ESTABLISHMENT AND VALIDATION OF LOOP-MEDIATED AMPLIFICATION FOR SPECIFIC DETECTION OF TOMATO BACTERIAL PATHOGEN *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS*

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Jarred H. Yasuhara-Bell

Dissertation Committee:

Anne Alvarez, Chairperson
Daniel Jenkins
Gernot Presting
Michael Melzer
Sally Miller
Sean Callahan

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ABSTRACT

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*) causes bacterial canker of tomato and pepper. The purpose of this study was to determine the potential role in disease outbreaks of seed- and plant-associated nonpathogenic *Clavibacter* strains, which gave false positives with currently available diagnostic tests. A loop-mediated amplification (LAMP) assay for specific detection of *Cmm* was designed to target the *clvA* gene, which is located in the clavicidin gene cluster that proved to be unique to and conserved in *Cmm*. PCR profiles of additional genes located on a pathogenicity island on the *Cmm* chromosome showed that a lack of some of these genes, but not all, resulted in a nonpathogenic phenotype. Strains with patterns 1-5 and 7 showed normal virulence, while strains with patterns 6 and 8 were non-pathogenic. LAMP detected all *Cmm* strains, even though some of the essential pathogenicity genes were missing. The *clvA* LAMP detected *Cmm* on tomato seed and infected tomato tissue, with and without an enrichment step. A collection of 348 *Cmm* strains, representing diversity in a worldwide population, was used to validate the assay. Included were two separate populations of seed-associated *Clavibacter* reclassified as two new *Clavibacter* subspecies, with proposed names of *Clavibacter michiganensis* subsp. *chilensis* subsp. nov. and *Clavibactermichiganensis* subsp. *californiensis* subsp. nov. These nonpathogenic strains were often associated with tomato tissue and seed, and cross-reacted with the standard Immunostrip® test, causing false positives. The LAMP assay discriminated them from *Cmm*, as well as other subspecies. In co-inoculation studies, these bacteria did not acquire virulence by gene exchange or synergistic complementation of their secreted enzyme repertoire. It is proposed that the LAMP assay be included in the standard seed testing regimen, with positives indicating contamination by true *Cmm*, and Immunostrip®-positive, LAMP-negative indicating potential presence of other *C. michiganensis* subspecies that should not pose a risk for international distribution of tomato seed. A positive LAMP result could be followed up by pathogenicity profiling using a panel of PCR tests described in this study. The LAMP assay is currently being used in greenhouse surveys for *Cmm* and may later be commercialized for tomato seed testing.

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1.	µg	–	microgram(s)
2.	µl	–	microliter(s)
3.	µM	–	micromolar
4.	A	–	adenine
5.	A	–	alanine
6.	ABC	–	ATP-binding cassette
7.	AFLP	–	amplified fragment length polymorphism
8.	AIC	–	Akaike Information Criterion
9.	aka	–	also known as
10.	AL	–	Alabama
11.	API	–	analytical profile index
12.	ASAP	–	automated simultaneous phylogenetics
13.	ATCC	–	American Type Culture Collection
14.	ATP	–	adenosine triphosphate
15.	AZ	–	Arizona
16.	BASF	–	Badische Anilin- und Soda-Fabrik (Baden Aniline and Soda Factory)
17.	BC	–	British Columbia
18.	BCCM	–	Belgian Coordinated Collections of Microorganisms
19.	BCT	–	bacterial canker of tomato
20.	BHQ	–	black hole quencher
21.	BIP	–	backward inner primer
22.	BL	–	bioluminescent
23.	BLAST	–	basic local alignment search tool
24.	bp	–	base pair(s)
25.	*Bst*	–	*Bacillus stearothermophilus*
26.	BUG	–	Biolog Universal Growth
27.	C	–	Celsius
28.	C	–	cysteine
29.	C	–	cytosine

30. CA – California
31. $CaCO_3$ – calcium carbonate
32. CAD – cadaverine
33. Cat. – catalog
34. CCD – charge-coupled device
35. cel – cellulase
36. CFBP – French Collection of Plant-associated Bacteria
37. CFU – colony forming unit(s)
38. chp – chromosomal homology to *pat*-1
39. CLSM – confocal laser-scanning microscopy
40. clv – clavicidin
41. cm – centimeter(s)
42. *Cmi* – *Clavibacter michiganensis* subsp. *insidiosus*
43. *Cmm* – *Clavibacter michiganensis* subsp. *michiganensis*
44. CMM1T – *Cmm* tris-buffered semiselective medium (Cmm1-Tris100)
45. *Cmn* – *Clavibacter michiganensis* subsp. *nebraskensis*
46. *Cmp* – *Clavibacter michiganensis* subsp. *phaseoli*
47. *Cms* – *Clavibacter michiganensis* subsp. *sepedonicus*
48. *Cmt* – *Clavibacter michiganensis* subsp. *tessellarius*
49. CNS – *Cm. nebraskensis* semiselective
50. Co. – Company
51. Cyclo – cyclohexamide
52. D – aspartic acid
53. d.p.i. day post inoculation/infection
54. Da – Dalton(s)
55. DAB – 2,4-diaminobutyric acid
56. DART – direct assessment in real time
57. ddH_2O – double-distilled water
58. DE – Delaware
59. DE – Deutschland (Germany)
60. Dha – dehydroalanine

61. Dhb – dehydrobutyrine
62. DNA – deoxyribonucleic acid
63. dNTP – deoxynucleotide triphosphate
64. DPG – diphosphatidylglycerol
65. dsDNA – double-stranded DNA
66. DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
67. *E. coli* – *Escherichia coli*
68. EDTA – ethylenediaminetetraacetic acid
69. (E)GFP – enhanced GFP
70. ELISA – enzyme-linked immunosorbent assay
71. ELP – extension-like protein
72. EPPO – European and Mediterranean Plant Protection Organization
73. EPS – exopolysaccharide
74. ERIC – enterobacterial repetitive intergenic consensus
75. ET – esculin trehalose
76. EtBr – ethidium bromide
77. ETI – effector-triggered immunity
78. FAM – carboxyfluorescein
79. FAO – Food and Agriculture Organization of the United States
80. Fe – iron
81. $FeCl_3$ – ferric chloride
82. $FeSO_4$ – ferrous sulfate
83. $FeSO_4 \cdot 7H_2O$ – ferrous sulfate heptahydrate
84. fg – femtogram(s)
85. FIP – forward inner primer
86. FL – Florida
87. FRET – fluorescence resonance energy transfer
88. FS – Fieldhouse-Sasser
89. G – glycine
90. g – gram(s)
91. G – guanine

92. GFP – green fluorescent protein
93. GL – glycolipids
94. GP – Gram-positive
95. *Gsp* – *Geobacillus* sp.
96. h – hour(s)
97. H – histidine
98. H_2O – water
99. H_2O_2 – hydrogen peroxide
100. H_2S – hydrogen sulfide
101. H_3BO_3 – boric acid
102. HCl – hydrochloric acid
103. HI – Hawai‘i
104. hLRT – hierarchical likelihood ratio test
105. HPLC – high-performance liquid chromatography
106. HR – hypersensitive response
107. I – isoleucine
108. I – ivory
109. IA – Iowa
110. ID – Idaho
111. ID – identification
112. IF – immunofluorescence
113. IF – inoculation fluid
114. ILD – incongruence length difference
115. IN – Indiana
116. Inc. – incorporated
117. ISF – International Seed Federation
118. ISHI – International Seed Health Initiative
119. ISSR – inter simple sequence repeat
120. ITS – internal transcribed spacer
121. K – lysine
122. K_2HPO_4 – potassium phosphate (dibasic)

123. kb – kilobase(s)
124. KCl – potassium chloride
125. kDa – kiloDalton(s)
126. KH_2PO_4 – potassium phosphate (monobasic)
127. KNO_3 – potassium nitrate
128. KS – Kansas
129. L – leucine
130. L – liter(s)
131. LAMP – loop-mediated amplification
132. LB – Luria Broth
133. LMG – Laboratorium voor Microbiologie - Universiteit Gent
134. LPPA – Phytopathology Laboratory of the Regional Service of Agrofood Research and Development in the Principality of Asturias
135. Ltd. – limited
136. Lys – lysine
137. M – methionine
138. M – molar
139. MA – Massachusetts
140. MALDI – matrix-assisted laser desorption/ionization
141. Mb – megabase(s)
142. MEGA – molecular evolutionary genetics analysis
143. mg – milligram(s)
144. $MgSO_4$ – magnesium sulfate
145. $MgSO_4 \cdot 7H_2O$ – magnesium sulfate heptahydrate (Epsom salts)
146. MI – Michigan
147. mic – michiganin
148. min – minute(s)
149. ml – milliliter(s)
150. ML – maximum-likelihood
151. MLSA – multilocus sequence analysis
152. mM – millimolar
153. mm – millimeter

154. MN – Minnesota
155. $MnSO_4 \cdot H_2O$ – manganese sulfate monohydrate
156. MO – Missouri
157. MP – maximum parsimony
158. MR-VP – methyl red Voges-Proskauer
159. na – not applicable
160. Na_2SO_3 – sodium sulfite
161. NaCl – sodium chloride
162. NC – North Carolina
163. NCBI – National Center for Biotechnology Information
164. NCPPB – National Collection of Plant Pathogenic Bacteria
165. nd – no dye
166. nd – not determined
167. NE – Nebraska
168. NEAR – nicking enzyme (or, nicking and extension) amplification reaction
169. ng – nanogram(s)
170. NH_4Cl – ammonium chloride
171. $NH_4H_2PO_4$ – ammonium phosphate (monobasic)
172. NIFA – National Institute of Food Agriculture
173. NINA – non-instrumented nucleic acid amplification
174. NJ – neighbor-joining
175. NL – Netherlands
176. No. – number
177. nov. – nova or novae, meaning new
178. nt – nucleotide(s)
179. O – orange
180. OD_{A600} – optical density of absorbance at 600 nm
181. OEPP – Organisation Européenne et Méditerranéenne pour la Protection des Plantes
182. OH – Ohio
183. OR – Oregon
184. Orn – ornithine

185. p – plasmid
186. P – proline
187. PA – Pennsylvania
188. PAGE – polyacrylamide gel electrophoresis
189. PAMP – pathogen-associated molecular pattern
190. PBS – phosphate buffered saline
191. PCR – polymerase chain reaction
192. PFGE – pulse-field gel electrophoresis
193. PG – phosphatidylglycerol
194. pg – picogram(s)
195. php – plasmid homology to *pat*-1
196. PI – pathogenicity island
197. pi – post inoculation/post infection
198. PIA – peptone iron agar
199. POCT – point-of-care testing
200. PR – pathogenesis-related
201. PRR – pattern recognition receptor
202. PS – peptone sucrose
203. PSA – peptone saccharose agar
204. PTI – plant-triggered immunity
205. PUT – putrescine
206. pv – pathovar
207. Q – glutamine
208. R – arginine
209. R – red
210. *R* – resistance
211. ^{r} – resistant
212. rep-PCR – repetitive sequence-based PCR
213. RIF – replication initiation factor
214. Rif – rifampicin
215. RNA – ribonucleic acid

216. RPA – recombinase-polymerase amplification
217. rpm – revolutions/rotations per min
218. s – second(s)
219. S – serine
220. S – sucrose
221. ^{s} – susceptible
222. SA – *Staphylococcus aureus*
223. SAMBA – simple amplification-based assay
224. SCRI – Specialty Crop Research Initiative
225. SDS – sodium dodecyl sulfate
226. SN2 – snakin-2
227. sp. – species (singular)
228. SPD – spermidine
229. SPM – spermine
230. spp. – species (plural)
231. ssDNA – single-stranded DNA
232. STAT – statistics
233. subsp. – subspecies
234. SYBR – Synergy Brands Inc.
235. T – threonine
236. T – thymine
237. t – tonne(s)
238. T – transmittance
239. T3SS – type-3 secretion system
240. *Taq* – *Thermus aquaticus*
241. TE – Tris EDTA
242. TOF – time-of-flight
243. TX – Texas
244. TZC – 2,3,5-triphenyl-tetrazolium chloride
245. U – unit
246. UK – United Kingdom

247. USA – United States of America
248. USDA – United States Department of Agriculture
249. UT – Utah
250. V – valine
251. v – variable
252. V – volt(s)
253. v – volume
254. VA – Virginia
255. VT – Vermont
256. w – weak
257. w – weight
258. W – white
259. WA – Washington
260. WI – Wisconsin
261. wk – weeks
262. WT – wild-type
263. Y – tyrosine
264. Y – yellow
265. YSC – yeast saccharose $CaCO_3$

PREFACE

Tomatoes (*Solanum lycopersicum*) are native to South America, originating from Peru and spreading around the world following the Spanish colonization of the Americas. There are approximately 7,500 tomato varieties that are grown worldwide for various purposes. According to FAOSTAT, tomatoes were the 4th largest crop commodity worldwide in 2012, behind rice, wheat, and soy, respectively. Total worldwide production of tomato reached 161,793,834 t in 2012, totaling $59,108,521,000 (FAOSTAT, 2012). The tomato industry is one of high significance. However, tomato production is affected by a variety of problems such as pests, viruses, bacteria and fungi. In particular, bacterial canker, caused by *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), can cause devastating agroeconomic losses during outbreaks. Bacterial canker was first reported by Erwin F. Smith in 1910 in Grand Rapids, Michigan. Since then, it has spread throughout the world and losses of 20-30% to as high as 70% have been reported (EPPO, 2005). There are no known resistant cultivars, therefore once the disease is found, means of eradicating the pathogen include solarization of the field and/or rotation to non-susceptible host crops, both of which take time and/or money, use of chemical bactericides, which may or may not be totally effective, or total sanitization. The most efficient way to avoid disease is to prevent the introduction of the pathogen into fields and greenhouses. The only real control measures that can accomplish this are making sure the planting area is disease free, purchasing certified pathogen-free seed and transplants, and maintaining phytosanitary cultural practices. This places a large emphasis on plant and seed testing to ensure that no pathogen is present before, during, and after the cultivation process. Current tests for *Cmm* suffer from false results, thus placing an urgent need on development of more efficient and accurate diagnostic tools.

