through unit 3. Each aliquot was passed through a separate 0.45 µm terminal membrane in unit 3, followed by a post-filtration rinse, as before. Smaller volumes were passed entirely through one 0.45 µm membrane. Terminal membranes were aseptically transferred to 47 mm absorbent cellulose pads saturated with liquid Cmm1 semiselective medium (Cmm1 medium without agar; Alvarez and Kaneshiro, 1999), and incubated for four days at 28°C.

A modification of the basic system was developed to filter smaller volumes (<5 ml) of seed extract. After prewetting, seed extract was passed directly through unit 3, followed by a post-filtration rinse. The terminal membrane was processed as above.
Between samples, filter units were disassembled and wiped down with 70% ethanol. After each experiment, units were soaked in 0.5% sodium hypochlorite, rinsed with distilled water, wrapped in aluminum foil, and autoclaved (20 p.s.i. for 15 min).

**Colonial blot immunoassay development.** A modified version of the tissue blot immunoassay (TBIA) developed by Hu et al. (1997) was used to detect *Cmm* colonies transferred onto nitrocellulose (NC) membranes from 0.45 µm terminal filtration membranes. NitroBind NC membranes (Osmonics, Inc., Minnetonka, MN) were used in all procedures. Concentrations of goat anti-mouse alkaline phosphatase conjugate (1:1000 dilution in PBS; Sigma-Aldrich, Inc., St. Louis, MO) and alkaline phosphatase substrate (Sigma Fast™ BCIP/NBT tablets; Sigma-Aldrich, Inc.) also remained constant. For optimization experiments, serial dilutions of *Cmm* suspended in PBS were spotted onto NC membranes. Membranes were dried and stored at room temperature until used.

**Antibody optimization.** MAb CMMI ascites were diluted in PBS and evaluated for use at 1:500, 1:1000, 1:2000, and 1:4000 dilutions. Three incubation times (30 min, 1 hour, and 2 hours) were tested. After incubation, NC membranes were rinsed in PBS-Tween 20 (0.05% Tween 20 in PBS), incubated in goat anti-mouse alkaline phosphatase conjugate for 1 hour, rinsed again in PBS-Tween 20, and developed for 15 min in alkaline phosphatase substrate. Reactions were stopped by rinsing membranes in distilled water.

**Enzyme inactivation.** Initial experiments with mixed bacterial cultures indicated that endogenous phosphatases, either produced by certain bacteria or present in the seed extract, gave false positive results with this assay. Three heat-treatment methods were evaluated to eliminate endogenous enzyme activity: oven incubation (dry heat), steam
incubation (wet heat), and liquid submersion (wet heat). For the liquid submersion method, hot PBS buffer versus boiling PBS was compared, and incubation times were determined. Treated NC membranes were incubated in MAb CMM1 (1:1000 dilution) and rinsed in PBS-Tween 20. Subsequent incubations in goat anti-mouse alkaline phosphatase and alkaline phosphatase substrate were as described above.

**Blocking optimization.** Powdered skim milk dissolved in PBS buffer was used for blocking. Three blocking methods were tested: filter submersion (incubation in blocking buffer), filter saturation (incubation of membrane face-up on an absorbent pad saturated with blocking buffer), and filter blotting (incubation of membrane face-down on a saturated absorbent pad). Variations in incubation time and milk concentrations were compared. Blocking was also evaluated for use before and after enzyme inactivation procedures. Treated NC membranes were rinsed in PBS-Tween 20, and incubated in MAb CMM1, goat anti-mouse alkaline phosphatase conjugate, and BCIP/NBT solution, as described above.

**Final colony blot immunoassay protocol.** After a four-day incubation following filtration, bacterial colonies on the 0.45 μm terminal membranes were transferred to 47 mm 0.45 μm NC membranes. NC membranes were gently pressed down onto the terminal membranes using the wide end of a 1 ml pipette tip to ensure adequate antigen transfer. An orientation notch was made with a sterile scalpel through both membranes. NC membranes were removed from the terminal membranes in one smooth motion using sterile forceps, and allowed to dry overnight at room temperature. Blotted terminal membranes were realigned on the cellulose absorbent pads, and reincubated for 24 hours
at 28°C for colony regrowth. Additional Cmm1 liquid medium was added to the absorbent pads prior to reincubation when necessary.

Completely dried NC membranes were blocked in 5% PBS-milk for 30 min, and then boiled in PBS for 10 min to eliminate endogenous enzyme activity. Boiled membranes were incubated sequentially in MAb CMM1 ascites (1:2000 dilution in PBS), and goat anti-mouse alkaline phosphatase conjugate (1:1000 dilution in PBS) for one hour each. After each incubation, membranes were washed three times for five minutes each in PBS-Tween 20. Membranes were developed in alkaline phosphatase substrate for 15 minutes, rinsed with distilled water and air-dried. Processed blots were examined using a dissecting microscope. Positive NC membranes were realigned with original terminal membranes for identification and isolation of reactive colonies.

**Immunoassay protocol selection.** Sensitivities of an indirect ELISA procedure and the colony blot immunoassay were evaluated for detecting *Cmm* in spiked seed extract regrown on terminal filter membranes. Seed extract was filtered following the final filtration protocol. For indirect ELISA, colonies were grown for five days on terminal membranes and then resuspended in CBC before being transferred in 100 μl aliquots to microtiter plate wells. Plates were dried, and indirect ELISA proceeded as previously described. The colony blot immunoassay was run as described above. Optimal filter incubation times and incubation medium were also determined.

**Comparison of spread plate and filtration assays for Cmm detection.** *Cmm*-infested seed samples were used to evaluate the sensitivity of the filtration-colony blot immunoassay procedure against a standard spread plating assay. Seed extract from each sample was generated as previously described. One hundred microliter aliquots of the
extract were spread plated to five Cmm1 and five SCM-Y semiselective media plates. The remaining extract was processed using the filtration and colony blot immunoassay protocol. *Cmm* and saprophyte colonies recovered from the Cmm1 spread plates were used to calculate initial concentrations of colony forming units (cfu) per ml extract and *Cmm*-to-saprophyte ratios. Additional seed lots were obtained to further test the filtration and immunoassay technique in a blind study.

**Results**

**Identification of infested seed lots for validation testing.** Nine seed lots were initially obtained for validation testing of the filtration-immunoassay protocol (Table 2.2), and were assessed using standard spread plating procedures. Lots 1 through 6 originated from naturally infected plants, while lots 7, 8, and 9 were produced by spray-inoculating flowering tomato plants with virulent *Cmm* strains, harvesting all fruits and collecting seeds after pectinase treatment. *Cmm* colonies were recovered at very low concentrations from two of the six naturally infested lots (they represented only 0.06% and 0.08% of the total bacteria per lot, respectively), and *Cmm* was not recovered in every assay. Over time, *Cmm* was no longer isolated from lot 1. Sporadic results and apparently low infestation rates excluded these seed lots from use for validation of the filtration system.

*Cmm* colonies were isolated from all three spray-infested seed lots. As in the naturally infested lots, recovery of *Cmm* from lot 7 was sporadic. Seed infestation percentages for lots 8 and 9 were calculated at 30% and 70%, respectively, from assays
of individual seeds. *Cmm* cfu per seed were variable, ranging from 0 to 139 cfu. Only lots 8 and 9 from this initial batch were used for further system validation testing.

Two additional lots were produced for system validation purposes by inoculating tomato pedicels with *Cmm* and collecting the infested seeds. Initial seed infestation for lots 19 and 20 were calculated at 83% and 100%, respectively. Initial *Cmm* concentrations per seed varied widely, from 0 cfu to $10^7$ cfu in lot 19 and $10^2$ cfu to $10^7$ cfu in lot 20. Unexpectedly in lot 19, a dramatic reduction was seen in both infestation percentage and *Cmm* concentrations per seed over time (Fig 2.2). Immediately after harvest, *Cmm* concentrations per seed averaged $10^5$ cfu. At one year (342-356 days) post-harvest, *Cmm* was recovered from only 20% (6 of 30) of the assayed seeds on Cmm1 medium with an average of 118 cfu/seed. In comparison, average *Cmm* concentrations from lot 20 seeds decreased from $10^6$ cfu/seed (average *Cmm* concentration at 0 months) to $10^5$ cfu/seed at one year post-harvest (data not shown). At two years (736-738 days) post-harvest, 20% of the assayed seeds from lot 19 again yielded recoverable *Cmm* on Cmm1 medium, but average *Cmm* concentrations dropped to 56 cfu/seed. The different survival rates exhibited by the pathogen in the two seed lots may be explained by differences in seed storage methods (lot 19 was stored at room temperature in a transparent petri plate; lot 20 was stored at room temperature in the dark). The discrepancy may also be due to the stage at which immature fruits were inoculated (2 cm diameter fruits for lot 19, 1 cm diameter fruits for lot 20).

**Development of a filtration and colony blot immunoassay system.** The combination of a Whatman 1 filter, a 3.0 μm membrane overlayed with a G8 prefilter, and a 0.45 μm membrane as the terminal filter gave the most consistent *Cmm* recovery
percentages and the highest flow rate of the methods tested. Use of the G8 prefilter, in particular, increased flow rate through the downstream 3.0 μm and 0.45 μm membranes during filtration of more turbid seed extracts. Three negative-pressure units were selected for the final filtration protocol. A 90 mm diameter Bucher funnel was selected for unit 1, since the larger surface area allowed better flow rate using increased seed extract volumes.

The optimized colony blot immunoassay procedure was highly sensitive, and a positive visual signal with the MAb occurred when the aliquotted spot on the NC membrane contained at least 1000 cfu. Boiling the NC membrane in PBS for 10 minutes eliminated nonspecific reactions with the alkaline phosphatase conjugate caused by the seed extract and/or certain bacterial colonies. Boiling also enhanced the intensity of the positive reaction. Since MAb CMM1 detects an extracellular polysaccharide (EPS) antigen, blocking prior to boiling was necessary to prevent a dark purple background on the membranes caused by redeposition of the reactive Cmm EPS.

