Virulence in Bacterial Plant Pathogens: Significance in Diversity of Populations That Cause Bacterial Canker of Tomato

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ABSTRACT

Methods to differentiate yellow seed saprophytes from *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) were assessed, and a potential etiological role for hypovirulent and nonvirulent *Cmm* was studied. Half of the *Cmm* strains isolated from seed were hypovirulent or nonvirulent, but ELISA using MAb Cmm1, MicroLog™ and rep-PCR still consistently differentiated these *Cmm* subpopulations from saprophytes. Co-inoculation of virulent and nonvirulent *Cmm* strains did not alter the capacity of either strain to colonize tomato stems, however, further studies are necessary to confirm these observations. Until it is established that hypovirulent and nonvirulent *Cmm* strains pose no threat to seed health, they cannot be ignored when developing new detection assays.

INTRODUCTION

Strains of the bacterial canker pathogen, *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), exhibit marked differences in virulence, regardless of geographic origin (5). Twenty-six percent of 236 *Cmm* strains isolated from around the world were hypovirulent or nonvirulent, implying that these subpopulations hold some significance. Nonvirulent strains of a related pathogen, which alone cause no symptoms, caused disease when co-inoculated into plants (7), and the epidemiological potential of such strains remains largely unknown. Current tomato seed assays require presumptive *Cmm* be confirmed with plant bioassays, ensuring that some hypovirulent and all nonvirulent strains are misidentified, with unknown consequences. The purpose of this study was to determine the prevalence of hypovirulent and nonvirulent strains from tomato seed, to find methods of separating *Cmm* subpopulations from yellow saprophytes, and to determine whether co-inoculation of strains of varying virulence affects colonization.

MATERIALS AND METHODS

Seed assays. Seed samples (10 – 25 g) from seven seed lots were soaked for 4 hr at room temperature in 30 – 75 ml 0.01 M phosphate buffered saline (PBS, pH 7.4) amended with 100 mg/L cycloheximide and 0.02% Tween 20, then agitated for 15 minutes in a laboratory blender (Stomacher 400, Seward, Inc.). Seeds from two highly contaminated lots were individually macerated in PBS. Sub-samples of extract (0.1 ml) were plated onto CMM semiselective medium (1). Plates were incubated at 28°C for 7 d then *Cmm*-like colonies (yellow to orange, semi-fluidal, convex, with entire margins) were spotted onto YSC medium (containing 10 g yeast extract, 20 g sucrose, 20 g calcium carbonate, and 17 g agar per liter) and tested by ELISA using MAb Cmm1 (clone 103-142)(2).

Characterization of bacterial strains. Virulence was determined on four-week-old tomato seedlings (cultivar 'Kewalo') by wound-inoculation of a 72-hour culture into the

stem between the cotyledons. Plants were observed for 21 d and rated on a scale of 0 - 7 (4). Endocellulase production was detected by the procedure of Nissinen et al. (7). PCR was done using Cm_3/Cm_4 (8) and CMM-5/CMM-6 (3), with conditions similar to those reported. Rep-PCR was performed using the BOXA1R primer (BOX-PCR), as described in Louws and Cuppels (6). Species identifications were obtained with MicroLog3™ (release 4.2.04, Biolog, Inc.) using the Gram positive database (release 6.11).

PCR for 16S rDNA sequence analysis was done using primers 264F (5'-GAT GAT CAG CCA CAT TGG GAC -3') and 1078R (5'-CCC AAC ATC TCA CGA CAC GAG -3'), with 27 µl Platinum PCR SuperMix high fidelity (Invitrogen), 1 µl each primer (10 µM), and 1 µl genomic DNA (50 ng). PCR conditions were: 5 min at 94 °C, followed by 35 cycles of 45 sec at 94°C, 45 sec at 55°C, and 45 sec at 72°C, and ending with 5 min at 72°C. Most significant alignments were obtained through BLASTN search of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/).

Coinoculation of virulent and nonvirulent strains. Two strains were selected based on differences in colony morphology, virulence, and reactivity with MAb Cmm1. Plants were inoculated with individual cultures or were co-inoculated by placing equal amounts of inoculum (10^6 CFU per strain) at the wound site. After 30 d, ten 1-cm stem sections taken from the inoculation point towards the apical meristem were individually ground in PBS buffer and dilution plated to CMM medium. Colonies recovered from plants inoculated with individual strains were enumerated after 5 d. Colonies of the MAb Cmm1-negative virulent strain from the co-inoculated plants were enumerated in this manner, and then plates were blotted with nitrocellulose membranes and run using an immunoblot (4) to enumerate colonies of the MAb Cmm1-positive nonvirulent strain.