CHAPTER 1
LITERATURE REVIEW

1. *CLAVIBACTER MICHIGANENSIS MICHIGANENSIS*

1.1 Classification

The genus *Clavibacter*, which belongs to the class *Actinobacteria* (Stackebrandt, Rainey, & Ward-Rainey, 1997) and the family *Microbacteriaceae* (Evtushenko & Takeuchi, 2006), was first defined by Davis et al. (1984). The *Clavibacter* species are Gram-positive, aerobic, non-spore forming, coryneform bacteria that were previously grouped within the *Corynebacterium* genus (Eichenlaub, Gartemann, & Burger, 2006). The production of exopolysaccharides (EPS) often causes them to display mucoid colony morphology (Evtushenko & Takeuchi, 2006). There exists only one recognized species of *Clavibacter*, *C. michiganensis*, which comprises five subspecies that are based on host specificity and other characteristics (Burger & Eichenlaub, 2003; Davis, et al., 1984; Eichenlaub & Gartemann, 2011; Gartemann et al., 2003). *Clavibacter* subspecies are generally pigmented yellow to orange, with the exception of *C. michiganensis* subsp. *sepedonicus*, which is non-pigmented. The known subspecies of *Clavibacter* are plant pathogens that cause disease in agriculturally important plants (Eichenlaub, et al., 2006; Evtushenko & Takeuchi, 2006; Gartemann, et al., 2003), usually infecting one primary host and perhaps some closely related species (Eichenlaub & Gartemann, 2011; Eichenlaub, et al., 2006). Non-pathogenic *Clavibacter* strains are isolated frequently from the environment (Nazina et al., 2002; Zaluga et al., 2014; Zinniel et al., 2002), though these strains are often referred to simply as *Clavibacter* sp.

Clavibacter michiganensis subsp. *insidiosus* (*Cmi*) causes wilting and stunting in alfalfa (*Medicago sativa*) (McCulloch, 1925). *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) causes bacterial canker of tomato (*Solanum lycopersicum*) (Davis, et al., 1984; Strider, 1969) and pepper (*Capsicum annuum* and *Capsicum frutescens*) (Lai, 1976; Zutra & Cohn, 1970). *Clavibacter michiganensis* subsp. *nebraskensis* (*Cmn*) causes wilt and blight of maize (*Zea mays*) (Schuster, 1975; Vidaver & Mandel, 1974). *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*) causes ring rot of potato (*Solanum tuberosum*) (Manzer & Genereux, 1981). *Clavibacter michiganensis* subsp. *tessellarius* (*Cmt*) causes leaf freckles and leaf spots in wheat (*Triticum aestivum*) (R.R. Carlson & A.K. Vidaver, 1982; R.R. Carlson & A.K. Vidaver, 1982). Recently, Gonáslez and Trapiello (2012, 2014) described a new subspecies, *Clavibacter michiganensis* subsp. *phaseoli* subsp. nov., that infects bean (*Phaseolus vulgaris* L.) and causes bacterial bean leaf yellowing.

1.2 Pathogenicity genes

The *Cmm* genome is comprised of one circular chromosome (3.298 Mb) with high G+C content (72.6%) (Gartemann et al., 2008) and two circular plasmids, pCM1 (27 kb) and pCM2 (70 kb), each with lower G+C content than the chromosome, at 67.56% for pCM1 and 66.50% for pCM2 (Gartemann, et al., 2008). The *Cmm* chromosome contains all the genes responsible for colonization of host plants (Gartemann, et al., 2008), while virulence genes associated with disease symptoms are plasmid-borne (Burger et al., 2005; Dreier, Meletzus, & Eichenlaub, 1997; Gartemann, et al., 2008; Jahr, Dreier, Meletzus, Bahro, & Eichenlaub, 2000; Meletzus, Bermpohl, Dreier, & Eichenlaub, 1993).

Each virulence plasmid contains a single gene that is considered sufficient for disease induction. pCM1 contains the *celA* gene that encodes for CelA, an endoglucanase (Jahr, et al., 2000; Meletzus, et al., 1993), while pCM2 contains the *pat-1* gene that encode Pat-1, a serine protease (Dreier, et al., 1997). Two proteins with homology to Pat-1 are also encoded on plasmid pCM2, PhpA and PhpB (plasmid homology to *pat*-1 A and B) (Burger, et al., 2005; Stork, Gartemann, Burger, & Eichenlaub, 2008), but no direct association with virulence has been demonstrated as with Pat-1 itself. Plasmid content varies among *Cmm* strains (Kleitman et al., 2008), as plasmids can be lost due to stress; for example, temperatures above 30°C (Meletzus, et al., 1993). Many possess both plasmids (Kleitman, et al., 2008); however strains with only one of the two plasmids can exhibit a hypovirulent phenotype and cause delayed wilting symptoms and canker formation compared to a virulent wild-type (Kaneshiro, 2003; Kaneshiro & Alvarez, 2001; Meletzus, et al., 1993). Loss of both plasmids results in a non-pathogenic phenotype that elicits *in planta* titers as high as a virulent wild-type without causing any disease symptoms (Meletzus, et al., 1993). The presence of these avirulent strains in nature is important, as reacquisition of the plasmids will restore virulence. Gentamicin (Jahr, et al., 2000) and neomycin (Gartemann, et al., 2003) resistance cassettes were used to demonstrate plasmid reacquisition, in conjunction with a plasmid-free mutant strain of *Cmm* (CMM100) carrying chloramphenicol resistance on the chromosome (Gartemann, et al., 2003; Kirchner, Gartemann, Zellermann, Eichenlaub, & Burger, 2001).

The chromosome of *Cmm* contains a 129 kb region of lower G+C content (~64.8%), relative to the rest of the chromosome, that encodes genes with functions relevant for pathogenicity, including colonization and evasion or suppression of host plant defense reactions (Gartemann, et al., 2008). This region is called the *chp*/*tomA* region and is considered a putative pathogenicity island, being that it is flanked by two 1.9 kb direct repeats, each with 99% nucleotide sequence homology, and deletions can occur through recombination within these

repeats (Gartemann, et al., 2008). This region is divided into two subregions, with the *chp* subregion coding for many proteases and other extracellular enzymes. The *chp* subregion also codes for proteases with homology to Pat-1, which were designated ChpA-G (chromosomal homology to *pat*-1) (Burger, et al., 2005; Gartemann, et al., 2008; Stork, et al., 2008). Additionally, the *chp* subregion codes for members of a second family of serine proteases (the Ppa family, PpaA-E) and one subtilase (SbtA) (Gartemann, et al., 2008). These proteins, as well as the Chp proteases, are predicted to be secreted (Gartemann, et al., 2008). Inactivation of *chpC*, as well as *ppaA* and *ppaC*, is associated with reduced colonization titers that result in a hypovirulence (Gartemann, et al., 2008; Stork, et al., 2008), thus demonstrating an essential role of serine proteases in the disease development process (Eichenlaub & Gartemann, 2011). The *tomA* subregion contains the *tomA* gene that encodes for tomatinase, which inactivates α-tomatine, an antibacterial/antifungal saponin present in all parts of a tomato plant (Kaup, Gräfen, Zellermann, Eichenlaub, & Gartemann, 2005). This region also codes for proteins involved in uptake or metabolism of carbohydrates (Gartemann, et al., 2008). As inactivation/loss of some or all the genes located within the *chp*/*tomA* region inhibits colonization and produces an avirulent phenotype, the presence of these strains in nature pose a serious risk for potential outbreaks, representing nonvirulent reservoirs for plasmids that could be transferred to the plasmid-free avirulent strains discussed previously, thereby restoring virulence (Eichenlaub & Gartemann, 2011).

1.3 Studying *Clavibacter michiganensis* subsp. *michiganensis*

The development of cloning vectors greatly aided molecular investigation of *Cmm*. These cloning vectors were designed to contain the origins of replication of *Cmm* plasmids pCM1 and pCM2, as well as that of the *Escherichia coli (E. coli)* vector pBR325 (Laine et al., 1996; Meletzus & Eichenlaub, 1991), and antibiotic resistance genes (Gartemann, et al., 2003). Meletzus and Eichenlaub (1991) demonstrated the first successful transformation of *Cmm*, the method for which has since been modified (Meletzus, Jahr, & Eichenlaub, 2000) and optimized (Gartemann, et al., 2003; Kirchner, et al., 2001) to produce high transformations rates. Improved transformation efficiency has also allowed successful transposon mutagenesis of *Cmm* (Gartemann & Eichenlaub, 2001; Gartemann, et al., 2003). Moreover, these vectors and transposons have application in other *Clavibacter* subspecies (Gartemann, et al., 2003; Kirchner, et al., 2001; Laine, et al., 1996; Meletzus & Eichenlaub, 1991).

Xu et al. (2010) used transposon mutagenesis to develop a bioluminescent strain of *Cmm* (BL-Cmm17) that utilized a *lux* reporter gene. The luciferase enzyme that catalyzes the bioluminescent reaction for light emission is encoded by *luxAB* (Engebrecht & Silverman, 1984). The remaining *lux* genes, *luxCDE*, code for enzymes necessary for producing a fatty aldehyde substrate that is required for the bioluminescence (Engebrecht & Silverman, 1984). Photo emission by cell expressing *lux* can be photographed using a sensitive charged-coupled device (CCD) camera (Hooper & Ansorge, 1990). BL-Cmm17 is a virulent strain that was used to investigate infection and colonization of tomato plants (X. Xu, et al., 2010). This strain provides an extremely useful tool to study all aspects of *Cmm* growth, infection, colonization, transmission, and control *in planta* in real-time (X. Xu, et al., 2010; X. Xu, Rajashekara, Paul, & Miller, 2012).

Taking another approach to real-time *in planta* studies of *Cmm*, Chalupowicz et al. (2012) developed an enhanced (E)GFP reporter plasmid (pK2-22) to produce fluorescent cells that can be visualized by confocal laser-scanning microscopy (CLSM). This plasmid is stable for at least one month *in planta* and was used to follow the colonization and movement of *Cmm*, thus showing the utility for other potential *in planta* studies (Chalupowicz, et al., 2012). Additionally, recent advances in whole genome sequencing have allowed assembly of the complete *Cmm* genome (Gartemann, et al., 2008), promoting bioinformatic and global analysis approaches, including proteome and transcriptome analyses (Flügel, Becker, Gartemann, & Eichenlaub, 2012; Savidor et al., 2012), to assess host-pathogen interactions.

1.4 Disease

Cmm naturally infects host plants though wounds, hydathodes, or via contaminated seed (Gartemann, et al., 2003). Once inside the plant, bacteria colonize the xylem vessels and spread systemically throughout the whole plant (Bryan, 1930), reaching bacterial titers of up to 10^9 CFU/g plant tissue (Gartemann, et al., 2003; Jahr, Bahro, Burger, Ahlemeyer, & Eichenlaub, 1999; Meletzus, et al., 1993), causing the characteristic wilting, stem canker, and vascular discoloration (Bryan, 1930). Disease symptoms usually begin as unilateral wilting of leaves, with wilting eventually spreading to all leaves. Disease progression results in canker lesions development on the stem, followed by plant death. Plants that become infected during late-stage development can survive and produce fruit, which develop spots, called “bird’s eyes.” Infection can then disseminate to seed (Bryan, 1930). Disease symptoms can vary depending on strain virulence (Kaneshiro, 2003; Kaneshiro & Alvarez, 2001; Kaneshiro, Mizumoto, & Alvarez, 2006; Kleitman, et al., 2008), plant age (Forster & Echandi, 1973; Sharabani, Shtienberg, et al., 2013),

temperature (Basu, 1966; R. J. Chang, S. M. Ries, & J. K. Pataky, 1992; Forster & Echandi, 1973; Kendrick Jr & Walker, 1948; Sharabani et al., 2014), relative humidity (X. Xu, et al., 2012), and cultivar of tomato (Çaliş, Bayan, & Çelik, 2012; Crinò et al., 1995; Emmatty & John, 1973; Forster & Echandi, 1973; Poysa, 1993; Thyr, 1971, 1976).

EPS produced by *Cmm* can block xylem vessels, inhibiting water transport, which leads to wilt symptoms (Denny, 1995; Eichenlaub & Gartemann, 2011; Gartemann, et al., 2003; Jahr, et al., 1999). EPS may shield *Cmm* from host plant defense reactions (Beiman, Bermpohl, Meletzus, Eichenlaub, & Bartz, 1992; Benhamou, 1991; Bermpohl, Dreier, & Eichenlaub, 1996; Jahr, et al., 1999; Kiraly, El-Zahaby, & Klement, 1997). Plants utilize pattern recognition receptors (PRRs) as basal defense against invading pathogens by recognizing pathogen-associated molecular patterns (PAMPs) (Jones & Dangl, 2006; Schwessinger & Zipfel, 2008). Virulent strains cause disease by overcoming this PAMP-triggered immunity (PTI) (Speth, Lee, & He, 2007). As *Cmm* does not possess a type-3 secretion system (T3SS) and/or corresponding effectors (Gartemann, et al., 2008), effector-triggered immunity (ETI) (Jones & Dangl, 2006) is not activated, and as a result there is no known resistance to *Cmm*.

Cmm is an especially dangerous pathogen due to the frequent occurrence of latent infections (Franc, 1999; Gitaitis, Beaver, & Voloudakis, 1991; X. Xu, et al., 2010) and their ability to invade seeds (Biddle, McGee, & Braun, 1990; Bugbee & Gudmestad, 1988; Franken, Kamminga, Snyders, Van Der Zouwen, & Birnbaum, 1993; McBeath & Adelman, 1986; Nemeth, Laszlo, & Emody, 1991; Samac, Nix, & Oleson, 1998; Tancos, Chalupowicz, Barash, Manulis-Sasson, & Smart, 2013; Tsiantos, 1987), which allows long-distance spread of the pathogen. Under favorable conditions, one infected seed in 10,000 can cause a disease outbreak (Gitaitis, et al., 1991). *Cmm* can access tomato seeds internally though the plant vasculature or externally by penetrating floral parts or fruits (Agarwal & Sinclair, 1997; Bryan, 1930; de León, Siverio, López, & Rodriguez, 2011; Singh & Mathur, 2004; Tancos, et al., 2013). Another important issue regarding maintenance of *Cmm* within the environment has recently come to light (Dutta, Gitaitis, Smith, & Langsten Jr., 2014). *Cmm* is able infect non-host plant flowers and seed and reside in non-host seedlings as epiphytes and/or endophytes (Dutta, et al., 2014). This poses a new potential risk for inadvertent movement and spread of *Cmm* on non-host plants and seed that requires further attention (Dutta, et al., 2014).

1.5 Control

Various control measures have been attempted to eradicate *Cmm* from infected soil and/or plants, though none are 100% effective. Application of copper compounds and bactericides can reduce bacterial titer (Hausbeck, Bell, Medina-Mora, Podolsky, & Fulbright, 2000; Werner, Fulbright, Podolsky, Bell, & Hausbeck, 2002); however copper compounds have phytotoxic effects and complete elimination of *Cmm* is not achieved. Additionally, various natural compounds, extracts and essential oils have been tested for activity against *Cmm* (Choi, Baek, & Moon, 2014; Hausbeck, et al., 2000; Iacobellis, Lo Cantore, & Capasso, 2005; Kotan et al., 2014; Li, Jin, Chen, & Lu, 2013; Madok, Torres, Wilkens, & Urzúa, 2004; Silva, Pascholati, & Bedendo, 2013; Soylu, Baysal, & Soylu, 2003; Utkhede & Koch, 2004), all with limited success.