After a four-day enrichment period, individual Cmm colonies were detected from spiked seed extract samples using the colony blot immunoassay in conjunction with the three-unit filtration system. Reactive colonies were typically pinpoint, and appeared dark purple with a distinctive “comet-tail” (Fig 2.3). Nonreactive colonies were colorless, light purple, or mauve.

Indirect ELISA using microtiter plates did not detect Cmm in seed extract samples initially spiked with $10^4$ Cmm cfu even after a five-day enrichment period, but did detect Cmm from enriched samples initially spiked with $10^7$ Cmm cfu. Elevated background
readings in the microtiter wells due to high saprophyte concentrations and/or seed particulate matter were problematic using this method.

Under optimal conditions (Cmm cells suspended in PBS buffer), the three-unit filtration system recovered at least one Cmm colony for every 10 cfu that entered the system (Fig 2.4). The average Cmm recovery rate for the three-unit system was 15%. The one-unit protocol used to assay smaller sample volumes (<5 ml) gave an average Cmm recovery of 85%.

Using the three-unit system, recovery of individual Cmm colonies steadily decreased when Cmm cells were mixed with increasing concentrations of a single seed saprophyte strain in PBS buffer (Fig 2.5). However, Cmm recovery rates were consistent with those established for the three-unit system using pure Cmm suspensions. Recovery of Cmm from increasing concentrations of the saprophyte strain using the one-unit system remained near optimal at Cmm-to-saprophyte ratios as low as 1:1700. Twice the number of saprophytes (1:3500) caused a marked decrease in Cmm recovery.

Optimal recovery with the one-unit system using pure Cmm suspensions was approximately 85%. However, when partially filtered extract (e.g., extract passed through units 1 and 2) was subsequently spiked with low Cmm concentrations (ca. 100 cfu) and passed through terminal membranes, Cmm recovery percentages ranged from 0% to 79% (data not shown). This reduction in Cmm recovery with the one-unit system using partially filtered seed extract might have resulted from reduced Cmm growth on the membranes due to competition with increasingly diverse populations of seed saprophytes or growth inhibition by undefined seed extract components remaining on the membranes after filtration. At a 5:1 Cmm-to-saprophyte ratio, 79% of the Cmm cells initially spiked
into the sample were recovered. At a 1:1 ratio, \textit{Cmm} recovery was 42%, whereas at a 1:9 ratio, \textit{Cmm} recovery was 0%.

Recovery of \textit{Cmm} from samples of spiked or infested seed extract (not partially filtered extract, as before) with the three-unit system indicated that mixed populations of saprophytes and seed-associated particulate matter did not affect \textit{Cmm} recovery percentages to the same extent using this system as with the one-unit system. Based on semiselective medium plate counts, \textit{Cmm} recovery with the three-unit system was between 0.3% and 25%. \textit{Cmm-to-saprophyte} ratios from these samples ranged from 1:1 to 1:149. When spiked seed extract was drawn directly through the 0.45 \textmu m membrane in the one-unit system, both the numbers and diameters of the recovered \textit{Cmm} colonies were substantially reduced and high backgrounds occurred on the developed NC blots due to particles in the extract. \textit{Cmm} recovery was 26%. The high background on the NC membranes due to the unfiltered seed extract made visual discrimination of the pinpoint \textit{Cmm} colonies difficult. These results confirmed that the three-unit system was suitable for larger seed lots (which are typically tested without prior cleaning and generally are “dirtier”), while the one-unit system was most suited for small, cleaner seed lots.

**Assay comparisons.** Twenty-one seed samples were used to compare the sensitivities of the filtration-colony blot immunoassay and that of standard spread plating assays on two semiselective media. Twelve infested seed samples were created by contaminating non-infested seed samples with a predetermined number of seeds from the four infested lots (lots 8, 9, 19, and 20). Seed samples from nine additional seed lots with unknown levels of infestation (lots 10 through 18) also were assayed.
*Cmm* colonies recovered from four seed samples mixed with 10, 10, 5, and 2 seeds from lot 20, respectively, were too numerous to count. Countable numbers of *Cmm* colonies were recovered from six seed samples infested with 10 seeds each from lots 8 and 9. No *Cmm* colonies were recovered from two seed samples mixed with 60 seeds each from lot 19. No *Cmm* colonies were recovered from any of the nine additional seed samples by either spread plating or filtration.

Of the six samples having countable numbers of *Cmm*, all were positive using the three-unit filtration protocol (Table 2.3). Four of six were positive with the spread plating method. *Cmm* recovery on Cmm1 and SCM-Y media were similar; however, 16 times more saprophytic colonies per ml were recovered on SCM-Y. Terminal membranes that received at least 1000 *Cmm* cfu (calculated by *Cmm* colony counts on Cmm1 media plates) had recovery rates of 11% to 26%, similar to the percentages recovered from pure *Cmm* suspensions by the three-unit filtration system. Greater *Cmm* recovery was obtained with seed samples exhibiting higher *Cmm*-to-saprophyte ratios (e.g., fewer saprophytes), but *Cmm* could still be recovered from samples with low *Cmm*-to-saprophyte ratios (1:149).

After processing NC membranes using the colony blot immunoassay, an effort was made to identify and recover MAb CMM1-positive colonies from the original mixed cellulose ester 0.45 μm terminal membranes. In most cases, recovery was unsuccessful due to the small size of the reactive colonies and the abundance and sizes of saprophytic colonies also present on the membranes. However, one reactive colony was recovered and later identified as *Cmm*.
Discussion

A filtration and colony blot immunoassay protocol was developed as a rapid, specific, and sensitive alternative to the semiselective media plating and IF techniques commonly used for Cmm detection (Fatmi and Schaad, 1989; OEPP/EPPO, 1998; ISHI, 2001). Present protocols are capable of testing only a fraction of the seed extract generated from each sample. Assay sensitivities are therefore primarily limited by the number of Cmm cells present per subsample, which can vary tremendously depending upon the initial degree of seed infestation. With filtration, the entire volume of seed extract generated can be passed through one or several terminal membranes, theoretically allowing recovery of single target cells from large extract volumes. A filter concentration protocol such as this was developed to detect as few as 3 cfu Xanthomonas campestris pv. vesicatoria per gram of tested tomato seed (McGuire and Jones, 1989). Filter concentration is also used widely in food microbiology, where a small number of pathogens often must be recovered from highly proteinaceous samples (Sharpe, 1994).

Under optimized conditions (e.g., using pure Cmm suspensions), the three-unit filtration system recovered 7% to 31% of Cmm cells from 50 ml buffer, and recovered at least one cfu per 50 ml buffer when a minimum of 10 cfu were present. Although these recovery rates were low compared to the 77% recovery of Salmonella achieved by Hoszowski et al. (1996), the three-unit filtration system could detect Cmm concentrations from pure suspensions at least 50 times lower than that needed for recovery of a single cfu on a culture plate containing semiselective medium. The percentage recovery of Cmm from infested seeds using the three-unit system was similar to that obtained from optimized conditions. Low overall recovery rates (15% average recovery under
optimized conditions) may occur due to cell loss during manual transfer of liquid between the filter units. Simpler alternatives to the three-unit system were therefore evaluated, but most were abandoned because of slow flow rates or reduced Cmm recovery. The one-unit modified system was initially favored because of its high Cmm recovery percentages under optimized conditions. However, further testing showed it to be less effective when saprophyte diversity or seed particulate matter was increased. The one-filter setup is useful for assaying small quantities of cleaner, valuable breeder stock. It also can be used for assaying seed with low saprophyte populations or in protocols where extraction methods do not require extensive maceration of the seed samples (OEPP/EPPO, 1998; ISHI, 2001).

Use of a colony blot immunoassay to detect Cmm from terminal membranes provided a way to identify small numbers of Cmm colonies from a large background of seed saprophytes. The combined filtration and immunoassay procedure took only six days to complete because it eliminated the need for visual colony morphology assessment. Spread plating assays can take up to 12 days depending upon media selection (Gleason et al., 1993). When insufficient time is given for colony development, Cmm cannot be distinguished from the numerous yellow Cmm-like bacteria commonly found on tomato seeds. Using the filtration protocol, individual Cmm colonies on the membranes gave positive reactions after four days of incubation (eight days less than for observations on semiselective media). Positive colonies were identified using a dissecting microscope and even pinpoint Cmm colonies could be visualized by this method, whereas they would have been visibly undetectable on semiselective media.
Few other antibody-based identification methods have been developed that allow pathogen detection directly in seed extract. A dot blot immunoassay for \textit{Xanthomonas campestris pv. phaseoli} was developed for seed assay purposes, but detection of the pathogen was hindered by seed extract components and the test evidently was abandoned (Malin et al., 1985). A previous study in our laboratory found that a similar dot blot assay adapted from Leach et al. (1987) for \textit{Cmm} detection produced high backgrounds even with pure bacterial suspensions (Alvarez, unpublished data). The IF technique is most commonly used in Europe, and is an accepted method for \textit{Cmm} detection (OEPP/EPPO, 1998). IF is specific, but sometimes requires the operator to distinguish single fluorescing cells in a background of autofluorescing seed compounds. A modified version of IF, termed immunofluorescence colony staining (IFC), utilizes an enrichment stage to confirm viability of the pathogen, and this improves upon IF sensitivity (Alvarez et al., 1997; van Vuurde, 1997). The IFC technique has been assessed for \textit{Cmm} detection potential in this laboratory, but was discontinued because the colony blot immunoassay requires less antibody.