RESULTS AND DISCUSSION

Over 27,000 bacterial colonies were isolated from the nine seed lots. After transfer to YSC plates and ELISA using MAb Cmm1, 60 ELISA-positive strains were identified. Presumptive *Cmm* were identified as *C. michiganensis* by 16S rDNA sequence analysis, and 32 were characterized further (Table 1). Additionally, 12 yellow seed saprophytes identified by 16S rDNA as non-*Clavibacter* species were tested to compare reactivities of the various methods with non-target strains. Sixteen of the 32 *Cmm* strains (52%) were either nonvirulent or hypovirulent, and reactivity with PCR and the endocellulase assay was variable. Virulent strains gave more uniform reactions with these methods. None of the saprophytes reacted with either primer set, but seven produced endocellulase. All 32 *Cmm* strains were identified as *C. michiganensis* using MicroLog™, whereas the saprophytes were not. BOX-PCR also differentiated the 32 strains from saprophytes based on three bands (296, ~500 and ~700 bp) present in *Cmm* fingerprints but absent from non-*Cmm* fingerprints. ELISA using MAb Cmm1, MicroLog™ and BOX-PCR were the most reliable of the methods tested for differentiating hypovirulent and nonvirulent *Cmm* from similar-appearing seed saprophytes.

Both virulent and nonvirulent *Cmm* strains colonized tomato stems when inoculated alone, with the nonvirulent strain doing so to a lesser extent (7 cm, compared to 10 cm for the virulent strain). Co-inoculation had no apparent effect on colonization by either the virulent or the nonvirulent strain, as colony counts remained unchanged between individually inoculated and co-inoculated plants. As these conclusions are based on limited studies of a few strains, further studies using new MAbs and strain-specific

primers must be undertaken to understand the nature of the interactions between different bacterial populations. Additional studies to assess the ability of hypovirulent and nonvirulent *Cmm* to infest and survive on seed will also give insight into the potential role these *Cmm* subpopulations play in bacterial canker epidemiology.

Table 1. Characterization of 32 presumptive *Cmm* strains isolated from tomato seed using selected phenotypic and genotypic tests.

Virulence	# of strains	MAb Cmm1	PCR reactivity		Endocellulase production	MicroLog™ (*Cm*)	rep-PCR
			Cm_3/ Cm_4	CMM-5/ CMM-6			
NV	6	+	-	-	-	+	+
NV	1	+	-	-	+	+	+
NV	8	+	+	+	+	+	+
H	1	+	-	-	+	+	+
H	1	+	+	-	+	+	+
V	1	+	+	-	+	+	+
V	14	+	+	+	+	+	+

LITERATURE CITED

1. Alvarez, A.M., Kaneshiro, W.S. and Vine, B.G. (2005) Diversity of *Clavibacter michiganensis* subsp. *michiganensis* populations in tomato seed: What is the significance? Acta Horticulturae (ISHS) 695:205-214.
2. Alvarez, A.M., Derie, M., Benedict, A., and Gabrielson, R. 1993. Characteristics of a monoclonal antibody to *Clavibacter michiganensis* subsp. *michiganensis*. Phytopathology 83:1405.
3. Dreier, J., Bermpohl, A., and Eichenlaub, R. 1995. Southern hybridization and PCR for specific detection of phytopathogenic *Clavibacter michiganensis* subsp. *michiganensis*. Phytopathology 85:462- 468.
4. Kaneshiro, W.S. 2003. Detection and characterization of virulent, hypovirulent, and nonvirulent *Clavibacter michiganensis* subsp. *michiganensis*. M.S. Thesis, University of Hawaii.
5. Kaneshiro, W.S. and Alvarez, A.M. 2001. Specificity of PCR and ELISA assays for hypovirulent and avirulent *Clavibacter michiganensis* subsp. *michiganensis*. Phytopathology 91:S46. Publication no. P-2001-0330-AMA.
6. Louws, F.J. and Cuppels, D.A. 2001. Molecular Techniques. Pages 321-333 in: Laboratory Guide for Identification of Plant Pathogenic Bacteria, 3rd Edition, N.W. Schaad, J.B. Jones and W. Chun, eds. American Phytopathology Society Press, St. Paul, MN.
7. Nissinen, R., Kassuwi, S., Peltola, R., and Metzler, M.C. 2001. *In planta*-complementation of *Clavibacter michiganensis* subsp. *sepedonicus* strains deficient in cellulase production or HR induction restores virulence. Eur. J. Plant Pathol. 107:175-182.
8. Sousa Santos, M., Cruz, L., Norskov, P., and Rasmussen, O.F. 1997. A rapid and sensitive detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seeds by polymerase chain reaction. Seed Sci. Technol. 25:581-584.