Cmm can persist in soil on dead plant tissues for some time. The application of formaldehyde to soil and solarization are measures used to eliminate *Cmm* from soil (Antoniou, Tjamos, & Pnangopoulos, 1995; Shelvin, Mahrer, Kritzman, & Katan, 2004), though these are only partially effective, as dust from infected soil from nearby areas can result in recontamination. *Cmm* does require the presence of plant tissue to persist in soil; therefore it is possible that over time *Cmm* can be eliminated. Switching to non-host crops for 3-5 years can help to accomplish this; however time and cost requirements often make this unfeasible, as well as the newly described problem of non-host *Cmm* persistence (Dutta, et al., 2014).

Although attempts have been made to breed tomato varieties resistant to *Cmm*, no resistant cultivars have been produced that are available for private or commercial use. Attempts have been made to create transgenic plants that exhibit resistance, using expression of *Clavibacter*-specific phage endolysins (Wittmann, Eichenlaub, & Dreiseikelmann, 2010) and over-expression of two tomato proteins, snakin-2 (SN2) and extensin-like protein (ELP) (Balaji & Smart, 2011). While these novel approaches show promise, further in-depth studies are required before these transgenic tomatoes will be made available to the public.

Cmm can ooze from cankers and/or hydathodes, during guttation, and spread along the same plant or to distal plants with the aid of wind and rain (Bryan, 1930; Sharabani, Manulis-Sasson, et al., 2013). Infections can also spread during routine cultural practices such as trimming, defoliation and harvesting. Grafting is a relatively recent advancement in tomato cultivation that is now commonplace during greenhouse production and poses a novel critical point of *Cmm* spread (C. Kubota, McClure, Kokalis-Burelle, Bausher, & Rosskopf, 2008; X. Xu, et al., 2010). The most effective means of controlling the spread on *Cmm* is to ensure no *Cmm* introduction into production areas and/or prevention of spread by practicing Good Seed and Plant

Practices (GSPP, http://www.gspp.eu/). To prevent *Cmm* introduction, the use of only seeds and cuttings certified to be *Cmm*-free is recommended, and detection of *Cmm* in latently infected plants is crucial. Disease severity, combined with lack of any guaranteed control measures, *Cmm* has been classified as a quarantine organism under the European Union Plant Health Legislation, as well as in many other countries (Eichenlaub & Gartemann, 2011; Jahr, et al., 1999).

2. COMMERCIAL SAMPLING AND DIAGNOSIS

2.1 Plant sample testing

The European Plant Protection Organization (EPPO) has published plant testing standards (EPPO, 2013). The plant testing procedure (Figure 1.1) involves taking a plant sample and soaking it for up to 30 min in sterile distilled water or PBS to allow bacterial diffusion and make a suspension. Bacterial suspensions are then dilution plated on nutrient or semiselective media and any suspect colonies are purified and subjected to identification tests, including morphological and/or biochemical characterization using methods by Dye & Kemp (1977) or BIOLOG, MALDI-TOF spectromass photometry (Zaluga et al., 2011), immunosfluorescence (IF) assay, enzyme-linked immunosorbent assay (ELISA), Immunostrip® (Agdia® Inc.), PCR, genomic fingerprinting by BOX-PCR, and barcoding (Coenye et al., 1999; Edwards, Rogall, Blöcker, Emde, & Böttger, 1989; Richert, Brambilla, & Stackebrandt, 2005; Zaluga, et al., 2011). Samples showing no *Cmm* colonies upon reisolation attempts or negative results by identification tests are considered to not contain *Cmm*. Samples yielding both colonies and positive results from at least two identification tests are further tested by pathogenicicty assay to confirm infection by *Cmm* (EPPO, 2013).

The current testing regimen is time consuming and utilizes many resources. The use of a culturing step during presumptive diagnosis adds at least 3-10 days to the time required before beginning identification testing, not including the additional 3-5 days for colony purification. Additionally, standard pathogenicity tests require at least 8-21 days for results. It is of note to remember that there are hypovirulent populations of *Cmm* that cause disease (Kaneshiro, 2003; Kaneshiro & Alvarez, 2001; Kaneshiro, et al., 2006; Kleitman, et al., 2008), but symptoms may not be visible within time allotted for the pathogenicity test. Additionally, there are nonvirulent plasmid-free strains that appear non-pathogenic during pathogenicity testing, but can become virulent by reacquisition of plasmids (Gartemann, et al., 2003; Kirchner, et al., 2001; Meletzus, et al., 1993). Another drawback to the current testing regimen is the potential for false results. Immunoassays are known to cross-react with non-target bacteria, thereby producing false positive results. PCR assays based on genes know to occur on mobile genetic elements can

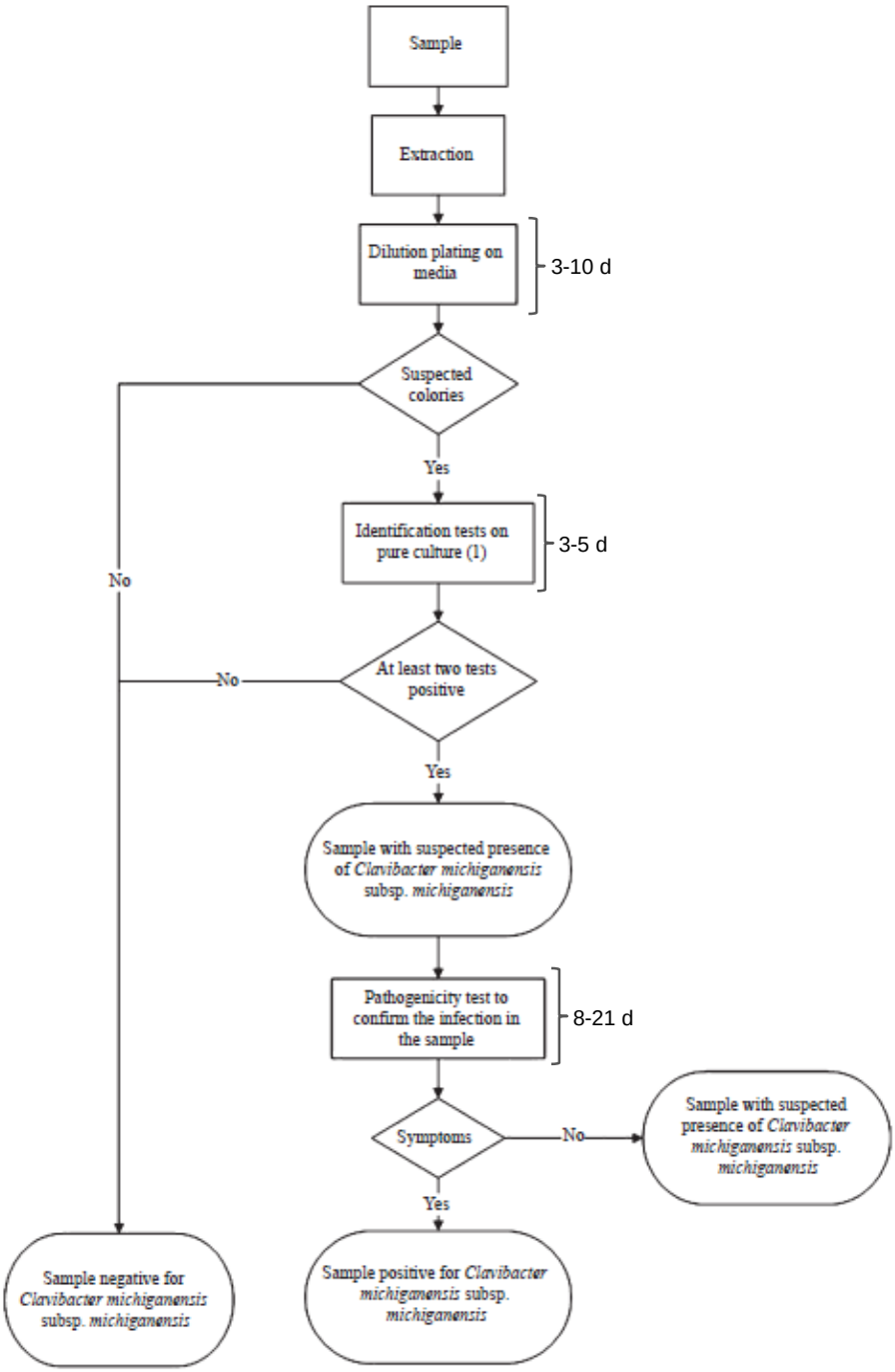


Figure 1.1. Scheme for detection and identification of *Clavibacter michiganensis* subsp. m*ichiganensis* in samples from symptomatic or symptomless tomato plants. Adapted and modified from "*Clavibacter michiganensis* subsp. m*ichiganensis*," by EPPO, 2013, *OEPP/EPPO Bulletin*, 43, p. 47 with permission. Copyright 2013 by the European and Mediterranean Plant Protection Organization.

produce both false positive and false negative results. Dilution plating on semi-selective media can also produce false negatives, depending on concentration of bacteria in the sample and the selective nature of the medium. More important are the existence of the *Cmm*-like bacteria (Jacques et al., 2012; Kaneshiro, et al., 2006; Nazina, et al., 2002; Zaluga, et al., 2011; Zaluga, Van Vaerenbergh, Stragier, Maes, & De Vos, 2013; Zinniel, et al., 2002) that are often found associated with tomato. These bacteria look like *Cmm* in culture and yield positive results with immunoassays, and therefore would be seen as a double positive during presumptive *Cmm* identification. These strains pose a major threat to commercial tomato producers, as presence of a bacterium that resembles *Cmm* and also reacts with immunoassays is cause for concern. Critical decisions have to be made regarding plant and seed health. Appearance of these bacteria during testing could have serious economic impacts, as entire crops/fields could be destroyed and production areas abandoned following presumptive *Cmm* identification.

3.2 Seed sample testing

The EPPO has also published seed testing standards (EPPO, 2013). There are two seed testing procedures (Figure 1.2) that have been validated. Procedure A is similar to the plant testing procedure, with an additional round of dilution plating. A seed sampling protocol has also been published by the International Seed Federation (ISF) (2011) and a minimum of 10,000 seeds is the recommended sample size. For Procedure A, seed extract is obtained by soaking and mechanical crushing (Franken, et al., 1993; Hadas, Kritzman, Kleitman, Gefen, & Manulis, 2005), while Procedure B only requires soaking. For Procedure B, IF is performed on seed extract and fluorescent cells with typical *Cmm* morphology are tested with PCR. PCR-positive samples are subjected to a bioassay (van Vaerenbergh & Chauveau, 1987), followed by reisolation of potential *Cmm.* IF- and PCR-negative samples are considered *Cmm*-free. Seed testing is also time consuming and resource exhaustive, and suffers from false results, as described previously.

3. DETECTION METHODS

3.1 Immunological

Several immunoassays are available for detecting *Cmm.* The SA-agglutination test uses specific antibodies adsorbed onto *Staphylococcus aureus* cells, which are used as the agglutinating agent. Available IF tests utilize different *Cmm*-specific antibodies from Loewe (DE, www.loewe-info.com), Agden (GB, www.agden.co.uk) and Plant Research International (NL, www.plant.wageningen-ur.nl). A variety of ELISAs can be performed using polyclonal

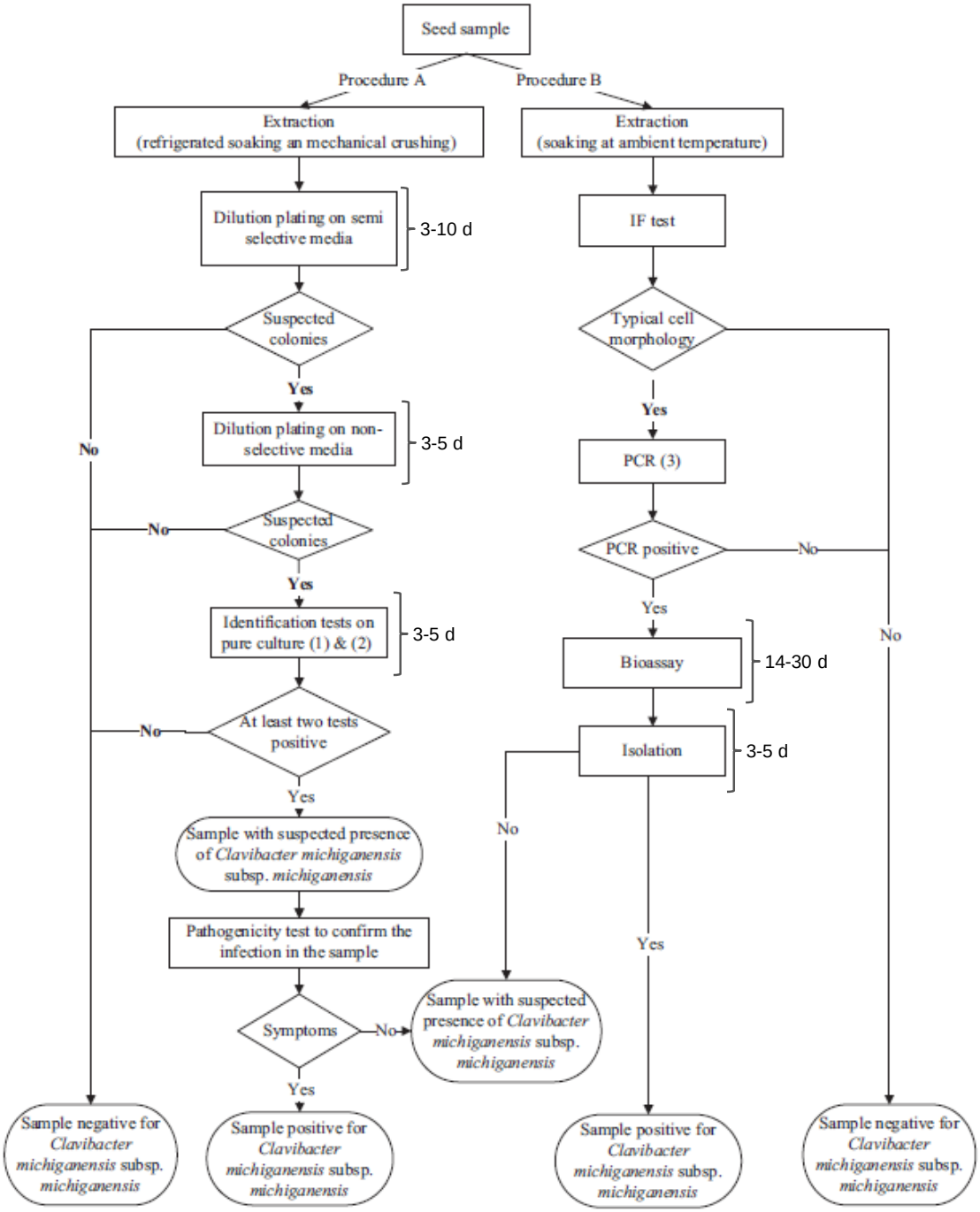


Figure 1.2. Scheme for detection and identification of *Clavibacter michiganensis* subsp. m*ichiganensis* in samples of tomato seed. Adapted and modified from "*Clavibacter michiganensis* subsp. m*ichiganensis*," by EPPO, 2013, *OEPP/EPPO Bulletin*, 43, p. 48 with permission. Copyright 2013 by the European and Mediterranean Plant Protection Organization.