The filtration and colony blot immunoassay protocol shows promise for detection of low numbers of \textit{Cmm} in seed extracts. The protocol is as sensitive and, in some cases, more sensitive, than standard spread plate assays. Two of six infested seed samples gave negative results using spread plating, but were positive with the colony blot immunoassay. All six samples were spiked with ten potentially infested seeds from lots 8 and 9, producing seed samples with infestation rates of at least 0.01% to 0.1%. Had this been an actual seed certification situation, these samples would have been declared noninfested by semiselective media plating, and the lots released for sale.
Before acceptance, a proposed assay method is tested in multiple laboratories to assess its reproducibility and reliability. However, due to the unavailability of naturally infested seed lots, a final evaluation of the filtration and colony blot immunoassay is not possible at this time. Results from this study show that Cmm cells are not distributed evenly among seeds in a lot, and that recoverable Cmm in seed decreases with prolonged storage. Methods to prevent loss of inoculum during storage, or alternative methods to standardize tests, must be developed to avoid the variability that will occur when multiple laboratories compare data. Use of standardized reference materials as proposed by van den Bulk et al. (1997; 1999) may be the best way to deal with the uneven distribution of the pathogen in infested seed lots.
Table 2.1. Tomato seed lots used in this study.

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<td>20</td>
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</table>

\(^a\) Place of origin or location of distributor.

\(^b\) Harvest year or date received for testing.

\(^c\) Produced in the greenhouse for this study.
Table 2.2. Seed lots tested for *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) infestation for use in filtration assay validation studies.

<table>
<thead>
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<th>Seed lot</th>
<th>Grams tested (assays)</th>
<th>Total colonies&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Colonies tested</th>
<th>Colonies reacting with MAb CMM1&lt;sup&gt;d&lt;/sup&gt;</th>
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</tr>
<tr>
<td>5</td>
<td>0.1 (1)</td>
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<tr>
<td>6</td>
<td>1 (1)</td>
<td>50</td>
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</tr>
<tr>
<td>7</td>
<td>100 (4)</td>
<td>139</td>
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<td>100</td>
</tr>
<tr>
<td>8</td>
<td>10 (2)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>730</td>
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</tr>
<tr>
<td>9</td>
<td>N/A&lt;sup&gt;f&lt;/sup&gt;</td>
<td>156</td>
<td>42</td>
<td>36</td>
</tr>
</tbody>
</table>

<sup>a</sup> Recovered on Cmm1 and SCM-Y semiselective media.

<sup>b</sup> Transferred from Cmm1 or SCM-Y semiselective media and observed for pigmentation on YSC.

<sup>c</sup> Transferred from YSC medium and tested by toothpick ELISA.

<sup>d</sup> MAb CMM1 = clone 103-142; tested using an indirect ELISA.

<sup>e</sup> In addition to 10 g, 10 seeds were also individually assayed.

<sup>f</sup> Ten seeds were individually assayed.
Table 2.3. Comparison of a spread plating procedure and the filtration-colony blot immunooassay technique for detection of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) in infested tomato seeds.

<table>
<thead>
<tr>
<th>Seed lot&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Spread plates&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Filtration / immunoassay&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Seed saprophytes recovered&lt;sup&gt;d&lt;/sup&gt;</th>
<th><em>Cmm</em>-to-saprophyte ratio&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>9</td>
<td>175</td>
<td>1667</td>
<td>270</td>
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<td>8</td>
<td>232</td>
<td>398</td>
<td>488</td>
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</tr>
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<td>5</td>
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<td>1</td>
<td>386</td>
<td>-&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Ten seeds per lot into 25 g non-infested seed.

<sup>b</sup> Colony forming units (cfu) recovered on *Cmm*<sub>1</sub> semiselective medium.

<sup>c</sup> Total reactive colonies on filters.

<sup>d</sup> Calculated using measured bacterial concentrations per ml from *Cmm*<sub>1</sub> medium colony counts.

<sup>e</sup> Ratio not calculated; *Cmm* colonies were not recovered on *Cmm*<sub>1</sub> plates.
Fig 2.1. Three-unit filtration system for recovery of *Clavibacter michiganensis* subsp. *michiganensis* from seed extract. Seed extract is clarified by passing through filters in units 1 and 2, and then drawn through a 0.45 μm terminal membrane in unit 3. Large volume filtrates are separated into at least three equal aliquots for individual filtration through 0.45 μm terminal membranes in unit 3. Terminal membranes are incubated for 4 days, and then blotted with nitrocellulose. Nitrocellulose membranes are processed using the colony blot immunoassay. For the one-unit system, smaller extract aliquots are passed only through unit 3. Terminal membranes are processed as above.
Fig 2.2. Decrease in recoverable populations of *Clavibacter michiganensis* subsp. *michiganensis* from seed lot 19 over two years. The 0 month assay was done 0-18 days post-harvest; 12 month assay was done 342-356 days post-harvest; 24 month assay was done 736-738 days post-harvest.
Fig 2.3. Photograph of a nitrocellulose (NC) membrane developed using the colony blot immunoassay procedure. After seed extract filtration, terminal 0.45 μm membranes are incubated for four days and then blotted with NC. *Clavibacter michiganensis* subsp. *michiganensis* colonies appear as pinpoint, dark purple spots (→). Many have distinct “comet tails” (←). Saprophytes are light purple or mauve.
Fig 2.4. Recovery of Clavibacter michiganensis subsp. michiganensis (Cmm) from Cmm-only liquid suspensions using the one- and three-unit filtration systems. The concentrations marked by an asterisk (*) represent 1, 2, and 3, and 0, 1, and 2 cfu Cmm colonies recovered by one- and three-unit filtration systems, respectively. No Cmm were detected at this initial concentration by spread plating.
Fig 2.5. *Clavibacter michiganensis* subsp. *michiganensis* recovery percentages from mixed liquid suspensions containing increasing concentrations of a single seed saprophyte strain.
Chapter 3

Identification of *Clavibacter michiganensis* subsp. *michiganensis* strains isolated from infested tomato seed

Abstract

Putative *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) strains isolated from tomato seed, initially identified by colony morphology and monoclonal antibody (MAb) CMM1 reactivity, were characterized using additional assays to confirm their identities. Strains were tested with the MicroLog™ identification system, rep-PCR, PCR using two *Cmm*-specific primer sets, an endoglucanase assay, and a tomato bioassay. All MAb CMM1-positive strains were confirmed as *Cmm* based on the results of this polyphasic testing scheme. A high percentage (81%) of MAb CMM1-positive colonies were hypovirulent or nonvirulent on tomato (*Lycopersicon esculentum* cv. Kewalo). Neither of the two PCR primer sets identified all hypovirulent and nonvirulent *Cmm* strains, and the endoglucanase assay was not specific for *Cmm*. Of the assays tested, MicroLog™ and rep-PCR were the most consistent in identifying MAb CMM1-positive strains as *Cmm*.

Introduction

Detection and identification of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) in tomato seed lots prior to commercial sale is critical for the prevention of pathogen dissemination. Seed lots are currently accepted or rejected on the basis of seed
assays that detect pathogen contamination either by direct culture or by other immunodiagnostic methods (Fatmi and Schaad, 1989; OEPP/EPPO, 1998; ISHI, 2001). Final confirmation that a seed lot is infested with Cmm is done with pathogenicity testing. The prevalence of yellow seed saprophytes in or on tomato seeds can affect accurate identification of actual Cmm strains.

Previously, 40 strains from tomato were identified that exhibited typical Cmm-type colony morphologies on Cmm1 semiselective medium and reacted strongly with the CMM1 monoclonal antibody (MAb; clone 103-142), but were hypovirulent or nonvirulent in tomato pathogenicity tests (Kaneshiro and Alvarez, 2001). These strains were subsequently identified as Cmm based on further testing using the MicroLog™ Microbial Identification System and rep-PCR using the BOXA1R primer (Versalovic et al., 1994). In Chapter 2, 59 strains from seed were isolated that exhibited Cmm-type colony morphologies on semiselective media and were MAb CMM1-positive. Thirty-one strains were tested further, and were also found to vary in virulence. Studies have shown that virulent, hypovirulent, and nonvirulent Cmm strains can be isolated from the same diseased plant tissues (Fulbright et al., 1998; Werner, 2001), but the significance of hypovirulent and nonvirulent Cmm strains in disease epidemiology is unclear.

The purpose of this study was to determine whether this subset of 31 MAb CMM1-positive strains from seed were indeed Cmm. This was accomplished by characterizing each using the MicroLog™ identification system, rep-PCR using the BOXA1R primer (Versalovic et al., 1994), determining reactivities with PCR primer sets CM3/CM4 (Sousa Santos et al., 1997) and CMM-5/CMM-6 (Dreier et al., 1995), and endoglucanase production on M9CMC medium (Meletzus and Eichenlaub, 1991).
addition, these identification tests were also assessed to determine which could be used to reliably differentiate virulent, hypovirulent, and nonvirulent *Cmm* strains from MAb CMM1-negative yellow seed saprophytes.

**Materials and Methods**

**Strain selection.** Thirty-one strains from seed were selected for further characterization based on typical colony morphology on semiselective media and reactivity with the CMM1 MAb. An additional 14 strains from a *Cmm* culture collection were selected as reference strains (Table 3.1) based on colony morphology, MAb CMM1 reactivity, and fingerprint patterns with rep-PCR.

**Tomato bioassay.** Tomato seedlings (*Lycopersicon esculentum* cv. Kewalo) at the 3 to 5 leaf stage were used to test the virulence of putative *Cmm* strains from seed. Plants were grown in a commercial potting mix, and maintained on a greenhouse bench for 3 to 4 weeks prior to inoculation. Bacterial strains were grown on YSC for 2-3 days at 28°C, and then inoculated into the seedlings by a single stab into the stem between the cotyledons using a sterilized scalpel dipped directly into the bacterial culture. Inoculated plants were maintained on a greenhouse bench and assessed for symptom development for a total of 21 days. Virulence was rated on a scale of 0 to 7, where “0” meant no symptom development, and “7” equaled a plant showing canker and complete wilting of the leaves. On this scale, hypovirulent strains that produced a canker at the inoculation site, but no leaf wilting, ranked “1”. Two plants were inoculated per strain at one time. In many cases, inoculations were repeated to confirm results.
**DNA extraction.** Bacterial strains were revived from −80°C storage and grown on yeast sucrose calcium carbonate agar (YSC) to confirm purity. Single colonies were subcultured to additional YSC plates and grown for another 2-3 days, after which a loopful of cells were transferred to yeast glycerol broth tubes (YG broth, consisting of 5 g yeast extract, 1 g dibasic potassium phosphate, 0.5 g magnesium sulfate, and 17 ml glycerol, per L) and incubated overnight on a rotary shaker (200 rpm at 30°C). DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI), following the manufacturer’s protocol for gram positive bacteria. DNA quantity was estimated using the Rapid Agarose Gel Electrophoresis (RAGE) apparatus (RGX-100 system; Cascade Biologics, Inc., Portland, OR). Before use, all electrophoresis solutions were cooled, and the RAGE apparatus placed in an ice bath to prevent the agarose gel from melting. One microliter samples were used per well, and DNA standards ranging from 10 ng/μl to 100 ng/μl were run with each gel. Gels were stained with ethidium bromide for 30 min (0.6 mg per ml in 1x Tris-acetate-EDTA [TAE] buffer), destained in distilled water for 30 min, and photographed with UV transillumination using Polaroid 667 film. Using the standards to estimate DNA concentration, working solutions of 50 ng/μl were prepared for use in both PCR and rep-PCR reactions.

**PCR.** PCR was done using two published *Cmm* primer sets, designated CM3/CM4 (Sousa Santos et al., 1997) and CMM-5/CMM-6 (Dreier et al., 1995). All primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). Each 50 μl reaction mixture consisted of 37.5 μl ultrapure water, 5 μl 10X PCR buffer (Promega or Applied Biosystems, Foster City, CA), 2.5 μl MgCl₂ (15 mM; Promega), 2.5 μl dNTP mix (200 μM; Amersham Pharmacia Biotech, Inc., Piscataway, NJ), 0.5 μl each
primer (10 μM), 0.5 μl AmpliTaq (5 units per μl, Applied Biosystems), and 1 μl DNA working solution (50 ng/μl). PCR was done using an MJ Research PTC-100 Programmable Thermal Controller equipped with a heated lid (MJ Research, Inc., Waltham, MA). PCR conditions were as follows: initial denaturing for 7 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at either 60°C (for CM3/CM4) or 55°C (for CMM-5/CMM-6), and 1 min at 72°C, and ending with a final extension of 5 min at 72°C.

PCR products were typically run on 1.2% agarose gels for 30 min at 60V, with a 1 kb marker (Gibco BRL, Life Technologies, Grand Island, NY) as a standard. Positive reactions showed bands of approximately 614 bp (CMM-5/CMM-6 product) or 645 bp (CM3/CM4 product). Reactions were repeated at least once. In several instances, it was necessary to repeat the reaction a third time to achieve reproducible results.

**rep-PCR genomic fingerprinting.** rep-PCR was performed on all strains using the BOXA1R primer (Versalovic et al., 1994), following the procedures of Louws and Cuppels (2001). The BOXA1R primer was provided by F. Louws, and was synthesized by the Macromolecular Structure, Sequence and Synthesis Facility at Michigan State University. Extracted DNA was used as a template for all reactions. Each reaction was run for 30 cycles in a PTC-100 Programmable Thermal Controller (MJ Research, Inc.). rep-PCR products were run for 14 hours at 70V and 4°C on an H4 horizontal gel apparatus (Gibco BRL) in 1.5% agarose gels. Gels were stained and destained as described above, and photographed on a UV transilluminator using Polaroid 665 film. rep-PCR products from Cmm reference strains corresponding to each of the four fingerprint types described by Louws et al. (1998) were included on each gel as
standards. Fingerprints generated from the seed strains were compared to the reference strains in order to determine whether they were *Cmm*.

**Endoglucanase testing.** An agar plate assay for endoglucanase production was used to indirectly establish the presence of *celA*, an endo-β-1,4-glucanase gene found to play a role in *Cmm* virulence (Meletzus et al., 1993). Semiquantitative measurements of endoglucanase production were taken according to the procedure of Nissinen et al. (2001), with slight modifications. Bacterial strains were grown for 2-3 days on YSC. Cells were suspended in 0.01M PBS and adjusted to an optical density of $A_{600} = 0.1$ (approximately $10^8$ cfu/ml). Ten microliter spots of each suspension were plated to M9CMC medium (Meletzus et al., 1993) containing sodium carboxymethylcellulose and slightly dried in a biological cabinet before incubation for 5 days at 28°C. Prior to staining plates, colonies were outlined with a permanent marker, in order to facilitate subsequent measurements. A positive reaction produced a clear, yellowish zone around the traced colony, while a negative reaction showed no clearing. Strains were tested at least twice. Positive and negative control strains were included on each plate.

**MicroLog™ identification.** An identification of each strain using metabolic profiling was obtained using the MicroLog™ Microbial Identification System (Biolog, Inc., Hayward, CA), following the manufacturer's protocol. GP2 plates (Biolog, Inc.) were used for all strains. At 16-24 hours, plates were read in a microplate reader using MicroLog™ release 4.2 software and results were confirmed by visual assessment of the plates. In a few cases, threshold levels and/or well reactions were adjusted to accurately reflect visual assessments.
Results

Characterization of *Cmm* reference strains. Fourteen representative nonvirulent, hypovirulent, and virulent *Cmm* strains from various geographical regions were characterized by colony morphology, MAb CMM1 reactivity, MicroLog™ identification, rep-PCR type, PCR primer set reactivity, and endoglucanase production (Table 3.2). All strains except for the virulent strain A4758 had typical colony morphologies on Cmm1 semiselective medium and YSC differential medium, and were MAb CMM1 positive. A4758 was a dry, yellow colony and was MAb CMM1 negative. Most reference strains were identified by the MicroLog™ system release 4.2 as *Clavibacter michiganensis* subsp. *tessellarius* (*Cmt*), a closely related subspecies that is pathogenic on wheat. One virulent strain was identified as *Clavibacter sp.*, while two nonvirulent strains were identified as *Cmm*. Prior rep-PCR fingerprinting analysis using the BOXA1R primer (BOX-PCR) identified all reference strains as *Cmm*, and placed each into one of four *Cmm* fingerprint types, A, B, C, or D. However, fingerprint patterns generated in this study for reference strains were highly polymorphic (Fig 3.1, lanes 2-9 and 18-23). Consistent banding patterns were not observed among strains within a fingerprint type (e.g., A type) that could be used to consistently differentiate them from strains belonging to other fingerprint types (e.g., B-D types). Most reference strains shared three common bands in the 700-bp, 517-bp, and 296-bp regions (Fig 3.1, arrows). Three nonvirulent strains, A4748, A4588, and A4598, did not exhibit the 296-bp band (Fig 3.1, lanes 20-22) although a faint band was observed for A4588 (Fig 3.1, lane 21). All seven virulent strains were positive with the CM3/CM4 primer set, and six of seven were positive with the CMM-5/CMM-6 primer set. The single hypovirulent
strain was positive with both primer sets. Most nonvirulent strains were also positive with one or both primer sets, whereas only a single nonvirulent strain did not react with either set. All strains produced endoglucanase in the agar plate assay.

**Characterization of putative Cmm strains from seed.** The 27,785 bacterial colonies isolated from nine seed lots (Chapter 2, Table 2.2) were observed for Cmm-type characteristics. Cell morphologies and Gram reaction, colony morphologies on Cmm1 and YSC media, and reactivity with the CMM1 MAb were used to differentiate putative Cmm from seed saprophytes, in order to select the most likely candidates for further characterization. Most seed strains exhibited non-Cmm colony morphology on Cmm1 and YSC media, or had different Gram reactions and/or cell morphologies. Some strains with typical Cmm colony morphology produced acid on YDC or YSC media and were not reactive with the CMM1 MAb. A few colonies initially exhibited Cmm-type colony morphology on YSC, but did not react with the CMM1 MAb, and with further colony development their morphologies no longer appeared typical. Only 59 strains from five seed lots were reactive with the CMM1 MAb. These were gram positive, pleomorphic rods, and exhibited Cmm-type colony morphology on Cmm1 and YSC medium. Thirty-one of these strains were selected for further characterization (Table 3.3).

Fifteen strains isolated from four of the five seed lots produced no symptoms on tomato plants 21 days post-inoculation and were considered nonvirulent. Ten additional strains from three seed lots caused canker formation alone, or canker formation with slight wilting on the lower leaves, and were classified as hypovirulent. Six virulent strains isolated from four seed lots caused canker formation, leaf necrosis and wilt.
The MicroLog™ system database release 4.2 consistently identified nonvirulent, hypovirulent and virulent seed strains as *Cmt*. Only one virulent strain was identified as *Cmm* (strain C131-A) while five were identified as *Cmt*. All but two nonvirulent and hypovirulent strains were also identified as *Cmt*. These two strains were identified as *Clavibacter sp.*

BOX-PCR fingerprinting of the putative *Cmm* seed strains generated many polymorphic bands in the 200 to 2000-bp regions (Fig 3.1, lanes 10-16 and 24-29). As with fingerprints generated from the reference strains, most putative *Cmm* seed strains exhibited three common bands in the 700-bp, 517-bp, and 296-bp regions (Fig 3.1, arrows). One virulent seed strain, C131-A, did not exhibit the 296-bp band (Fig 3.1, lane 13), but induced canker symptoms on tomato. Comparison of fingerprints of reference strains (Fig 3.2, lanes 2-5), putative *Cmm* seed strains (Fig 3.2, lanes 17-23), and other seed saprophyte strains (Fig 3.2, lanes 7-15) indicated that the presence of the 700-bp and 296-bp bands could consistently differentiate *Cmm* from other seed bacteria which were all negative with MAb CMM1. All putative *Cmm* from seed were therefore identified as *Cmm* based on these common bands.