antibodies from Neogen Europe, or a high-quality monoclonal antibody (Cmm1) (Alvarez, Derie, Benedict, & Gabrielson, 1993) that is commercially available from Agdia® Inc. Agdia® Inc. has also commissioned Cmm1 into an easy-to-use, portable, rapid Immunostrip® assay. All available antibodies for specific detection of *Cmm* cross-react with non-target bacteria. The Neogen Europe antibody cross-reacts with other *Clavibacter* subsp. (*Cmi, Cms* and *Cmn*), *Curtobacterium flaccumfaciens*, *Dickeya* sp., *Pantoea dispersa*, *Pantoea agglomerans*, *Pectobacterium atrosepticum, Pectobacterium carotovorum* subsp. *carotovorum* and *Rahnella aquatilis* (Kokošková, Miráz, & Fousek, 2010; Krämer & Griesbach, 2005). The Cmm1 antibody used in the commercial Immunostrip® cross-reacts with all known subspecies of *Clavibacter,* tomato seed- and plant-associated *Cmm*-like strains, *Ochrobactrum* sp. and *Microbacterium* sp. (Alvarez, et al., 1993; Alvarez & Kaneshiro, 1999; Alvarez, Kaneshiro, & Vine, 2005; Kaneshiro & Alvarez, 2001; Kaneshiro, et al., 2006; Sudarshana, May, Kurowski, & Thomas, 2012).

3.2 Molecular

3.2.1 Polymerase chain reaction

Cmm-specific PCR primers have been designed to amplify a variety of molecular targets, including RNA genes, repetitive sequences, a ferredoxin reductase gene, and known virulence genes (Cho et al., 2012; Dreier, Bermpohl, & Eichenlaub, 1995; Kokošková, et al., 2010; Lee, Bartoszyk, Gunderson-Rindal, & Davis, 1997; Louws et al., 1998; Pastrik & Rainey, 1999; Sousa Santos, Cruz, Norskov, & Rasmussen, 1997). Two sets of primers have been designed to amplify the two known plasmid-borne genes, pathogenicity-associated gene *pat-1* on pCM2 (Cmm5: 5'-GCGAATA**C**GCCCATATCAA-3' and Cmm6: 5'-CGTCAGGAGGTCGCTAATA-3') (Dreier, et al., 1995) and *ppaJ* on pCM 1 (CM_3: 5'-CCTCGTGAGTGCCGGGAACGTATCC-3' and CM_4: 5'-CCACGGTGGTTGATGCTCGCGAGAT-3') (Sousa Santos, et al., 1997). One primer set was designed to amplify the *tomA* gene of the putative pathogenicity island (Cmm1F: 5'-GACAAGCACCTCTACACCTGG-3' and Cmm1R: 5'-TTGATCCCTGACTTCAGCGT-3') (Kokošková, et al., 2010). As these genes are located on mobile genetic elements, false negatives have been reported for PCR assays targeting these genes (Alvarez, et al., 2005; Kaneshiro & Alvarez, 2001; Kaneshiro, et al., 2006; Kleitman, et al., 2008).

To avoid false negatives cause by targeting genes on mobile genetic elements, several PCR primer sets were designed to target genes located on stable portions of the chromosome. One primer set was designed to amplify the 16S-23S intergenic spacer region of *Cmm* (PSA-4: 5'-TCATTGGTCAATTCTGTCTCCC-3' and PSA-R: 5'-TACTGAGATGTTTCACTTCCCC-3') (Pastrik & Rainey, 1999). Recently, a primer set was developed to target the ferredoxin reductase

gene (Cmm141F: 5'-CAGGCGTCCGTCGGTGAGGTGGTC-3' and Cmm141R: 5'-GCGGGAGAGCGGTGCGGGAATG-3'), which was highly specific for *Cmm* (Cho, et al., 2012).

PCR analyses can also be used to characterize *Cmm* strains. BOX-PCR and ERIC-PCR can be used to generate DNA fingerprints for *Cmm* strains using the BOXA1R primer (5'-CTACGGCAAGGCGACGCTGACG-3') (Louws, et al., 1998; Louws, Fulbright, Stephens, & de Bruijn, 1994) or two ERIC primers (ERIC1R 5'-ATGTAAGCTCCTGGGTATTCAC-3' and ERIC2 5'-AAGTAAGTGACGGCAAGGCGACGCTGACG-3') (Hulton, Higgins, & Sharp, 1991; Kawaguchi, Tanina, & Inoue, 2010), respectively.

3.2.2 Nicking enzyme amplification reaction

Nicking enzyme (or, nicking and extension) amplification reaction (NEAR) is a novel isothermal nucleic acid amplification technology (Ionian Technologies Inc., 2009a; Maples et al., 2009a, 2009b; Van Ness, Van Ness, & Galas, 2003). NEAR utilizes a strand-displacing polymerase in conjunction with a nicking enzyme to isothermally amplify DNA and RNA targets (Ionian Technologies Inc., 2009a; Maples, et al., 2009a, 2009b; Van Ness, et al., 2003). The sensitivity and specificity of NEAR is comparable to, if not greater than, current PCR methods. NEAR is fast and cheaper than PCR, and its isothermal nature makes it applicable for field diagnostics as a portable test. Since its initial development and publication (Van Ness, et al., 2003), NEAR has been patented (Ionian Technologies Inc., 2009a; Maples, et al., 2009a, 2009b) and licensed to EnviroLogix™ (Ionian Technologies Inc., 2009b). EnviroLogix™ acquired exclusive rights to NEAR for the agricultural sector and has marketed this technology as DNAble® (EnviroLogix, 2013b), with a specific (Spenlinhauer et al., 2011), highly sensitive, portable, real-time fluorescence monitoring format available for detection of *Cmm* (EnviroLogix, 2013a). As NEAR is proprietary technology, it is not readily available for use in research.

3.2.3 Recombinase-polymerase amplification

Recombinase polymerase amplification (RPA) is another novel isothermal nucleic acid amplification technology developed by TwistDX Ltd (Piepenburg, Williams, Stemple, & Armes, 2006; TwistDX). RPA utilizes a polymerase in conjunction with recombinases, which directs primers to homologous sequences within dsDNA, to isothermally amplify DNA, as well as RNA targets, when used with reverse-transcriptiase (Agdia, 2011; Piepenburg, et al., 2006; TwistDX). RPA doesn't require nucleic acid amplification, making it a suitable for field use as a rapid (Craw & Balachandran, 2012), highly specific, highly sensitive, portable test for *Cmm* identification that

is available in a real-time monitoring platform (Euler et al., 2013; Kersting, Rausch, Bier, & von Nickisch-Rosenegk, 2014; Lutz et al., 2010; Mark et al., 2010; Shen et al., 2011; TwistDX). Agdia® Inc. has licensed RPA technology to develop, manufacture, and commercialize rapid DNA and RNA test kits (AmplifyRP™) for detection of plant pathogens, including *Cmm* (Agdia, 2011). As RPA is proprietary technology, it is also not readily available for use in research.

4. Point-of-care testing

Point-of-care testing (POCT) is useful in the field, as suitable facilities and supplies may not be available (Peeling & Mabey, 2010; Yager, Domingo, & Gerdes, 2008). Bringing a test directly into the field allows results acquired faster and prevents any potential sample degradation associated with transportation of samples to a testing facility. Portable handheld instruments and test kits exemplify POCT. The advancement of molecular tools, such as the development of PCR (Mullis & Faloona, 1987), has led to novel nucleic amplification methods for detecting pathogens. Isothermal amplification technologies are well-suited for POCT because they circumvent the need for expensive thermocycling equipment by operating at a constant temperature that can be maintained by relatively simple devices (Labarre et al., 2010). A non-instrumented nucleic acid amplification (NINA®) device was developed for field use (R. Kubota, LaBarre, Singleton, Beddoe, & Weigl, 2011; Labarre, et al., 2010). Fluorescent probes and intercalating dyes (Holland, Abramson, Watson, & Gelfand, 1991; Vitzthum & Bernhagen, 2002) allow real-time monitoring of isothermal amplification reactions, though more complex devices are required to interpret results (Craw & Balachandran, 2012). Recently, Diagenetix Inc. (Honolulu, HI) developed a more advanced POCT device for portable real-time fluorescence monitoring of isothermal amplification reactions (Jenkins, Kubota, Dong, Li, & Higashiguchi, 2011). This patent-pending technology was termed DART™ (Direct Assessment in Real Time) and has potential application with all real-time fluorescence-based diagnostic reactions, allowing potential quantitative analyses.

4.1 Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (LAMP) was developed in Japan at Eiken Chemical Co. Ltd. LAMP is a highly specific and rapid isothermal process that can amplify DNA and RNA targets (R. Kubota, Alvarez, Su, & Jenkins, 2011; R. Kubota, Vine, Alvarez, & Jenkins, 2008; Mori, Hirano, & Notomi, 2006; Mori, Nagamine, Tomita, & Notomi, 2001; Mori & Notomi, 2009; Nagamine, Hase, & Notomi, 2002; Nagamine, Watanabe, Ohtsuka, Hase, & Notomi, 2001; Notomi et al., 2000). Unlike NEAR and RPA, LAMP utilizes only one enzyme, a

polymerase with strand displacement activity, and a unique set of primers (Mori & Notomi, 2009; Notomi, et al., 2000). Four specifically designed primers recognize six distinct DNA sequences to ultimately produce a "dumbbell" structure that is the key to LAMP amplification (Notomi, et al., 2000). Through priming and self-priming reactions, this "dumbbell" structure provides rapid nucleic acid amplification that produces long concatenated stem-loop structures containing inverted repeats of the target DNA, while recycling the original "dumbbell" structure (Nagamine, et al., 2001; Notomi, et al., 2000).

Several modifications to LAMP have been made over the years. The addition of betaine (a DNA-helix destabilizing chemical) to LAMP reactions increased speed and selectivity (Notomi, et al., 2000). Introduction of two additional primers, loop primers, which target the stem loops of the "dumbbell" structure, more than double reaction speed (Nagamine, et al., 2002). Additionally, newly developed polymerases provide increase speed, sensitivity, and resistance to inhibitors (New England BioLabs, 2013a, 2013b; OptiGene, 2013a, 2013b). LAMP products can be detected using a variety of strategies, including gel electrophoresis, real-time turbidimetry, and fluorescence probes (R. Kubota, Alvarez, et al., 2011; Mori, et al., 2006; Mori, Kitao, Tomita, & Notomi, 2004; Mori, et al., 2001; Tomita, Mori, Kanda, & Notomi, 2008). Real-time fluorescence monitoring of LAMP reactions was made possible by the development of a FRET (fluorescence resonance energy transfer)-based assimilating probe (R. Kubota, Alvarez, et al., 2011).

LAMP technology is public domain and is therefore the most widely studied isothermal amplification technology (Craw & Balachandran, 2012), being used for detection of several microorganisms, including clinical and plant-associated bacterial (Enosawa et al., 2003; Hanaki et al., 2011; Harper, Ward, & Clover, 2010; Koide et al., 2010; R. Kubota, LaBarre, et al., 2011; R. Kubota, et al., 2008; Lalande et al., 2011; Lin et al., 2011; McKenna et al., 2011; Misawa et al., 2007; Neonakis, Spandidos, & Petinaki, 2011; Temple & Johnson, 2011; Temple, Stockwell, & Johnson, 2007; Yamazaki, Seto, Taguchi, Ishibashi, & Inoue, 2008), fungal (Lucas et al., 2010; Niessen & Vogel, 2010; Sun, Najafzadeh, Vicente, Xi, & de Hoog, 2010), viral (Curtis, Rudolph, & Owen, 2008, 2009; Dinh, Le, Vuong, Hasebe, & Morita, 2011; Fujino et al., 2005; Hatano et al., 2011; Imai et al., 2006; Kurosaki, Grolla, Fukuma, Feldmann, & Yasuda, 2010; Okafuji et al., 2005; Parida, Posadas, Inoue, Hasebe, & Morita, 2004), and parasitic (Bakheit et al., 2008; Lau et al., 2010; Matovu, Kuepfer, Boobo, Kibona, & Burri, 2010; Njiru et al., 2008; Nkouawa et al., 2010; Poon et al., 2006) pathogens. Additionally, the fact that primer design software (PrimerExplorer V4) is available online (http://primerexplorer.jp/elamp4.0.0/index.html), makes LAMP a suitable choice for developing new real-time diagnostics for detection of *Cmm.*

5. MICHIGANIN A

Many bacteria produce antibiotic compounds. Bacteriocins are antimicrobial peptides that help bacteria gain a competitive advantage over other bacteria within the same ecological niche (Eijsink et al., 2002; Riley & Gordon, 1999; Vidaver, 1983). Bacteriocins inhibit microbial growth by various mechanisms, with membrane pore formation being most notable (Bauer & Dicks, 2005; Chatterjee, Paul, Xie, & van der Donk, 2005; Moll, Konings, & Dreissen, 1999). Little is known about bacteriocins from Gram-positive bacteria, with even less from Gram-positive plant pathogens, as most have been found in Gram-negative bacteria (Chuang, Chien, & Wu, 2007; Feil et al., 2005; Ochiai, Inoue, Takeya, Sasaki, & Kaku, 2005; Van Sluys et al., 2003), Bacteriocins from Gram-positive bacteria vary in structure and function, though several are known to be cell-wall hydrolyzing enzymes, such as lysostaphin (Bastos, Coutinho, & Coelho, 2010).