BOX-PCR of nonvirulent and hypovirulent/virulent strains generated distinctly different fingerprint patterns. Fingerprints from the hypovirulent and virulent seed strains exhibited banding patterns in the 1,000 to 1,300-bp region similar to two *Cmm* standard strains (Fig 3.1, lanes 3 [A type] and 4 [B type]). PCR fingerprints for these seed strains (Fig 3.1, lanes 10-16) were nearly identical, although a few polymorphic bands still differentiated individual strains from one another. Two virulent strains, C219 (Fig 3.1, lane 14) and C221 (not shown), were identical, and produced slightly different banding
patterns compared to the other strains. Fingerprints from the nonvirulent seed strains exhibited more pattern variation (Fig 3.1, lanes 24-29), although all had the three characteristic bands identifying them as *Cmm*. Nonvirulent strains isolated from the three seed lots obtained from spray-inoculated field tomatoes exhibited fingerprint patterns similar to each other, and shared a strong resemblance with two reference strains (Fig 3.1, lanes 9 and 23 [both D type]). The six strains from the naturally infested California seed lot, on the other hand, showed a high degree of polymorphism (Fig 3.1, lanes 24-26).

All virulent strains, ten hypovirulent strains, and eight nonvirulent strains were positive with the CM3/CM4 primer set. One hypovirulent strain and six nonvirulent strains were negative. All virulent strains, eight hypovirulent strains, and eight nonvirulent strains were positive with the CMM-5/CMM-6 primer set. Three hypovirulent and six nonvirulent strains were negative. A second band of approximately 1000-bp occurred in 9 of the 22 strains that reacted with the CMM-5/CMM-6 primers (Fig 3.3, lanes 5-7). This band occurred with both virulent and hypovirulent strains, was always fainter than the expected 614-bp band, and was reproducible in repeated PCR reactions.

All virulent and hypovirulent strains produced endoglucanase. Nine of fourteen nonvirulent strains also produced this enzyme. Nonvirulent strains that showed no endoglucanase production appeared to grow as well on M9CMC medium as did those strains that were endoglucanase positive.
Discussion

Accurate identification of *Cmm* strains isolated from seed depends primarily upon the specificity of an assay for differentiating *Cmm* subpopulations from similar-appearing, nonvirulent seed saprophytes. It was found that strain identification based on pathogenicity testing alone was not sufficient for differentiating nonvirulent *Cmm* strains from seed saprophytes. On the other hand, the CMM1 MAb was highly specific for *Cmm*, including virulent, hypovirulent, and nonvirulent strains (Alvarez and Kaneshiro, 1999; Kaneshiro and Alvarez, 2001). In Chapter 2, colonies isolated from seed were identified as potential *Cmm* strains based on their colony morphologies on Cmm1 and YSC media and their reactivity with the CMM1 MAb. Of the additional identification methods tested here, the MicroLog™ system and rep-PCR using the BOXA1R primer (BOX-PCR; Versalovic et al., 1994; Louws et al., 1998) were the most consistent at identifying these MAb CMM1-positive, yellow strains from seed as *Cmm*.

The MicroLog™ system release 4.2 consistently identified virulent, hypovirulent, and nonvirulent *Cmm* seed strains to the species level. Subspecific identifications were mainly *Cmt*, a pathogen of wheat (Carlson and Vidaver, 1982). Even typical, virulent *Cmm* reference strains were identified as *Cmt*. Previous characterization studies using an older MicroLog™ system release (3.5) identified *Cmm* strains of all virulence types as *Cmm* or *Clavibacter* sp., or placed them in the closely-related *Curtobacterium* genus as *Curtobacterium* sp. or *C. flaccumfaciens* pv. *flaccumfaciens* (Alvarez and Kaneshiro, 1999; Kaneshiro and Alvarez, 2001). With MicroLog™ release 4.01, strain identifications improved and the correct species identification was consistently obtained. However, most strains were identified as *Cmt*. As with release 4.2, even typical, virulent
*Cmm* strains were identified as *Cmt*. Different *C. michiganensis* subspecies affect different hosts, so the presence of other subspecies in infested or infected tomato plant materials was highly unlikely. Positive reactions with D-melibiose (well C8), D-ribose (well D7), succinic acid mono-methyl ester (well F7), and adenosine (well H1) often appeared to influence the identification of strains as *Cmt* rather than *Cmm*. The MicroLog™ system was therefore used for species confirmation only. As such, the database was found reliable in identifying the strains as *Clavibacter michiganensis*.

BOX-PCR fingerprinting consistently identified the MAb CMM1-positive seed strains as *Cmm* based on banding similarities to *Cmm* reference strains within the 296-bp to 700-bp region. This conclusion was supported by other data, including MicroLog™ identifications and MAb CMM1 reactivity. BOX-PCR could also separate *Cmm* from seed saprophyte strains, which were not identified as *Cmm* by MicroLog™ and were not MAb CMM1 reactive. The complex banding patterns generated by this technique also allowed for comparison between *Cmm* and seed saprophytes, as opposed to standard PCR primer sets that would have differentiated the two groups by either the presence or absence of single bands.

Interestingly, *Cmm* fingerprint patterns obtained in this study did not correspond to those reported by Louws et al. (1998). Reference strains were selected from their study and from a group of strains previously typed in the laboratory of D. Fulbright in order to compare the test strains with A, B, C, and D type reference fingerprints. Unexpectedly, BOX-PCR fingerprints generated from these reference strains revealed a higher number of band polymorphisms than previously reported. Banding patterns in the 1000-bp region that were originally used to differentiate A, B, C, and D type fingerprints
by Louws et al. (1998) were not easily distinguished in this study. Instead, the 1000-bp region patterns obtained here were highly similar among the fingerprint types, and were reproducible using either extracted genomic DNA (Fig 3.3, lanes 2 to 9 and lanes 18 to 23) or whole bacterial cells (data not shown) as template.

It is unknown why the fingerprint patterns observed by Louws et al. were not reproducible in this work, even when using strains from their study. In other studies, BOX-PCR fingerprinting was used successfully to characterize genomic diversity of *Cmm* field isolates (Fulbright et al., 1998; Werner, 2001). Werner (2001) observed slight variations in banding patterns of specific strains between agarose gel electrophoresis runs, but these polymorphisms did not affect the characteristic banding patterns that differentiated A, B, C, and D type *Cmm* fingerprints. Differences in DNA extraction methods did not appear to affect the fingerprint patterns obtained, and standardized BOX-PCR protocols were used. Further testing is needed to determine why additional bands were obtained before the BOX-PCR can be used to identify A, B, C, and D subgroups of *Cmm* in our laboratory.

Neither PCR primer set CM3/CM4 (Sousa Santos et al., 1997) or CMM-5/CMM-6 (Dreier et al., 1995) was adequate for identifying strains as *Cmm*. Primer set CM3/CM4 was developed by "shotgun" cloning of a virulent Hungarian *Cmm* strain, and was tested against five *Cmm* strains of unidentified virulence and from unidentified origins (Sousa Santos et al., 1997). These primers identified most *Cmm* strains in this study, but did not react with all nonvirulent and hypovirulent strains. Primer set CMM-5/CMM-6 was developed to amplify a portion of the *pat-1* region on plasmid pCM2, and specifically identified virulent *Cmm* strains. Sixteen virulent European *Cmm* strains and 7
nonvirulent strains from Europe and North America were tested with this primer set (Dreier et al., 1995). In the current study, CMM-5/CMM-6 could not distinguish between virulent, hypovirulent, and nonvirulent *Cmm* types.

The endoglucanase plate test was used to check indirectly for the presence of *celA*, the only *Cmm* endoglucanase gene identified to date and a known virulence factor (Jahr et al., 2000). This test identified as many *Cmm* strains as did the CM3/CM4 primer set; however, since it is only designed to test for enzymatic activity, it is not specific for *Cmm* and would only be useful in a polyphasic testing scheme.

The combined results from this study indicated that all 31 MAb CMM1-positive strains from seed were indeed *Cmm*. MAb CMM1 reactivity, MicroLog™ identification, and BOX-PCR fingerprinting were the most reliable methods of those tested for identifying virulent, hypovirulent, and nonvirulent *Cmm* from tomato seed. However, the MicroLog™ database had some difficulties in identifying the seed strains down to the subspecies level, and highly polymorphic banding patterns generated by BOX-PCR made grouping the strains into *Cmm* fingerprint types complicated. Identification with the CMM1 MAb was simple, rapid, and provided straightforward results. Therefore, this method still appears to be the most efficient for rapid identification of *Cmm*.
Table 3.1. *Clavibacter michiganensis* subsp. *michiganensis* reference strains.

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</tr>
<tr>
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<td>123&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>D. Fulbright</td>
</tr>
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<tr>
<td>A4833</td>
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<td>D. Fulbright</td>
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<sup>a</sup> Strains included in Louws et al. (1998).
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<th>MAb CMM1 reactivity</th>
<th>MicroLog™ ID&lt;sup&gt;b&lt;/sup&gt;</th>
<th>BOX-PCR type</th>
<th>CM&lt;sub&gt;3&lt;/sub&gt;CM&lt;sub&gt;4&lt;/sub&gt;</th>
<th>CMM-5/CMM-6</th>
<th>Endoglucanase production</th>
<th>Virulence on tomato</th>
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</tr>
<tr>
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<td>Cmt</td>
<td>A&lt;sup&gt;e&lt;/sup&gt;</td>
<td>+&lt;sup&gt;e&lt;/sup&gt;</td>
<td>-&lt;sup&gt;e&lt;/sup&gt;</td>
<td>+</td>
<td>Nonvirulent&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
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<td>Cmt</td>
<td>A&lt;sup&gt;d&lt;/sup&gt;</td>
<td>+&lt;sup&gt;e&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>Hypovirulent</td>
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<td>A&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>+</td>
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<td>-&lt;sup&gt;e&lt;/sup&gt;</td>
<td>-&lt;sup&gt;e&lt;/sup&gt;</td>
<td>+</td>
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<td>B&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>+</td>
<td>+</td>
<td>Virulent&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>C&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>D&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>+</td>
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<sup>a</sup> Clone 103-142; reactivity using an indirect ELISA.