Bacteriocins are usually classified based on structural and functional characteristics (Bierbaum & Sahl, 2009; Eijsink, et al., 2002). Class I bacteriocins have characteristic posttranslational modifications (Holtsmark, Mantzilas, Eijsink, & Brurberg, 2006a) that include dehydration of threonine residues to dehydrobutyrine (Dhb) and serine residues dehydroalanine (Dha).). Class I bacteriocins are called lantibiotics because these modified residues, in turn, can form β-methyllanthionine or lanthionine ring structures, respectively, by reacting with the sulfhydryl groups of cysteine residues (Bierbaum & Sahl, 2009; Eijsink, et al., 2002). Class II bacteriocins do not possess these modifications (Holtsmark, et al., 2006a). Lantibiotics are further subdivided into two groups, with Group A (type-1) containing elongated peptides that are cationic, flexible, and have pore-forming and cell wall synthesis-blocking activities, while Group B (type-II) contains compact noncationic globular molecules (Holtsmark, et al., 2006a).

Holtsmark et al. (2006a; 2006b) identified three different compounds with antimicrobial activity produced by *Cmm*. The lantibiotic michiganin A (2,145 Da) is heat- and protease-resistant, and has activity against *Cms* (Holtsmark, et al., 2006b). An analysis of the *Cms* genome showed that it does not contain the gene cluster responsible for michiganin A production (Eichenlaub & Gartemann, 2011), thus supporting findings by Holtsmark et al. (2006a, 2006b). Michiganin A is a group-B actagardine-like lantibiotic (Holtsmark, et al., 2006a). The fact that there are relatively few other known group-B lantibiotics, michiganin A is located in the *Cmm* chromosome in a stable manner, and that michiganin A acts on a closely related subspecies (*Cms*) suggests that this region has potential as novel selectable marked for specific detection of *Cmm*.

CHAPTER 2

LOOP-MEDIATED AMPLIFICATION OF THE *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS micA* GENE IS HIGHLY SPECIFIC

1. ABSTRACT

Loop-mediated amplification (LAMP) was used to specifically identify *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), causal agent of bacterial canker of tomato. LAMP primers were developed to detect *micA*, a chromosomally stable gene that encodes a type-II lantibiotic, michiganin A, which inhibits growth of other *C. michiganensis* subspecies. Four hundred and nine bacterial strains (351 *Cmm* and 58 non-*Cmm*) from a worldwide collection were tested with LAMP to determine its specificity. LAMP results were compared to genetic profiles established using PCR amplification of seven genes (*dnaA, ppaJ, pat*-1, *chpC, tomA, ppaA* and *ppaC*). *Cmm* strains produced eight distinct profiles. The LAMP reaction identified all *Cmm* strains and discriminated them from other *C. michiganensis* subspecies and non-*Clavibacter* bacteria. LAMP has advantages over immunodiagnostic and other molecular detection methods because of its specificity and isothermal nature, which allows for easy field application. The LAMP reaction is also not affected by as many inhibitors as PCR. This diagnostic tool has potential to provide an easy one-step test for rapid identification of *Cmm*.

2. INTRODUCTION

Clavibacter michiganensis subspecies *michiganensis* (*Cmm*) is the causal agent of bacterial wilt and canker of tomato (*Solanum lycopersicum*) (Davis, et al., 1984; Strider, 1969) and is one of the most important bacterial pathogens of tomato (Eichenlaub & Gartemann, 2011; Gartemann, et al., 2003). This worldwide industry, valued at $59 billion in 2012 (FAOSTAT, 2012), can suffer substantial economic losses from *Cmm* outbreaks.

Most *Cmm* strains harbor two circular plasmids pCM1 (27 kb) and pCM2 (70 kb) that are involved in virulence, with varying plasmid content found among *Cmm* isolates (Kleitman, et al., 2008). Dreier et al. (1997) and Jahr et al. (2000) showed that the plasmid-encoded pathogenicity genes *celA* (on pCM1) and *pat*-1 (on pCM2) are sufficient for disease induction. The *celA* gene encodes an endo-β-1,4-glucanase, which is a 78 kDa protein (746 amino acids) belonging to the glycosyl hydrolase family 5 (cellulase family A_1) (Jahr, et al., 2000; Meletzus, et al., 1993). The *pat*-1 gene encodes a serine protease, which is a 29.7 kDa protein (280 amino acids) (Dreier, et al., 1997) having significant homology to serine proteases of the chymotrypsin type with serine

and histidine in the catalytic triad (Eichenlaub & Gartemann, 2011). The virulence plasmids can be lost by stress (e.g., growth at temperatures above 30°C), resulting in a reduced-virulence phenotype, and curing of both plasmids results in total loss of virulence (Meletzus, et al., 1993). Plasmid-free strains may attain *in planta* titers as high as the virulent wild-type strains but do not elicit symptoms (Meletzus, et al., 1993), indicating that all functions required for host recognition, invasion, suppression of host defenses, and colonization of the host are encoded on the *Cmm* chromosome. Cured strains that maintain one of the two plasmids, either pCM1 or pCM2, still produce a virulent phenotype, however wilting symptoms are delayed with respect to the wild-type (Meletzus, et al., 1993).

The *Cmm* chromosome includes a genomic island of approximately 129 kb, which has a significantly lower G+C content than that typically found in *Cmm* and occurs close to the origin of replication. The genomic island is flanked by two direct repeats of 1.9 kb and represents a putative pathogenicity island (PI) (Gartemann, et al., 2008), since a deletion of this region can occur by recombination within these repeats. This PI, termed the *chp/tomA* region, is subdivided into subregions based on structural features (Eichenlaub & Gartemann, 2011). Proteins encoded by the *chp* subregion of the chromosome share sequence homology with the Pat-1 protein and were designated *chpA-G* (chromosomal homology to *pat*-1). These genes encode proteases belonging to serine protease family S1A and display the same general structure (Burger, et al., 2005; Stork, et al., 2008). Inactivation of *chpC,* as well as *ppaA* and *ppaC,* leads to a reduction in colonization titer, which results in a reduced virulence phenotype (Gartemann, et al., 2008; Stork, et al., 2008). The *tomA* subregion carries the *tomA* gene that encodes for tomatinase, which deglycosylates the antifungal/antibacterial saponin α-tomatine (Kaup, et al., 2005). TomA of *Cmm* is specific for α-tomatine and cleaves off the tetrasaccharide β-lycotetraose. The *tomA* mutants of *Cmm* show higher sensitivity to α-tomatine than the wild type in plate assays, however *tomA* mutants show no reduction in virulence (Kaup, et al., 2005). Perhaps in wild species of tomato or in cultivars with high concentrations of tomatine, *tomA* may have a protective effect in that detoxification of tomatine may suppress plant defense mechanisms.

Avirulent mutants (cured derivatives lacking pCM1 and pCM2) are able to colonize the tomato plant without producing symptoms and reacquisition of the plasmids restores virulence (Eichenlaub & Gartemann, 2011; Gartemann, et al., 2003). Thus, it is possible that loss of plasmids in a natural *Cmm* population during high temperatures associated with tomato harvest results in frequent isolation of avirulent strains from tomato stems and seed. Whether or not these populations can be complemented in the infection court or restored to virulence by the reacquisition of plasmids from other *Cmm* cells carrying the plasmids is unknown. There is little

information on the epidemiology and the population structure of *Cmm* in areas where outbreaks are common (Eichenlaub & Gartemann, 2011), and the role of avirulent strains during infections is inconclusive (Kaneshiro, 2003).

Contaminated seed is the major inoculum source leading to outbreaks of bacterial canker in agriculture (Gartemann, et al., 2003). As a result, *Cmm* is classified as a quarantine organism in many countries, with cuttings and seeds of tomato requiring certification as *Cmm*-free. The European Plant Protection Organization (EPPO) has released minimal standards for the identification of *Cmm* in plants and seeds (EPPO, 2013). Immunodiagnostic and molecular methods based on primers developed from ribosomal RNA genes, repetitive sequences or known virulence genes are available to confirm identity of *Cmm,* following isolation of suspect bacteria by growth on semiselective media (Dreier, et al., 1995; Lee, et al., 1997; Louws, et al., 1998; Pastrik & Rainey, 1999; Sousa Santos, et al., 1997). Pathogenicity tests are time-consuming and unreliable because avirulent populations occur frequently in natural *Cmm* populations. Additionally, PCR assays based solely on virulence genes may produce false results (Alvarez & Kaneshiro, 1999; Alvarez, et al., 2005; Kaneshiro, 2003; Kleitman, et al., 2008). In this paper, we evaluate an alternative diagnostic method for a genetically diverse population of *Cmm* strains from an international collection. Loop-mediated amplification (LAMP) was utilized as the diagnostic tool for this study, with primers being designed to detect a single gene, *micA,* in order to specifically detect all *Cmm* strains, both virulent and avirulent.

3. MATERIALS AND METHODS

3.1 Bacterial strains and culture conditions

A total of 409 bacterial strains were tested in this study. Strains with K, A and C numbers (100, 11, and 23, respectively) were from the Pacific Bacterial Collection at the University of Hawai‘i at Mānoa and determined to be *Cmm*; A and C strains were previously characterized by bacteriological, genetic, and pathogenicity tests (Kaneshiro, et al., 2006) and K strains were also characterized by *dnaA* sequence analysis (Schneider, Marrero, Alvarez, & Presting, 2011). Another set of 233 strains, designated by T numbers, were acquired from OmniLytics Inc. (Salt Lake City, UT) and subcultured. Strains T001-T016 were positive with *Cmm*-specific Immunostrips® (unpublished report from OmniLytics Inc.) but did not have the cultural morphology of *Cmm*. When analyzed by 16S PCR they had closest identity with *Ochrobactrum* sp. (T001-T002 and T013-T015) and *Microbacterium* sp. (T003- T012 and T016). All other T strains were identified as *Cmm* by phenotypic tests. Forty-two additional non-*Cmm* strains were tested (two *Clavibacter michiganensis* subsp. *sepedonicus* [K090 and *Cms* from the Netherlands],

two *Clavibacter michiganensis* subsp. *insidiosus* [K091 and *Cmi* from the Netherlands], *Clavibacter michiganensis* subsp. *nebraskensis* [*Cmn* (from the Netherlands)], *Clavibacter michiganensis* subsp. *tessellarius* [*Cmt* (from the Netherlands)], *Actinobacter* sp., *Agromyces* sp., *Burkholderia* sp., *Cellulosmicrobium* sp., *Enterobacter* sp., *Micrococcus* sp., *Pectobacterium* sp., 5 *Ralstonia* spp., 3 *Staphylococcus* spp., 17 *Clavibacter* spp. from tomato seed and 2 additional *Microbacterium* spp.). Strains were removed from -80°C, plated onto TZC-S medium (17 g/L agar, 10 g/L peptone, 5 g/L sucrose and 0.001% 2,3,5-triphenyl-tetrazolium chloride (TZC)) and then incubated at 26°C (± 2°C). Isolated colonies were streaked onto YSC medium (17 g/L agar, 10 g/L yeast extract, 20 g/L sucrose and 20 g/L $CaCO_3$) and incubated at 26°C (± 2°C) for large quantity production. Bacterial cells were harvested from YSC plates (approximately 50-250 µl worth) and put into 1.5 ml microfuge tubes for DNA extraction and/or storage at -20°C for later use, or added to cryotubes containing Luria Broth (LB) (10 g/L tryptone, 2.5 g/L NaCl and 5 g/L yeast extract) with 25% glycerol for storage at -80°C as subcultures. For pathogenicity testing, strains were removed from -80°C, plated onto PSA medium (17 g/L agar, 10 g/L peptone and 5 g/L sucrose) and then incubated at 26°C (± 2°C).

3.2 DNA extraction

A Chelex® DNA extraction was performed on samples contained in the 1.5 ml microfuge tubes. Briefly, 0.75-1.0 ml of 40% Chelex® 100 resin (Bio-Rad, Hercules, CA, Cat. No. 142-1253) in 1X TE buffer (10 mM Tris HCl and 1 mM EDTA at pH 8) with 10% Triton™ X-100 (Sigma-Aldrich, St. Louis, MO, Cat. No. T-6878) was added to each tube. Samples were mixed with vigorous pipeting and vortexing and then heated to 95°C for 10 min on a digital heat block. Samples were stored at 4°C for at least 24 h to allow for separation of the DNA.

3.3 Loop-mediated amplification (LAMP)

A LAMP diagnostic reaction was developed for *Cmm* using the LAMP primer design program (PrimerExplorer) located on the Eiken Chemical Co., Ltd (Tokyo, Japan) website (http://primerexplorer.jp/e/). Primers were designed to amplify the *micA* (michiganin A) region of the *Cmm* genome (Table 2.1). LAMP reactions were performed in triplicate and contained 5 µl bacterial DNA (~5 pg) and 20 µl LAMP reaction master mix (micALAMP2-F3 (0.2 µM), micALAMP2-B3 (0.2 µM), micALAMP2-FIP (1.6 µM), micALAMP2-BIP (1.6 µM), micALAMP2-LoopF (0.8 µM), micALAMP2-LoopF Probe (0.08 µM), and Quencher probe (0.16 µM), dNTP mix (1.2 mM), 10X ThermoPol Reaction Buffer (New England Biolabs, Ipswich, MA, Cat. No. B9004S) (2 mM), betaine (1 M), $MgSO_4$ (4 mM), *Bst* DNA polymerase

(New England Biolabs, Ipswich, MA, Cat. No. M0275L) (8 U) and ddH_2O). Negative controls contained 5 µl ddH_2O and 20 µl LAMP reaction master mix. LAMP reactions were run and analyzed using the iQ^{TM}5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, Cat. No. 170-9780) under the following conditions: 65°C for 1h, with fluorescence readings being taken at 1-min intervals, and then a final 5 min at 85°C.

Table 2.1. LAMP primers used in this study.

Oligonucleotide Primer	Sequence (5'-3')	Source/Reference
micALAMP2-F3	CGACAACAGGAACACAGGT	This Study
micALAMP2-B3	GCCACATTCGATGGTGAGC	This Study
micALAMP2-FIP	GAGCAGCATGTCCCACCGGGACACGATGAACGACATCCTC	This Study
micALAMP2-BIP	CGTCCGTCCAGACCCAGATCGCTGGACATGTACGGGCTCA	This Study
micALAMP2-LoopF	TGACCATGACGGGGGTCT	This Study
micALAMP2-LoopF Probe	/56-FAM/ ACGCTGAGGACCCGGATGCGAATGCGGATGCGGATGCCGATGACCATGACGGGGGTCT	This Study
Quencher probe	TCGGCATCCGCATCCGCATTCGCATCCGGGTCCTCAGCGT/3BHQ_1/	Kubota et al., 2011a

/56-FAM/: 5' 6- carboxyfluorescein.
/3BHQ_1/: 3' Black Hole Quencher 1

3.4 Polymerase chain reaction (PCR)

PCR amplifications were carried out using six primer pairs designed to amplify six different regions of the *Cmm* genome (1 region on each plasmid and 4 chromosomal regions). Two of the PCR primer pairs are commercially available and were designed to detect pathogenicity plasmids pCM1 (CM_3/CM_4 - *ppaJ*) (Sousa Santos, et al., 1997) and pCM2 (Cmm5/Cmm6 - *pat*-1) (Dreier, et al., 1995). Four primers were designed to amplify *tomA* (tomatinase A) and *chpC* (chromosomal homology of *pat*-1), along with pathogenicity genes *ppaA* and *ppaC*, contained within the putative PI of the chromosome. Primers used to detect the *dnaA* gene were RIF (replication initiation factor) primers developed by Schneider et al. (2011). The primer sequences are listed in Table 2.2. The primer sequence used for Cmm5 was developed by Dreier et al. (1995), with a 1 nt change by Kokošková et al. (2010). PCR reactions for all primers except *dnaA*F/R were performed in a 25 µl reaction volume containing 5 µl bacterial DNA and 20 µl PCR reaction master mix [primers (0.2 µM each), 5X Green GoTaq® Reaction Buffer (Promega, Madison, WI, Cat. No. M7911) (2 mM), dNTP mix (0.2 mM), MangoTaq^{TM} DNA Polymerase (Bioline, Randolph, MA, Cat. No. BIO-21078) (1 U), and ddH_2O)]. For *dnaA*F/R, reactions were performed in a 25 µl reaction volume containing 5 µl bacterial DNA and 20 µl PCR reaction master mix [primers (1 µM each), 5X Green GoTaq® Reaction Buffer (2 mM), dNTP mix (0.2 mM), MangoTaq^{TM} DNA Polymerase (1 U), and ddH2O)]. PCR reaction conditions for all primers used can be found in Table 2.2. PCR products were resolved using

1.5% agarose gel electrophoresis. Gels were analyzed using the Foto/Analyst® Express System (Fotodyne Inc., Hartland, WI).

Table 2.2. PCR primers and conditions used in this study.

Primer	Sequence (5'-3')	Cycle Conditions (35 cycles of)	Size (bp)	Source/Reference
Cmm5* Cmm6	GCGAATACGCCCATATCAA CGTCAGGAGGTCGCTAATA	94°C for 7 min, (94°C for 1 min, 55°C for 1 min, 72°C for 1 min), 72°C for 5 min	614	Dreier et al., 1995; Kokoskova et al., 2010
CM_3 CM_4	CCTCGTGAGTGCCGGGAACGTATCC CCACGGTGGTTGATGCTCGCGAGAT	94°C for 7 min, (94°C for 1 min, 60°C for 1 min, 72°C for 1 min), 72°C for 5 min	645	Sousa Santos et al., 1997
chpC-F3 chpC-B3	TCCGGAATCTCCCGGAAG CGGAGCATATATGCCCAACC	95°C for 5 min, (95°C for 15 s, 55°C for 30 s, 72°C for 30 s), 72°C for 7 min	203	This Study
tomA-F3 tomA-B3	ATCACAGTGGTGAAGTGCTC TTCAATGGCCTTTCTCCCG	95°C for 5 min, (95°C for 15 s, 55°C for 30 s, 72°C for 30 s), 72°C for 7 min	229	This Study
ppaAF ppaAR	CTGGTTCTGGTTTGCGGGC TGCTGCTGGTCCTCCTGGTA	95°C for 5 min, (95°C for 30 s, 55°C for 30 s, 72°C for 30 s), 72°C for 7 min	496	This Study
ppaCF ppaCR	TGGTCGTCGTTGAGGAGGCA CTGTCGTCGGAGCGTCGGAT	95°C for 5 min, (95°C for 30 s, 55°C for 30 s, 72°C for 30 s), 72°C for 7 min	140	This Study
dnaAF dnaAR	TACGGCTTCGACACCTTCG CGGTGATCTTCTTGTTGGCG	94°C for 5 min, (94°C for 30 s, 61°C for 1 min, 72°C for 30 s), 72°C for 10 min	933	Schneider et al., 2011

*C indicates the change made by Kokošková et al. (2010).

3.5 DNA sequencing

PCR products for dnaAF/R were purified using a MinElute PCR Purification Kit (Qiagen, Valencia, CA, Cat. No. 28006), and the products were sequenced at the University of Hawai‘i sequencing facilities, using the same forward and reverse primers, according to their specifications.

3.6 Phylogenic analyses

Sequence alignment and phylogenetic analyses were performed using MEGA5 version 5.05 (Tamura et al., 2011). Sequence data for *Cmm* strains NCPPB 382, K0074, K0079, K0084, K0087, K0428, K0448, K0449, K0465 and K0469 (Accession: AM711867.1, HM181168.1, HM181170.1, HM181247.1, HM181274.1, HM181201.1, HM181213.1, HM181216.1, HM181227.1 and HM181230.1, respectively), *Cmi* strain K0091 (Accession: HM469685.1), *Cms* strain K0090 (Accession: HM181287.1) and out-group *Aquifex aeolicus* (Accession: AE000657.1) were obtained from NCBI GenBank. Sequences were trimmed to 661 nt and multiple alignments were made using ClustalW, taking into account the corresponding amino acid alignments for protein-coding genes. Maximum-parsimony (MP) analysis was used to construct trees with the closest neighbor interchange at search level 3 (Nei & Kumar, 2000). Neighbor-joining (NJ) analyses (Saitou & Nei, 1987) were performed using the Jukes-Cantor method (Jukes & Cantor, 1969) to compute evolutionary distances. Confidence intervals were assessed using the bootstrap method with 1000 replications (Felsenstein, 1985).

3.7 Nucleotide accession numbers

The *Clavibacter* sp. partial *dnaA* sequences have been deposited into the GenBank Database with accession numbers KC416011-KC416025.

3.8 Pathogenicity testing

A sterile scalpel was used to make a vertical incision (~1 mm) on the stem, between the cotyledons, of juvenile tomato plants (*Solanum lycopersicum* cv. 'Kewalo') that were approximately 3-4 weeks old. Bacteria (~10^9 CFU/ml) were applied to the wound, using the scalpel tip. Negative controls were made by applying 50 µl ddH_2O to the wound. Inoculated plants and controls were then carefully placed into plastic bags, closed using twist-ties, and incubated at room temperature for 24 h. After the initial incubation, plants were removed from the bags and allowed to grow for 4 wk in a growth room (30°C) under Philips F40/AGRO Agro-Lite™ fluorescent lamps (Philips, Amsterdam, NL, Cat. No. 392282). Plants were checked periodically for symptoms and pathogenicity was determined at 4 wk post-inoculation, relative to controls. Wilt and/or necrosis of true leaves and/or canker at the inoculation site were indicative of pathogenicity. Two plants were used per test for each strain, with the entire set of tests being repeated three times.

4. RESULTS

4.1 Molecular identification and bacterial characterization using LAMP and PCR

The utility of the LAMP reaction was evaluated by molecular tests performed on 409 bacterial strains that were also characterized by PCR. Three hundred and fifty-one strains (85.81%) were LAMP-positive and formed eight distinct genetic patterns, as determined by PCR (Table 2.3 and Appendix A). Three strains (0.85%) [A4598, A4820 and A4830] were positive for LAMP, but did not match any of the above profiles and were designated Patterns 9 and 10 in Table A.1. All non-*Cmm* strains tested were negative for the LAMP reaction (Table 2.4). Two *Ralstonia* strains (A3292 and A3293), five *Ochrobactrum* strains and 13 *Microbacterium* strains were positive for CM_3/CM_4, but were negative for all other PCR tests. Interestingly, one strain of *Cms* (K90) was also positive for CM_3/CM_4. Five *C. michiganensis* strains (2 *Cmi*, *Cmn* and 2 *Cms*) were positive for *dnaA*, while the *Cmt* strain did not produce a reaction with the *dnaA* PCR test, suggesting that this strain may not be a true *Cmt*. This study included 17 *Clavibacter* sp. strains that were isolated from tomato seed, twelve of which were from the Netherlands. These strains were subjected to the same tests and all strains, except X-4, were positive only for

dnaA, suggesting that they are in fact *Clavibacter* spp. Strain X-4 was subjected to 16S rDNA analysis and showed the closest match with *Agromyces* sp. (99% max. ID). The remaining strains were then subjected to *dnaA* sequence analyses.

Table 2.3. Representative groups of 351 *Clavibacter michiganensis* subsp. *michiganensis* strains[a] as determined by LAMP, PCR and pathogenicity tests.

Strain #	% Population	dnaA	Cmm5/6	$CM_{3/4}$	chpC	tomA	ppaA	ppaC	LAMP	Pathogenicity	Pattern #
252	71.79	+	+	+	+	+	+	+	+	+	1
26	7.41	+	-	+	+	+	+	+	+	+	2
19	5.41	+	+	-	+	+	+	+	+	+	3
4	1.14	+	-	-	+	+	+	+	+	+	4
18	5.13	+	+	+	-	+	+	+	+	+	5
5	1.42	+	+	+	-	-	+	+	+	-	6
5	1.42	+	+	+	+	+	-	+	+	+	7
19	5.41	+	+	+	-	-	-	-	+	-	8

[a]Patterns of three LAMP-positive *Cmm* strains (A4598, A4820, and A4830) were not identical to any of the above and are shown in Appendix A.

Table 2.4. Non-*Clavibacter michiganensis* subsp. *michiganensis* strains evaluated by LAMP and PCR tests.

Strain #	Other Designation	dnaA	Cmm5/6	$CM_{3/4}$	chpC	tomA	ppaA	ppaC	LAMP
1	*Acinetobacter* sp.	-	-	-	-	-	-	-	-
1	*Acgromyces* sp.	-	-	-	-	-	-	-	-
1	*Burkholderia* sp.	-	-	-	-	-	-	-	-
1	*Cellulosmicrobium* sp.	-	-	-	-	-	-	-	-
17	*Clavibacter* sp. from tomato seed	+	-	-	-	-	-	-	-
2	*Cm.* subsp. *insidiosus*	+	-	-	-	-	-	-	-
1	*Cm.* subsp. *nebraskensis*	+	-	-	-	-	-	-	-
1	*Cm.* subsp. *sepedonicus*	+	-	-	-	-	-	-	-
1	*Cm.* subsp. *sepedonicus*	+	-	+	-	-	-	-	-
1	*Cm.* subsp. *tessellarius*	-	-	-	-	-	-	-	-
2	*Curtobacterium* sp.	-	-	-	-	-	-	-	-
1	*Enterobacter* sp.	-	-	-	-	-	-	-	-
13	*Microbacterium* sp.	-	-	+	-	-	-	-	-
1	*Micrococcus* sp.	-	-	-	-	-	-	-	-
5	*Ochrobactrum* sp.	-	-	+	-	-	-	-	-
1	*Pectobacterium* sp.	-	-	-	-	-	-	-	-
1	*Ralstonia eutropha*	-	-	-	-	-	-	-	-
2	*R. solanacearum*	-	-	+	-	-	-	-	-
2	*R. solanacearum* (*fliC* -)	-	-	-	-	-	-	-	-
3	*Staphylococcus* sp.	-	-	-	-	-	-	-	-

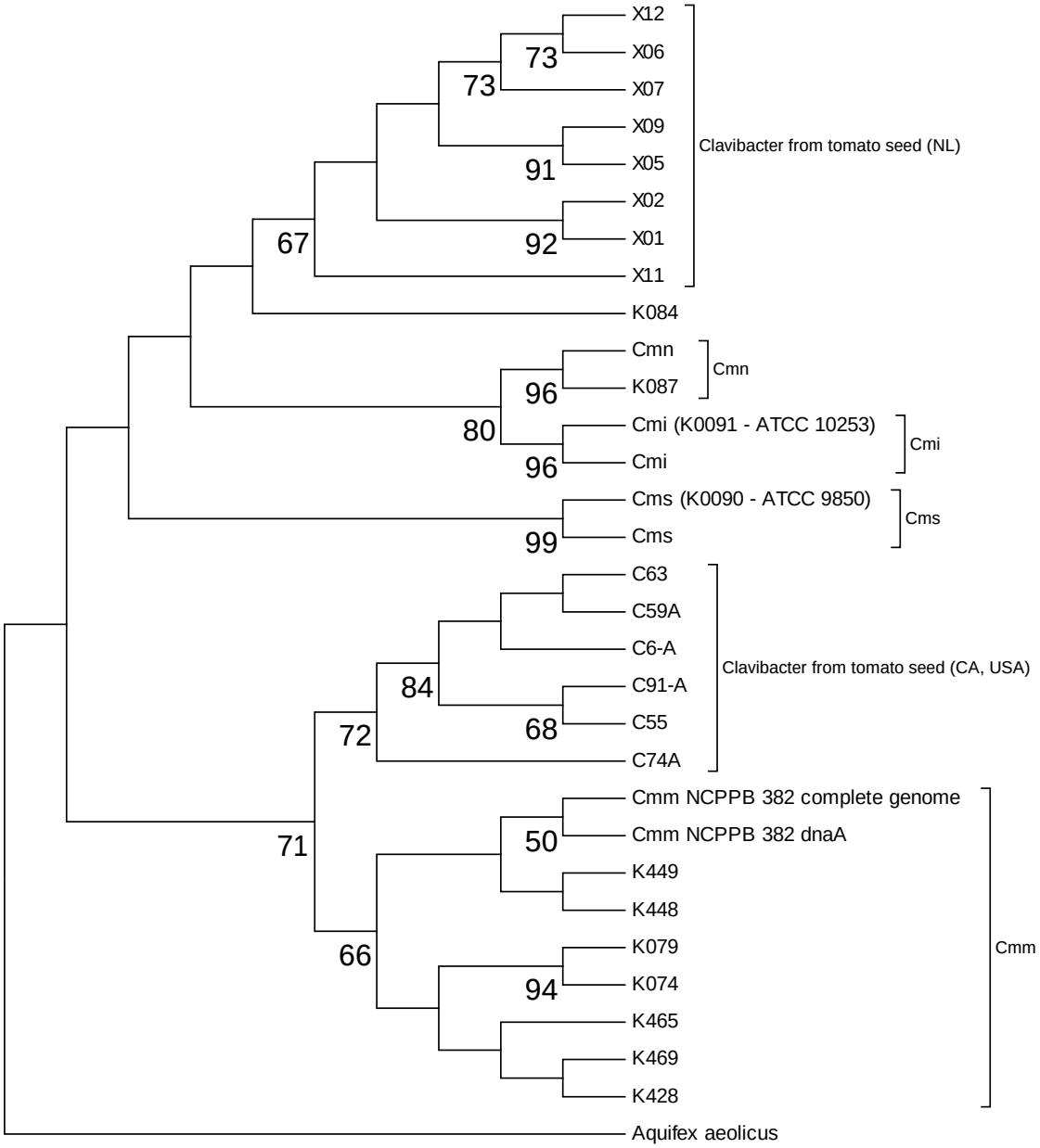


Figure 2.1. Phylogenetic analysis of *Clavibacter* strains based on *dnaA* sequences. Maximum-parsimony (MP) tree. *dnaA* sequences were trimmed to 661 nt and multiple alignments were made using ClustalW, taking into account the corresponding amino acid alignments for protein-coding genes. MP analysis was used to construct trees with the closest neighbor interchange at search level 3. Confidence intervals were assessed using the bootstrap method with 1000 replications.