<sup>b</sup> MicroLog™ release 4.2; Cmm = *Clavibacter michiganensis* subsp. *michiganensis*; Cmt = *Clavibacter michiganensis* subsp. *tessellarius*.

<sup>c</sup> Data from Louws et al. (1998).

<sup>d</sup> Identifications by J. Bell and D. Fulbright (Michigan State University).

<sup>e</sup> PCR reactions done by H. Koenraadt (NAKG, the Netherlands).

All other data were collected in this laboratory.
Table 3.3. Characterization of 31 monoclonal antibody (MAb) CMM1-positive strains isolated from five infested seed lots.¹

<table>
<thead>
<tr>
<th>Lot origin³</th>
<th>Year</th>
<th>Strain</th>
<th>MicroLog™ ID⁴</th>
<th>BOX-PCR type⁵</th>
<th>PCR reactivity⁶</th>
<th>Endoglucanase production⁷</th>
<th>Virulence on tomato⁸</th>
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</thead>
<tbody>
<tr>
<td>California</td>
<td>2000</td>
<td>C6-A</td>
<td>Clavibacter sp.</td>
<td>Cmm</td>
<td>-</td>
<td>-</td>
<td>NV (0)</td>
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<tr>
<td></td>
<td></td>
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<td>Cmt</td>
<td>Cmm</td>
<td>-</td>
<td>-</td>
<td>NV (0)</td>
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<tr>
<td></td>
<td></td>
<td>C63</td>
<td>Cmt</td>
<td>Cmm</td>
<td>-</td>
<td>-</td>
<td>NV (0)</td>
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<tr>
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<td></td>
<td>C74-A</td>
<td>Cmt</td>
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<td>-</td>
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<td></td>
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<td>-</td>
<td>-</td>
<td>NV (0)</td>
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<tr>
<td>Netherlands</td>
<td>2000</td>
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<td>Cmt</td>
<td>Cmm</td>
<td>+</td>
<td>+*</td>
<td>H (1)</td>
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<td></td>
<td></td>
<td>C117</td>
<td>Cmt</td>
<td>Cmm</td>
<td>+</td>
<td>+*</td>
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<td></td>
<td>C131-A</td>
<td>Cmm</td>
<td>Cmm</td>
<td>+</td>
<td>+*</td>
<td>V (3)</td>
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<tr>
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<td>2001</td>
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<td>Clavibacter sp.</td>
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<td>+</td>
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<td></td>
<td></td>
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<td>H (2)</td>
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Table 3.3. (Continued) Characterization of 31 MAb CMM1-positive strains isolated from five infested seed lots.\(^a\)

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<thead>
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<th>Lot origin(^b)</th>
<th>Year</th>
<th>Strain</th>
<th>MicroLog(^TM) ID(^c)</th>
<th>BOX-PCR type(^d)</th>
<th>PCR reactivity(^e)</th>
<th>Endoglucanase production(^f)</th>
<th>Virulence on tomato(^g)</th>
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<td>Oregon</td>
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<td>+</td>
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<td></td>
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<td>Cmt</td>
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<td>+</td>
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<td>Cmm</td>
<td>+</td>
<td>+*</td>
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</table>

\(^{a}\) All strains were selected based on colony morphology and reactivity with the CMM1 monoclonal antibody (clone 103-142).

\(^{b}\) Seed lots from California and the Netherlands were naturally infested. Lots from Ohio and Oregon were collected from plants that were spray inoculated at the flowering stage with Cmm.

\(^{c}\) MicroLog\(^TM\) release 4.2; Cmm = Clavibacter michiganensis subsp. michiganensis; Cmt = Clavibacter michiganensis subsp. tessellarius.

\(^{d}\) rep-PCR using the BOXA1R primer.

\(^{e}\) Primer sets were tested in separate reaction mixtures. + = positive; - = negative. An asterisk indicates a strain that produced a double band.

\(^{f}\) Endoglucanase production after a 5 day incubation. + = halo around colony outline after staining with 0.1% Congo red; - = no halo.

\(^{g}\) NV = nonvirulent (no canker or wilting); H = hypovirulent (cancer with no/slight wilting); V = virulent (cancer with extensive wilting). Virulence ratings in parentheses are based on a 7-point scale where 0 = no cancer and no wilt, and 7 = cancer and all leaves wiling.
Fig 3.1. Agarose gel electrophoresis of BOX-PCR products from *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) reference strains and putative *Cmm* strains from seed. DNA fingerprints of putative *Cmm* were compared to *Cmm* reference strains previously characterized as PCR types A, B, C, or D. Fingerprints were classified as *Cmm* types based on bands at 296, 517, and ~700 bp (arrows). Lanes 1, 17, and 30 contain 1 kb markers. Lanes 2-16 are fingerprints from strains that were hypovirulent or virulent on tomato, and consist of *Cmm* reference strains A4004, A4810, A4825, A4827, A2058, A4758, A4830, and A4833 (Lane 2-9, respectively); and seed strains C117, C202, C215, C131-A, C219, C222, and C225 (Lanes 10-16, respectively). Lanes 18-29 are fingerprints from strains that were nonvirulent on tomato, and consist of *Cmm* reference strains A4818, A4820, A4748, A4588, A4598, and A4775 (Lanes 18-23, respectively); and seed strains C6-A, C55, C91-A, C210, C216, and C223 (Lanes 24-29, respectively). Strains A4830 and A4833 (lanes 8 and 9, respectively) were virulent in the study by Louws et al. (1998), but nonvirulent in this study. Strain A4818 (lane 18) was nonvirulent in the study by Louws et al. (1998), but hypovirulent here. These strains are grouped by their initial virulence, as reported by Louws et al. (1998). Strain A4758 (Lane 7) was included as a virulent, C type reference strain, but did not react with the CMM1 MAb (Alvarez and Kaneshiro, 1999).
Fig 3.2. Agarose gel electrophoresis of BOX-PCR products generated from seed saprophytes and putative *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) seed strains. *Cmm* fingerprints exhibited bands at 296-bp, 517-bp, and ~700-bp (arrows). Various *Cmm*-type strains that were nonreactive with the CMM1 monoclonal antibody (MAb; lanes 7-11) and other common seed saprophytes (lanes 12-15) did not produce the 296-bp and ~700-bp bands. Lanes 1, 16, and 24 contain 1 kb markers. Lanes 2-5 consist of virulent *Cmm* reference strains A4810, A4825, A4830, and A4833, representing PCR types A, B, C, and D, respectively. Lanes 17-23 contains putative *Cmm* strains of mixed virulence, and consist of nonvirulent (NV) strains C55, C91-A, and C210 (lanes 17-19), hypovirulent (H) strains C117 and C215 (lanes 20 and 21), and virulent (V) strains C219 and C222 (lanes 22 and 23).
Fig 3.3. Agarose gel electrophoresis of PCR amplification products from selected strains of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) using the CMM-5/CMM-6 primer set (Dreier et al., 1995). Bright bands in each lane correspond to expected PCR product size (614-bp). Note faint bands in lanes 5-7 (approximately 1,000-kb). Lanes 1 and 8 contain 1 kb markers; lanes 2-7 represent amplification products from strains C208, C217, C219, C204, C131-A, and C225, respectively. Letters over lane numbers indicate virulence status of each strain (NV = nonvirulent, H = hypovirulent, V = virulent).
**Chapter 4**

*In planta* interactions between coinoculated virulent and nonvirulent *Clavibacter michiganensis* subsp. *michiganensis*

**Abstract**

Nonvirulent *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) strains are commonly associated with tomato seed, but their role in epidemics is unknown. *In planta* interactions between virulent and nonvirulent strains were thus examined. Plants were inoculated at the 4 to 6 leaf stage with liquid suspensions of the appropriate bacteria, and incubated for 30 to 60 days. Ten 1-cm stem sections were taken per plant, proximal to the inoculation point. Stem extracts were dilution plated and the recovered bacteria were enumerated by plate count and/or colony blot immunoassay using the CMM1 monoclonal antibody. Virulent strain A4758 multiplied to high titers (ca. $10^8-10^9$) in all assayed stem sections. Nonvirulent strains moved only a few cm up the stem and were recovered at titers similar to those inoculated. Neither multiplication rates nor stem colonization was affected by coinoculation of virulent A4758 and nonvirulent A4748. Further assessment is required to determine whether this lack of interaction is strain-specific or is a phenomenon that is reproducible with other strains.
Introduction

*Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) is a serious seedborne pathogen and, as such, is a restricted quarantine organism. Many countries require that phytosanitary certificates accompany imported tomato seeds to prove the initial lot was tested for selected seedborne bacterial pathogens, including *Cmm*. However, continued bacterial canker outbreaks show that these screening procedures cannot fully guarantee pathogen-free material.

Assay protocols for detection of *Cmm* in seed lots vary depending upon the testing organization, but typically end in the recovery of putative *Cmm*-like colonies, which are then inoculated into tomato plants (*Lycopersicon esculentum*) to observe development of bacterial canker symptoms. Confirmation of a single virulent strain can result in destruction of the entire seed lot. In most accepted seed testing protocols, nonvirulent *Cmm*-like strains are disregarded (Fatmi and Schaad, 1989; OEPP/EPPO, 1998; ISHI, 2001).

In Chapter 3, 31 *Cmm* strains from seed were isolated and characterized. The majority of these strains exhibited reduced virulence, or were nonvirulent, and were identified as *Cmm* using a polyphasic testing scheme. Recovery of nonvirulent or hypovirulent *Cmm* strains from natural populations has also occurred in other studies (Fulbright et al., 1998; Louws et al., 1998; Werner, 2001). Whereas hypovirulent strains may pose a quarantine risk because slow-developing symptoms may not appear during the bioassay duration, the significance of nonvirulent strains remains unknown.