4.2 Phylogenetic analysis based on *dnaA* sequence data.

The subset of nine *Cmm* strains used for this analysis clustered separately from six unnamed *Clavibacter* spp. isolated from tomato seed from California (Kaneshiro, 2003) and from eight unnamed *Clavibacter* spp. isolated from tomato seed in the Netherlands (this study) (Figure 2.1). The *Cmm* strains also clustered separately from all other known subspecies of *Clavibacter michiganensis* (*Cmi*, *Cms* and *Cmn*) (Figure 2.1). Neighbor-joining analyses produced consensus trees with analogous topology (Appendix B, Figure B.1).

4.3 Pathogenicity of strains representative of each distinct population group

Results of pathogenicity tests, some of which have already been reported (Kaneshiro, et al., 2006), are shown in Figure B.1. Strains having Patterns 1, 2, 3, 4, 5 and 7 produced typical canker symptoms, mostly within 6 to 14 d, whereas strains from Patterns 6 and 8 produced no symptoms within the observation period (28 d after inoculation) (Table 2.3 and Appendix C). The three strains comprising Patterns 9 and 10 as well as the *Clavibacter* spp. strains isolated from seed also were non-pathogenic on tomato.

5. DISCUSSION

Clavibacter michiganensis subsp. *michiganensis* is an agroeconomic bacterium that can cause devastating losses to tomato production during outbreaks. The severity of the disease and the problems in controlling the spread of this pathogen have resulted in its classification as a quarantine organism under the European Union Plant Health Legislation, as well as in many other countries (Eichenlaub & Gartemann, 2011). Extensive control measures are taken to limit the number of outbreaks, including constant monitoring and testing of field samples, as well as only allowing the distribution of seed that has been certified to be *Cmm*-free. EPPO standards for *Cmm* testing have been published (EPPO, 2013). Plants samples are taken and rapid tests for presumptive diagnosis (SA-agglutination, IF, or PCR) are performed, along with dilution plating for bacterial isolation. If the rapid tests are not positive and plating shows no typical colonies, *Cmm* is said to not have been detected. If at least one rapid test is positive, but no typical colonies are found, the tests are repeated. If the rapid test is positive and typical colonies are detected, colonies are purified by subculture and subjected to further identification tests. These pure cultures are identified by testing for pathogenicity and at least one of the following: biochemical characteristics, SA-agglutination, IF, ELISA, PCR, genomic fingerprinting or SDS-PAGE. If two tests are positive, *Cmm* is said to have been detected. This testing regimen is time consuming and utilizes many resources, but has been deemed necessary for proper identification of *Cmm*. It

would be advantageous to at least have a rapid diagnostic system that could detect all presumptive *Cmm* while safely permitting negative samples to pass through the screening procedure. Workers could then definitively say whether or not *Cmm* is present and focus their efforts on isolation and subsequent pathogenicity testing for positives only, thereby eliminating the need for large-scale plating, bacterial isolations and potential retesting.

Loop-mediated amplification (LAMP) is a relatively new molecular detection method that involves an isothermal reaction (65°C). LAMP utilizes *Bst* DNA polymerase, which has DNA-displacement activity. At 65°C, the DNA helix is unstable, allowing the *Bst* polymerase to invade the helix and displace the two strands as it polymerizes, thus eliminating the need for denaturation. The isothermal nature of this reaction is advantageous as there is no need for expensive thermal cycling equipment, as required for PCR. LAMP has adequate sensitivity, and can be highly specific through proper primer design. The LAMP reaction is also relatively fast, with majority of reactions starting exponentially beTween-20 to 30 min, and requiring a maximum reaction time no greater than 1 hour. A positive LAMP reaction can be observed visually via turbidity (R. Kubota, et al., 2008), making it suitable for field application, using a NINA® device (R. Kubota, LaBarre, et al., 2011; Labarre, et al., 2010). LAMP reactions have been adapted for visualization in real-time with fluorescent probes (R. Kubota, Alvarez, et al., 2011). LAMP reactions also can be used directly in the field via SMART-DART™ technology (Jenkins, et al., 2011). Currently, LAMP technology is being applied to detection of several bacterial plant pathogens, including *Ralstonia solanacearum* (R. Kubota, LaBarre, et al., 2011; R. Kubota, et al., 2008) *Xylella fasidiosa* (Harper, et al., 2010) and *Erwinia amylovora* (Temple & Johnson, 2011; Temple, et al., 2007).

In this study, a LAMP reaction was designed to amplify the *micA* region of the *Cmm* chromosome, which encodes an antibiotic. Many bacteria produce antibiotic compounds that are either nonribosomally synthesized antibiotics or ribosomally synthesized proteins, also termed bacteriocins. These compounds are thought to be involved in the competition among bacteria for nutrients and specific habitats. The *micA* gene encodes for a heat- and protease-resistant peptide of 2,145 Da (AMP-II), the lantibiotic michiganin A (Holtsmark, et al., 2006a, 2006b). Michiganin A is a type-II actagardine-like lantibiotic with activity against *Cms*, the potato ring-rot pathogen. *Cms* lacks the gene cluster responsible for the production of michiganin A (Eichenlaub & Gartemann, 2011), suggesting that these compounds are utilized for population control of closely-related species. This region of the *Cmm* genome was chosen because other genes targeted for molecular detection are either virulence factors that exist on plasmids or genes located in a PI within the chromosome. Molecular detection tests based on a single pathogenic

determinant may produce false results. For example, a detection aimed at only one of the two plasmid-borne genes may give a negative result in a pathogenic strain, producing a false negative. In contrast, strains possessing both virulence plasmids could be non-pathogenic if missing the *chpC, tomA, ppaA* and *ppaC* region. Though detected by the corresponding PCR assay, the pathogenicity test would be negative and thus the assay would be considered a false positive by PCR. The observation that *micA* is present on the chromosome in an area not prone to deletions suggested that this gene should be present in all *Cmm* strains, regardless of pathogenicity. In addition, michiganin A inhibits closely related subspecies, such as *Cms*, so logically this region would be absent from the other *Clavibacter* subspecies.

Kleitman et al. (2008) found varying plasmid content among *Cmm* isolates. Twenty *Cmm* strains were tested for *celA, pat*-1, *chpC, ppaA* and *tomA*, as well as pathogenicity. Fifteen strains (75%) were pathogenic and positive for all five genes tested. The remaining 5 strains (25%) were non-pathogenic (Kleitman, et al., 2008). Two of the 5 non-pathogenic strains were negative only for the *chpC* region, thus confirming that the *chpC* gene of the *chp/tomA* PI alone is sufficient to produce a non-virulent phenotype, regardless of the presence of the pathogenicity plasmids. Two other non-pathogenic strains were negative for both *pat*-1 and *chpC*. The final non-pathogenic strain was only negative for *ppaA*, thus confirming this gene's role in pathogenicity. There are few known studies that include the *ppaA,* even though it has been attributed to a low colonization titer that results in reduced virulence. Additionally, no studies have been published that test for *ppaC.* In our study, we examined a large number of strains from widely separated geographical origins in order to gain a better understanding of the kinds of populations that occur in nature and to demonstrate the variety of gene content within strains that hamper current molecular detection. These results demonstrate the utility of the LAMP reaction.

In this study, all *Cmm* strains reacted with the LAMP, while all non-*Cmm* bacteria failed to react, thus displaying the LAMP's potential to eliminate false positives. LAMP reactions were observed at 20-30 min, which is typical for a LAMP reaction (Harper, et al., 2010; Jenkins, et al., 2011; R. Kubota, et al., 2008; Temple & Johnson, 2011; Temple, et al., 2007). Interestingly, several non-*Cmm* species produced a positive reaction with the CM_3/CM_4 primers used to detect the presence of a gene on plasmid pCM1. These results demonstrate the potential occurrences of false positives with available primers. The majority of the *Cmm* strains tested in this study (252 - 71.79%) were positive for all regions tested, corresponding closely with the results by Kleitman et al. (2008) who reported that 15/20 (75%) of *Cmm* strains were positive in PCR tests for all five genes analyzed. The virulence factor composition of these strains predicts that they will possess full pathogenicity. This prediction was confirmed by pathogenicity tests

(Table 2.3 and Appendix C). Strains positive only for $CM_{3/4}$ or Cmm5/6 are predicted to be pathogenic; however, they may have a slightly reduced or delayed pathogenicity (Eichenlaub & Gartemann, 2011; Gartemann, et al., 2003; Kleitman, et al., 2008). This prediction was also confirmed by the pathogenicity tests (Table 2.3 and Appendix C). Strains negative for both plasmid-encoded genes are predicted to be non-pathogenic. However, under the conditions of the pathogenicity tests performed here, these strains were pathogenic (Table 2.3 and Appendix C), which contradicts findings by other groups. Some strains were negative for only *chpC* or *ppaA*, which would suggest that these strains would be non-pathogenic. Again, these strains were pathogenic under the conditions of the pathogenicity tests performed here (Table 2.3 and Appendix C). It was unusual to find that strains missing the *chpC* or *ppaA* gene were pathogenic, as these results contradict the findings by other groups. A possible explanation as to why the pathogenicity tests yielded positives for Patterns 5 and 7 may be the high inoculum titers. These strains possess the pathogenicity plasmids, however lack the ability to colonize plants, due to the absence of key colonization genes located on the chromosome. At high inoculum levels, they may be able to produce canker symptoms without fully colonizing the plant. Further tests that allow quantification of inoculum titers for precise consistency between tests may be needed, along with testing more strains from each pattern. It is unknown as to why strains of Patterns 4, 5, and 7 produced a pathogenic phenotype. The remaining strains were negative for both *chpC* and *tomA*, or *chpC, tomA, ppaA* and *ppaC*, which would suggest that these strains would be non-pathogenic. These strains were non-pathogenic (Table 2.3 and Appendix C), thus confirming previous reports. It is interesting to note that these results, along with those from Kleitman et al. (2008), indicate that *chpC, tomA, ppaA* and *ppaC*, which are all located in the same putative pathogenicity island, can be lost independently of each other. Three strains were positive for LAMP, but did not match any of the 8 different profiles. Strains A4598 and A4820 (Pattern 9) appeared to be derived from Pattern 8 and strain A4830 (Pattern 10) appeared to be from Pattern 6, however none of the strains reacted with Cmm5/6 and may reflect a loss of pCM2. It is possible that stress caused the curing of pCM2, while pCM1 remained, possibly due to its more stable nature (Eichenlaub & Gartemann, 2011; Gartemann, et al., 2003; Jahr, et al., 2000).

The elevated temperatures during warm summer harvests or during the seed drying process may stimulate the conversion of virulent strains into plasmid-free non-virulent strains. An unforeseen cohabitation of tomato seed or plant with two non-virulent strains, one being a plasmid-free *chpC-ppaA-ppaC*-positive strain and the other possessing the virulence plasmids but missing *chpC, ppaA* and/or *ppaC*, is a potentially dangerous situation with possible devastating consequences. Should the non-virulent plasmid-free strains reacquire one or both plasmids from

the other non-virulent population, restoration to full virulence would produce an outbreak. This scenario demonstrates the need for reevaluating the way these non-virulent populations are viewed. Non-virulent strains cannot be simply disregarded during routine seed and plant tests, and such strains need further study.

The *Clavibacter* sp. strains isolated from tomato seed were morphologically similar to *Cmm*, and were positive with Immunostrips® (Alvarez, et al., 2005; Kaneshiro, 2003; Kaneshiro & Alvarez, 2001; Kaneshiro, et al., 2006) but negative for pathogenicity. Phylogenetic analysis grouped these strains separately from *Cmm* and other known subspecies of *C. michiganensis*. Similar findings were recently reported by Zaluga et al. (2011) and Jacques et al. (2012). Such strains look like *Cmm* in culture and often produce false positives when using current immunodiagnostic methods; however, the LAMP assay described here was able to distinguish such strains from true *Cmm.* This study validates the use of the *micA* gene for specific identification of *Cmm*, using LAMP as the diagnostic test.

CHAPTER 3

GENES *clvA*, *clvF* AND *clvG* ARE UNIQUE TO *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS* AND HIGHLY CONSERVED

1. ABSTRACT

Bacterial canker of tomato, caused by *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), occurs worldwide. This study focuses on three genes, *clvA* (clavicidin) and two associated genes (*clvF* and *clvG*, encoding a putative ABC-type multidrug transport system ATPase and permease component, respectively). Loop-mediated amplification of *clvA* and PCR of *clvA*, *clvF* and *clvG* showed positive reactions to only in *Cmm* and no other *Clavibacter* species/subspecies, as well as no other genera of plant-associated bacteria. Sequences of *clvA*, *clvF* and *clvG* from 48 geographically diverse strains of *Cmm* were analyzed phylogenetically to determine sequence variation. Maximum parsimony (MP), neighbor-joining (NJ) and maximum likelihood (ML) analyses placed strains into subgroups irrespective of their geographical origins. Multi-locus sequence analysis (MLSA) of these three genes produced the same results. All three *clv* genes are unique and conserved in *Cmm*, further supporting the use of the *clvA* gene for identification of *Cmm*, using either a previously designed LAMP assay or PCR with additional primers developed in this study. The genes investigated here are novel targets for specific detection of *Cmm* and have numerous molecular diagnostic applications.

2. INTRODUCTION

Clavibacter michiganensis subspecies *michiganensis* (*Cmm*) is the causal agent of bacterial wilt and canker of tomato (*Solanum lycopersicum*) (Davis, et al., 1984; Strider, 1969) and is one of the most important bacterial pathogens of tomato (Eichenlaub & Gartemann, 2011; Gartemann, et al., 2003). Several methods have been developed to identify and characterize *Cmm*, including conventional isolation, pathogenicity tests, biochemical characterization, metabolic profiling (BIOLOG), immunodiagnostic and molecular methods. Primers developed from ribosomal RNA genes, repetitive sequences or known virulence genes, are available to confirm the identity of *Cmm* (Alvarez, et al., 2005; Dreier, et al., 1995; Kaneshiro, et al., 2006; Lee, et al., 1997; Louws, et al., 1998; Milijaevšić-Marčić et al., 2012; Pastrik & Rainey, 1999; Rijlaarsdam et al., 2004; Sousa Santos, et al., 1997). However, many molecular methods involve sequence analysis, which requires time, money, and expertise, and PCR assays based solely on virulence

genes may produce both false positive and false negative results (Alvarez & Kaneshiro, 1999; Alvarez, et al., 2005; Kaneshiro, 2003; Kleitman, et al., 2008).