Work with gram negative plant pathogens has revealed that at least two types of interactions can occur when nonvirulent strains are present *in planta*. Many gram
negative pathogens possess a suite of hypersensitive response and pathogenicity (hrp) genes that are necessary for pathogenicity on host plants (Lindgren, 1997). Virulent, wild type strains possessing functional hrp genes are also capable of producing a hypersensitive response (HR) on nonhost plants. Although hrp genes have not yet been found in gram positive organisms, Cmm and the closely related C. michiganensis subsp. sepedonicus (Cms) are also capable of eliciting HR, and have been found to secrete HR-inducing proteins (Alarcón et al., 1998; Gitaitis, 1990; Nissinen et al., 1997). Studies with hrp mutants exhibiting attenuated in planta growth and wild type strains of Pseudomonas syringae pv. syringae (Hirano et al., 1999) and Xanthomonas campestris pv. campestris (Kamoun and Kado, 1990) showed that coinoculation allowed the mutant strains to multiply to near wild type levels. This phenomenon was hypothesized to occur due to exocellular components secreted by the wild type strains that complemented the defective gene in the mutant. Other studies focusing on coinoculation of two nonvirulent strains exhibiting different phenotypic and/or genotypic characteristics showed that, together, the strains were capable of causing symptom expression on the host (Bellemann and Geider, 1992; Hirano et al., 1999). The nonvirulent strains caused no symptom expression when inoculated individually, indicating that each strain contributed some factor(s) the other lacked to cooperatively cause disease. Interestingly, in planta complementation leading to symptom expression has also been shown to occur between two nonvirulent Cms strains, each possessing either one of two pathogenicity indicators (HR ability or cellulase production; Nissinen et al., 2001).

The purpose of this study was two-fold: first, to reveal whether coinoculation of nonvirulent and virulent Cmm strains would result in enhanced in planta movement
and/or multiplication of the nonvirulent strains, and second, to determine whether interactions between the strains could be monitored using a combination of dilution plating and colony blot immunoassays using the CMM1 monoclonal antibody (MAb, clone 103-142; Alvarez et al., 1993). If both purposes were fulfilled, this system would be used for future in planta studies; if not, alternate systems would be explored. The two nonvirulent Cmm strains used in this study were impaired in their ability to colonize and multiply in planta, and did not produce HR on four o'clock (Mirabilis jalapa, Gitaitis, 1990). However, they reacted with the CMM1 MAb, and therefore could be monitored using the colony blot immunoassay (Chapter 2). In order to use this MAb as a marker, however, the virulent coinoculated strain had to be nonreactive. Only one virulent strain has been identified previously that did not react with CMM1 (Alvarez and Kaneshiro, 1999); however, it was atypical in colony morphology, and was unable to elicit HR on four o’clock. Since only HR-positive virulent strains were used in the previous studies (Hirano et al., 1999; Kamoun and Kado, 1990), it was unknown whether complementation would be observed in this system.

Materials and Methods

Strain selection. Nonvirulent and virulent Cmm strains isolated from tomato were selected out of a collection of Cmm cultures from various geographical regions initially based on their reactivity to the CMM1 MAb (Alvarez et al., 1993), their reactivity with CM3/CM4 (Sousa Santos et al., 1997) and CMM-5/CMM-6 (Dreier et al., 1995) primer sets and their virulence on tomato (Lycopersicon esculentum cv. Kewalo). Virulence was determined by stab inoculations of three- to four-week old tomato plants,
as previously described (Chapter 3). Initial experiments were conducted to track the
nonvirulent strains through inoculated plants using a modification of the stem imprinting
technique developed by Gitaitis et al. (1991). One-cm stem sections taken from the
inoculation point to the apical meristem were pressed onto Cmm1 semiselective medium
(Alvarez and Kaneshiro, 1999), and then incubated to observe for colony development.
The same stem sections also were imprinted into microtiter wells, which were then filled
with 100 µl carbonate bicarbonate buffer (CBC; pH 9.6) and dried down for an indirect
ELISA using the CMM1 MAb (described in Chapter 3). Two nonvirulent strains
exhibited limited \textit{in planta} movement (as shown by reduced growth on Cmm1 medium
with increased distance from the inoculation point), and apparently attenuated \textit{in planta}
multiplication (as evidenced by weak to no reactivity with the CMM1 MAb from
imprinted stem sections exhibiting colony development on agar). These strains, A4748
and A4868, were selected for further study. Similar initial experiments were done to
assess the growth of virulent strain A4758. At 21 days, this virulent strain showed
extensive colonization in the inoculated stems.

Both selected nonvirulent strains were MAb CMM1-positive and were negative
using the two \textit{Cmm}-specific primer sets (Table 4.1). Virulent A4758 did not react with
the CMM1 antibody and was positive with both primer sets. Nonvirulent A4748 and
A4868 were previously identified in the laboratory of D. Fulbright by repetitive
sequence-based (rep)-PCR fingerprinting as \textit{Cmm} types B and C, respectively (Kaneshiro
and Alvarez, 2001).

\textbf{Preparation of inocula.} Stock cultures of the bacteria were preserved at \(-80^\circ\text{C}\)
and regrown before each experiment on yeast sucrose calcium carbonate agar (YSC) at
28°C for three days. Immediately before plant inoculations, suspensions of the virulent and nonvirulent strains were individually prepared in phosphate buffered saline (PBS; 0.01M, pH 7.4) and adjusted to A₆₀₀ = 0.1 (equivalent to 10⁸ cfu/ml). Mixed inocula were prepared by combining equal volumes of the adjusted suspensions from nonvirulent A4748 and virulent A4758 in a single tube. Initial bacterial concentrations for each strain were estimated by dilution plating.

**Plant inoculations.** Tomato plants at the four- to six-leaf stage of growth were used for all inoculations. Depending upon the experiment, between 30 µl to 50 µl of the prepared inocula were injected into the plant stems between the cotyledons using a 27-gauge insulin syringe. Individual plants were inoculated with either single-strain suspensions (A4758, A4748, or A4868), with the mixed inocula (A4748 + A4758), or with PBS buffer as negative controls. Inoculated plants were incubated on a greenhouse bench. Experiments were terminated approximately 2 weeks after symptoms were first observed on plants inoculated with the virulent Cmm strain.

**Plant sampling and determination of in planta bacterial titers.** Four to five plants were sampled for each single strain inoculation treatment. Two plants were sampled for the virulent-nonvirulent strain pair. Ten stem sections were taken per plant in 1 cm increments from the inoculation point towards the apical meristem, and immediately processed. Cut sections were weighed and surface sterilized with a 5 min soak in 0.5% sodium hypochlorite solution followed by two consecutive rinses in sterile distilled water. Each section was macerated in 1 ml PBS buffer using a sterile mortar and pestle, and the collected extract serially diluted in PBS buffer. Extract from each dilution tube was plated in three 33.3 µl spots to Cmm1 semiselective medium (Alvarez and
Kaneshiro, 1999) and incubated for up to 5 days at 28°C for colony development. For plants inoculated with single strains, colonies were visually counted and colony forming units (cfu) per stem section were calculated. To identify MAb CMM1-positive, nonvirulent A4748 colonies recovered from coinoculated plants, dilution plate spots were blotted with 0.45 μm ‘Nitrobind’ nitrocellulose membranes (Osmonics, Inc., Minnetonka, MN) and processed using the colony blot immunoassay procedure described in Chapter 2. MAb CMM1-positive colonies, visualized as dark purple spots on the developed nitrocellulose membranes, were counted using a dissecting microscope, and cfu per stem section were calculated.

**Data analysis.** Average initial and recovered cfu per plant were calculated for the single strain inoculation and coinoculation treatments. Values for recovered bacterial concentrations represented the sum of bacteria present in ten sampled stem sections per plant. Confidence intervals were determined for each treatment. Average recovered cfu per stem section were also calculated. Data comparing bacterial recovery from individual stem sections in the single strain-inoculated and coinoculated treatments were analyzed using the Student’s T-test.

**Results**

**Enumeration of virulent and nonvirulent Cmm from single strain-inoculated plants.** Three separate experiments were done to determine the extent of *in planta* movement and multiplication of the virulent and nonvirulent *Cmm* strains. Four to five plants were sampled for each single strain treatment. Sampling occurred between 30 to 60 days after inoculation, depending upon the rate of symptom development on virulent
strain-inoculated plants. As was seen in previous tomato bioassays using the stab-
inoculation technique, plants inoculated with suspensions of either nonvirulent strain 
A4748 or A4868 showed no canker or leaf wilt at sampling, although a slight vascular 
discoloration was observed in the lower stem sections of a few samples. Nonvirulent 
bacteria could not be recovered higher than 7 and 6 cm from the inoculation point, 
respectively. Total cfu counts in the stem sections assayed were similar to calculated 
initial inoculum concentrations (ca. 10^6 cfu; Table 4.2). These data confirm that the 
nonvirulent bacteria used in this study are impaired in their ability to move and multiply 
in tomato plants, but are capable of survival in planta for at least 60 days.

In comparison to plants inoculated with the nonvirulent strains, all plants 
inoculated with virulent strain A4758 developed a spreading canker at the inoculation 
point, while a few also developed characteristic one-sided wilting of the leaves. When 
dissected, stem sections were mealy and had a brown-colored vasculature. Colonies of 
strain A4758 were recovered from all stem sections tested. Bacterial titers were generally 
between 10^8 and 10^9 cfu per stem section, representing an increase of at least three orders 
of magnitude from the calculated initial inoculum concentration (Table 4.2).

**Enumeration of MAb CMM1-positive, nonvirulent A4748 from coinoculated 
plants.** Concentrations of virulent A4758 from coinoculated plants were estimated by 
counting colonies on the Cmm1 dilution plates. Nonvirulent A4748 colonies were not 
recovered at the higher plated dilutions (e.g., 10^4 to 10^7-fold dilutions), and colonies 
could not be visually identified on the Cmm1 plates at lower dilutions (e.g., 10^6 to 10^3-
of fold dilutions) due to the confluent overgrowth by the coinoculated virulent strain. For 
this reason, the colony blot immunoassay was employed to detect individual colonies of
the MAb CMM1-positive, nonvirulent strain in high backgrounds of the MAb CMM1-negative, virulent strain.

The colony blot immunoassay allowed the enumeration of pinpoint colonies of the MAb CMM1-positive, nonvirulent strain A4748 among high concentrations (ca. $10^5$ to $10^7$ cfu, estimated) of the CMM1-negative strain (Fig 4.1). Strain A4748 showed severely restricted colony development when the MAb CMM1-negative strain was present at titers ranging from $10^7$ to $10^8$ cfu per 33.3 μl spot. Reactive colonies gradually increased in size, as well as decreased in number, as the extract was diluted. Recovery of these putative MAb CMM1-positive colonies was attempted, but was unsuccessful due to the overgrowth of the virulent strain on the plates.

**Coinoculation of virulent and nonvirulent Cmm.** Two coinoculated plants were sampled to determine whether *in planta* coincubation affected the movement or growth characteristics of the paired strains; specifically, whether nonvirulent A4748 showed growth or colonization enhancement when incubated with virulent A4758. Coinoculated plants had at least a shallow canker at the inoculation point and a brown discoloration of the vasculature, but these symptoms were not as extensive as those from plants inoculated with the virulent strain alone. As with the single-strain treatments, recovery of bacteria from the coinoculated plants showed virulent A4758 present at high titers in all sampled stem sections, whereas nonvirulent A4748 appeared to be incapable of extensive *in planta* movement or multiplication (Fig 4.2). *In planta* coinoculation with virulent A4758 did not confer any growth enhancement to nonvirulent A4748 or increase its movement through the stem. Likewise, simultaneous inoculation of nonvirulent A4748 did not reduce the growth or colonization capabilities of virulent A4758.
Coinoculation, therefore, did not appear to alter *in planta* growth or colonization characteristics of either strain.

**Discussion**

The *Cmm* strains used in this study did not appear to interact with each other when coinoculated into tomato plants. However, the monitoring system used in this study restricted the choice of strains that could be detected simultaneously in coinoculation studies.

Although similar in focus, the *hrp* mutant studies by Hirano et al. (1999) and Kamoun and Kado (1990) differed significantly from our study in methodology. Antibiotic markers were used in those studies to differentiate the strains when recovered from coinoculated plants. Additionally, only total cfu per plant were recorded. In our study, the CMM1 MAb was used to follow the upward movement of the nonvirulent strains, while the Cmm1 semiselective medium was used to track the virulent strain. Although a novel approach to *in planta* monitoring, use of this MAb to trace a nonvirulent strain through the plant immediately eliminated all but one virulent *Cmm* strain from coinoculation consideration. This virulent strain, however, exhibited atypical colony morphology and did not elicit HR on *M. jalapa*. Previous studies have only shown enhanced growth of nonvirulent strains *in planta* when coinoculated with typical wild type strains.

It would be worthwhile to repeat this experiment using a different marker for *in planta* tracing, such as another MAb that would allow us to select a typical wild type *Cmm* for coinoculation, differential antibiotic resistance, or, possibly, tagging the
nonvirulent strain with a green fluorescent protein (GFP). Using any of these systems would permit a typical virulent strain to be coinoculated, while, at the same time, also allowing accurate monitoring of the nonvirulent strain. Only once a system is found that meets these parameters can an understanding of typical \textit{in planta} interactions between nonvirulent and virulent \textit{Cmm} be reached.
Table 4.1. Strains of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) used in this study.

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>CMM1 reactivity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CM&lt;sub&gt;3&lt;/sub&gt;/CM&lt;sub&gt;4&lt;/sub&gt;</th>
<th>CMM-5/CMM-6</th>
<th>Virulence&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A4748</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Nonvirulent</td>
<td>Kenya</td>
</tr>
<tr>
<td>A4868</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Nonvirulent</td>
<td>California</td>
</tr>
<tr>
<td>A4758</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Virulent</td>
<td>China</td>
</tr>
</tbody>
</table>

<sup>a</sup> Clone 103-142; reactivity in an indirect ELISA assay.

<sup>b</sup> Assessed 21 days post-inoculation.

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Table 4.2. Comparison of bacterial populations in plant tissues pre- and post-inoculation.<sup>y</sup>

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Initial&lt;sup&gt;z&lt;/sup&gt;</th>
<th>Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virulent A4758 x Nonvirulent A4748</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Individual A4748</td>
<td>6.46 ± 0.02 a</td>
<td>6.69 ± 0.27 a</td>
</tr>
<tr>
<td>Coinoculated A4748</td>
<td>6.14 ± 0.00 a</td>
<td>6.23 ± 0.62 a</td>
</tr>
<tr>
<td>Individual A4758</td>
<td>6.05 ± 0.38 a</td>
<td>9.87 ± 0.39 b</td>
</tr>
<tr>
<td>Coinoculated A4758</td>
<td>6.09 ± 0.00 a</td>
<td>9.78 ± 0.25 b</td>
</tr>
</tbody>
</table>

<sup>y</sup> Data are expressed as numbers of bacteria (log [colony forming units]) per sampled stem sections. Data for recovered individual strains represent the average sums of the bacteria recovered from ten 1-cm sections, plus confidence intervals based on 5 and 4 plants for A4748 and A4758, respectively. Values for recovered coinoculated strains were calculated as above, with confidence intervals based on 2 plants.

<sup>z</sup> Values followed by the same letter within the same rows do not differ significantly (95% confidence interval).
Fig 4.1. Enumeration of nonvirulent *Clavibacter michiganensis* subsp. *michiganensis* strain A4748 recovered from coinoculated plants. Dilution plates exhibiting confluent growth of virulent strain A4758 (A) were blotted with nitrocellulose, and a colony blot immunoassay was applied to the dried membranes. Pinpoint purple spots (B) indicated the presence of the nonvirulent strain, which is monoclonal antibody CMM1-positive.
Fig 4.2. Populations of nonvirulent (A4748) and virulent (A4758) Clavibacter michiganensis subsp. michiganensis recovered from single strain-inoculated (■) and coinoculated (■) tomato stems, sampled for 10 cm up the stem in 1-cm increments, starting at the inoculation point (1). Vertical bars represent standard error. Section marked with an asterisk (*) indicates a significant difference ($p<0.05$) between treatments.
Chapter 5

Conclusions

Bacterial canker outbreaks can be prevented only if seed assays are both sensitive and specific for identifying *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*)-infested seed lots. An improved seed assay employing a filtration concentration step and a colony blot immunoassay was developed that detected *Cmm* from samples containing 10 colony forming units (cfu) per 50 ml (equaling 0.2 cfu per ml), a 50-fold increase in sensitivity over a current seed extract plating method. The filtration and immunoassay method identified infested seed samples that contained *Cmm* concentrations lower than the detection threshold of the seed extract plating protocol. Detection and identification of *Cmm* occurs concurrently with this method, and should reduce current assay times by up to 3 weeks.

A high percentage (81%) of the *Cmm* strains isolated from tomato seed were hypovirulent or nonvirulent. Occurrences did not appear to correlate with the geographic origin of the seed lots. These *Cmm* strains were also morphologically indistinguishable from virulent strains on semiselective or differential media. Like the virulent *Cmm* strains, hypovirulent and nonvirulent strains were positive with the CMM1 monoclonal antibody (MAb), were identified to the species level with the MicroLog™ system, and exhibited common bands using rep-PCR that identified them as *Cmm*. Many, but not all, of the hypovirulent and nonvirulent *Cmm* strains were positive with the CMM-5/CMM-6 PCR primer set, which amplifies a portion of the plasmid-borne *pat-1* pathogenicity locus.
(Dreier et al., 1995), and for endoglucanase production, which indicates the presence of the plasmid- or chromosomally-borne celA endo-β-1,4-glucanase gene (Meletzus et al., 1993; Jahr et al., 2000). Since these are the only identified Cmm virulence factors to date, the presence of both in fully nonvirulent strains suggests the existence of other, as yet uncharacterized, genes or loci involved in virulence expression.

The significance of hypovirulent and nonvirulent subpopulations in disease development is unknown. Presence of these less virulent Cmm were previously established in symptomatic tomato plants obtained from various geographic locations (Fulbright et al., 1998; Louws et al., 1998; Werner, 2001), indicating that they are common components of natural Cmm populations in diseased tissues. Interestingly, only nonvirulent strains that did not react to the CMM-5/CMM-6 primer set and did not produce endoglucanase were isolated from a seed lot harvested from symptomatic plants, suggesting a loss of both known virulence factors in planta. Whether these nonvirulent strains are capable of becoming virulent with the in planta transfer of virulence plasmids from coinfected virulent strains is an important, yet unanswered, question. While nonvirulent, endophytic Cmm can be transformed into virulent pathogens with the insertion of the pat-1 locus, virulence cannot be induced in non-endophytic Cmm strains (Dreier et al., 1997). In planta movement of these nonvirulent strains isolated from seed was not addressed in this study, but should be the next step to establish their potential role in symptom development.

In planta tracing of two CMM-5/CMM-6 negative, endoglucanase positive nonvirulent strains in tomato stems indicated that movement of both were restricted, and in planta multiplication did not occur. However, these strains were recovered up to 60
days post-inoculation, indicating that, while they appeared incapable of \textit{in planta} multiplication, survival was not reduced. Results in other pathosystems have indicated that \textit{in planta} coinoculation of a virulent strain and a nonvirulent, non-endophytic strain can increase movement and multiplication of the nonvirulent strain with no observable genetic transfer (Kamoun and Kado, 1990; Hirano et al., 1999). \textit{In planta} coinoculation with a virulent \textit{Cmm} strain and a non-endophytic, nonvirulent \textit{Cmm} strain showed that coinoculation did not affect the growth characteristics of either strain. This experiment should be repeated using other virulent-nonvirulent strain combinations to determine if this lack of interaction is a general phenomenon, or occurs only between specific strains. As of yet, there is no evidence that nonvirulent \textit{Cmm} strains in seed play a role in bacterial canker epidemiology. However, their continued isolation from diseased and infested tissues, and their importance in other pathosystems, suggests some significance and warrants further investigation.


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