Recently, a loop-mediated amplification (LAMP) reaction was designed to amplify the *micA* (hereafter referred to as *clvA*) region of the *Cmm* chromosome (Yasuhara-Bell & Alvarez, 2012; Yasuhara-Bell, Kubota, Jenkins, & Alvarez, 2013), which encodes for a type-II actagardine-like lantibiotic called Michiganin A (Holtsmark, et al., 2006a, 2006b) (hereafter referred to as clavicidin) that has activity against other *Clavibacter* subspecies. Numerous strains collected from widespread geographic regions were examined and the LAMP reaction specifically detected all *Cmm* (Yasuhara-Bell & Alvarez, 2012; Yasuhara-Bell, et al., 2013). However, since bacteria may undergo independent evolution in distinct geographic locations, there was concern that tests based on the *clvA* gene and associated genes may fail to detect some *Cmm* strains, or alternatively, react with nonpathogenic *Cmm*-like strains and other genera of bacteria associated with tomato seeds and plant materials. This study examined whether *clvA* and two additional genes, *clvF* and *clvG,* are sufficiently conserved in *Cmm* for global diagnostic application.

3. MATERIALS AND METHODS

3.1 Bacterial strains and culture conditions

Forty-eight *Cmm* strains from the Pacific Bacterial Collection at the University of Hawai‘i at Mānoa were used in this study (Table 3.1). Strains were selected from a larger collection (Kaneshiro, et al., 2006) that represent a diverse geographical distribution, and are a subset of strains examined previously with LAMP (Yasuhara-Bell, et al., 2013). Also included in this study were *Cm.* subsp. *insidiosus* (*Cmi* - K0091, aka A1149 and ATCC10253), *Cm.* subsp. *sepedonicus* (*Cms* - A2041, aka R8) and the seed-associated *Cmm*-like strains C55, C59-A, C63, C6-A, C74A and C91-A from the Pacific Bacterial Collection at the University of Hawai ‘i at Mānoa. *Cm.* subsp. *nebraskensis* (*Cmn*) strains NCPPB 2579 (aka LMG 3698), 20037 and 200800460 were from A.K. Vidaver at the University of Nebraska, Lincoln. A strain of *Cm.* subsp. *tessellarius* (*Cmt* - LMG 7294, aka ATCC 33566) and the seed-associated *Cmm*-like strains ZUM3064, ZUM3065, ZUM3936, ZUM4206, ZUM4207, ZUM4209, ZUM4210 and ZUM4211 were provided by B. Woudt at Syngenta Seeds B.V. (Enkhuizen, NL). Ten different genera of plant-associated bacteria, including *Sphingomonas* sp., *Cupriavidus* sp., *Acinetobacter* sp., *Agrobacterium* sp., *Leifsonia* sp., *Curtobacterium* sp., *Pantoea* sp., *Herbaspirillum* sp., *Enterobacter* sp. and *Pseudomonas* sp., as previously determined by 16S PCR, were isolated from the environment and tested. Six tomato-pathogenic bacteria, including *Agrobacterium*

tumefaciens (C58), *Xanthomonas campestris* pv. *vesicatoria* (A3788, aka Xv138), *Pseudomonas syringae* pv. *tomato* (CC36), *Pseudomonas syringae* pv. *syringae* (A3830, aka 164), *Ralstonia solanacearum* (A3450, aka 30), and *Pectobacterium carotovorum* (A5371, aka CC26), were also tested. *Clavibacter* strains were removed from -80°C, plated onto TZC-S medium [17 g/L agar, 10 g/L peptone, 5 g/L sucrose and 0.001% 2,3,5-triphenyl-tetrazolium chloride (TZC)] and then incubated at 26°C (± 2°C). Isolated colonies were streaked onto YSC medium (17 g/L agar, 10 g/L yeast extract, 20 g/L sucrose and 20 g/L $CaCO_3$) and incubated at 26°C (± 2°C). Bacterial cells were harvested from YSC plates and put into 1.5 ml microfuge tubes for DNA extraction.

3.2 DNA extraction

A Chelex® DNA extraction was performed on samples contained in 1.5 ml microfuge tubes. Briefly, 0.75-1.0 ml of 40% Chelex® 100 resin (Bio-Rad, Hercules, CA) in 1X TE buffer (10 mM Tris HCl and 1 mM EDTA at pH 8) with 10% Triton™ X-100 (Sigma-Aldrich, St. Louis, MO) was added to each tube. Samples were mixed by pipeting vigorously and vortexing and then heated to 95°C for 10 min on a digital heat block. Samples were stored at 4°C for at least 24 h to allow for separation of the DNA.

3.3 PCR and LAMP

PCR amplifications were carried out using 3 primer pairs designed to amplify 3 different regions of the *Cmm* genome: *clvA* – clavicidin, a putative type II lantibiotic; *clvF* – putative ABC-type multidrug transport system ATPase component; *clvG* – putative ABC-type multidrug transport system permease component. The primer sequences are listed in Table 3.2. PCR reactions for all primers were performed in a 10 µl reaction volume containing 1 µl bacterial DNA and 9 µl PCR reaction master mix [0.5 µl of each primer (10 µM), 5.0 µl JumpStart™ REDTaq® ReadyMix™ (Sigma-Aldrich, St. Louis, MO), and 3 µl DNase/RNase free water]. PCR reaction conditions for all primers were as follows: an initial denaturing at 95°C for 7 min, followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 58°C for 30 s, and elongation at 72°C for 1 min, with a final elongation at 72°C for 5 min. PCR products were resolved using 1.5% agarose gel electrophoresis. The expected product sizes for clvA-F/R, clvF-F/R and clvG-F/R were 338 bp, 1166 bp, and 1178 bp, respectively. Gels were analyzed using the Foto/Analyst® Express System (Fotodyne Inc., Hartland, WI). LAMP reactions for *clvA* were performed according to methods established previously (Yasuhara-Bell, et al., 2013).

Table 3.1. *Clavibacter michiganensis* subsp. *michiganensis* strains tested in this study.

Strain[a]	Accession ID[b]	Original ID	Location	Source
K073	A2058	H-160	Idaho, USA	Azad, H.
K074	A4758	N 212	China	Hoyos, G.
K075	A4763	N 7388A	Morocco	Hoyos, G.
K077	A4691	cmm462	Portugal	Santos, M.S.
K078	A1749	A 518-5	Hawaii, USA	Chun W.
K080	A4918	E3	Ohio, USA	Ivey, M.
K081	C222	C222	Oregon, USA	di Nitto, L.
K082	A4791	71421	China	Coutu, D.
K083	A1753	A 438-1	Hawaii, USA	Chun W.
K085	A2645	S47	California, USA	Bolkan, H.
K086	A4588	cmm016	Washington, USA	Derie, M.
K088	A5131	ZUM 3036	Netherlands	Woudt, B.
K089	C217	C217	Oregon, USA	di Nitto, L.
K093	A4750	IPO 545	South Africa	de Vries, I.
K094	A4780	71169	China	Coutu, D.
K385	A1746	A 518-1	Hawaii, USA	Chun W.
K386	A1949	B-125	California, USA	Watterson, J.
K387	A2069	CM95	Ohio, USA	Nameth, S.
K388	A2071	CM97	Ohio, USA	Nameth, S.
K389	A2072	CM98	Ohio, USA	Nameth, S.
K390	A2073	CM99	Ohio, USA	Nameth, S.
K392	A2297	CM5	Ohio, USA	Coplin, D.
K393	A2626	C12	California, USA	Bolkan, H.
K394	A2627	C19	California, USA	Bolkan, H.
K395	A2644	S44	California, USA	Bolkan, H.
K399	A2696	CM36	North Carolina, USA	Beagle-Ristiano, J.
K400	A2697	CM33	North Carolina, USA	Beagle-Ristiano, J.
K402	A2700	CM Kyukendall	North Carolina, USA	Beagle-Ristiano, J.
K404	A3994	DR73	Iowa, USA	Braun, E.
K406	A3997	1(A)	Ohio, USA	Clevenstine, R.
K407	A3998	8(A-B)	Ohio, USA	Clevenstine, R.
K410	A4001	72	Ohio, USA	Clevenstine, R.
K439	A4042	DR59	Iowa, USA	Braun, E.
K440	A4043	BR4	Iowa, USA	Braun, E.
K460	A4589	cmm018	Washington, USA	Derie, M.
K461	A4591	cmm024	Washington, USA	Derie, M.
K462	A4596	cmm035	Washington, USA	Derie, M.
K463	A4597	cmm037	Washington, USA	Derie, M.
K465	A4690	cmm461	Portugal	Santos, M.S.
K467	A4744	IPO 500	United Kingdom	de Vries, I.
K470	A4747	IPO 542	Italy	de Vries, I.
K471	A4749	IPO 544	Hungary	de Vries, I.
K473	A4752	IPO 1799	Chile	Hoyos, G.
K476	A4755	N 202A	Chile	Hoyos, G.
K477	A4756	N 202B	Chile	Hoyos, G.
K478	A4757	N 211	China	Hoyos, G.
K479	A4759	N 213	Chile	Hoyos, G.
K480	A4769	N 713P	Chile	Hoyos, G.

[a] The K numbers are associated with the *dnaA* phylogenies by Schneider, et al. (2011).
[b] Accession numbers in the Pacific Bacterial Collection at the University of Hawai'i at Mānoa.

Table 3.2. PCR primers used in this study.

Primer	Sequence (5'-3')	Source/Reference
clvA-F	GCGACAACAGGAACACAGGT	This Study
clvA-R	CGGTGATCGTCACGTCGGAT	This Study
clvF-F	TGCATCTTGCGGAACTCGAT	This Study
clvF-R	GGCACATCCTCACCAAGCTC	This Study
clvG-F	AGAAGAGCAGGCCGTAGAAC	This Study
clvG-R	GCTGTTCGAGCACTCCATCC	This Study

3.4 DNA sequencing

PCR products were cleaned for sequencing using ExoSAP-IT® (Affymetrix®, Santa Clara, CA) according to the manufacturer's instructions. Cleaned PCR products were sequenced at the University of Hawai'i sequencing facilities, using each forward and reverse primer, according to specifications.

3.5 Phylogenetic analyses

Sequence alignment and phylogenetic analyses were performed using MEGA5 version 5.05 (Tamura, et al., 2011). Sequence data for *Cmm* strain NCPPB 382 (Accession: AM711867.1) was obtained from NCBI GenBank. Sequences for *clvA*, *clvF* and *clvG* were trimmed to 207 nt, 883 nt, and 753 nt (100%, 98.22% and 100% coverage), respectively. Multiple alignments were made using ClustalW, taking into account the corresponding amino acid alignments for protein-coding genes. Maximum-parsimony (MP) analysis was used to construct trees with the closest neighbor interchange at search level 3 (Nei & Kumar, 2000). Neighbor-joining (NJ) trees (Saitou & Nei, 1987) were constructed using the Jukes-Cantor method (Jukes & Cantor, 1969) to compute evolutionary distances. The model of evolution for maximum likelihood (ML) analysis was determined using Modeltest 3.7 in PAUP* (Swofford, 2002). Both the hierarchical likelihood ratio test (hLRT) and the Akaike Information Criterion (AIC) were used to evaluate model scores. Phylogenetic trees and bootstrap values for the nucleotide sequences of each gene fragment and of concatenated sequences were obtained with PhyML (Guindon et al., 2010) using an online bioinformatics platform, available at http://www.atgc-montpellier.fr/phyml/. ML trees were edited using TreeGraph 2 graphical editor for phylogenetic trees (Stöver & Müller, 2010) and final ML trees were drawn using MEGA5. Confidence intervals were assessed using the bootstrap method with 1,000 replications (Felsenstein, 1985). The mid-point rooting method was used to root resulting phylogenies (A.-M. Vandamme, 2003).

The ILD (incongruence length difference) test was used to assess the congruence of gene partitions for concatenation (Farris, Källersjö, Kluge, & Bult, 1994; Mickevich & Farris, 1981) using the ASAP program/Perl script (Sarkar, Egan, Coruzzi, Lee, & DeSalle, 2008). Multi-locus sequence analysis was performed by first concatenating 2 or more gene sequences and then performing alignment and phylogenetic analyses.

3.6 Nucleotide accession numbers

The *Clavibacter michiganensis* subsp. *michiganensis* sequences have been deposited into the GenBank Database with accession numbers KC262879-KC262926, KC262927-KC262974, and KC262975-KC263022 for the *clvA*, *clvF*, and *clvG* gene sequences, respectively.

4. RESULTS

4.1 Analysis of *clvA*, *clvF* and *clvG* gene sequences

PCR reactions for all 48 *Cmm* strains tested produced the expected products of approximately 338 bp, 1166 bp and 1178 bp, for the *clvA*, *clvF*, and *clvG* genes, respectively. No PCR products were observed for any of the non-*Cmm* strains tested, including the *Cmm*-like strains. The *clvA* LAMP produced the same results. DNA sequences of these three genes from all *Cmm* tested were input into NCBI BLAST and found to have 99% maximum identity to *Cmm* strain NCPPB 382 (Accession: AM711867.1). The *clvA* gene sequences only matched with one other sequence, *Cmm* michiganin A precursor gene (Accession: DQ458780.1), while the *clvF* gene only matched with the aforementioned sequence (Accession: AM711867.1). The *clvG* gene sequence matched with seven other unrelated bacterial species; however these matches were based on 18% or less query coverage. The *clv* genes tested were unique to *Cmm* and found in no other bacteria in GenBank, therefore no outgroup sequence was available for use in phylogenetic analyses.

Analysis of aligned sequence data (Table 3.3, Appendix D) revealed that the gene sequences obtained from the strains tested in this study had 5/207 (2.42%) (Appendix D, Figure D.1), 8/883 (0.906%) (Appendix D, Figure D.3) and 15/753 (1.99%) (Appendix D, Figure D.5) nucleotide variability for *clvA*, *clvF*, and *clvG*, respectively, when compared to the published sequence from strain NCPPB 382 (Accession: AM711867.1). These nucleotide changes corresponded to 3/69 (4.35%) (Appendix D, Figure D.2), 2/294 (0.68%) (Appendix D, Figure D.4) and 6/251 (2.93%) (Appendix D, Figure D.6) amino acid variability for *clvA*, *clvF*, and *clvG*, respectively (Table 3.3). Phylogenetic analyses were performed for each gene. MP, NJ and ML all showed varying separations of strains; however no outgroup was available to polarize

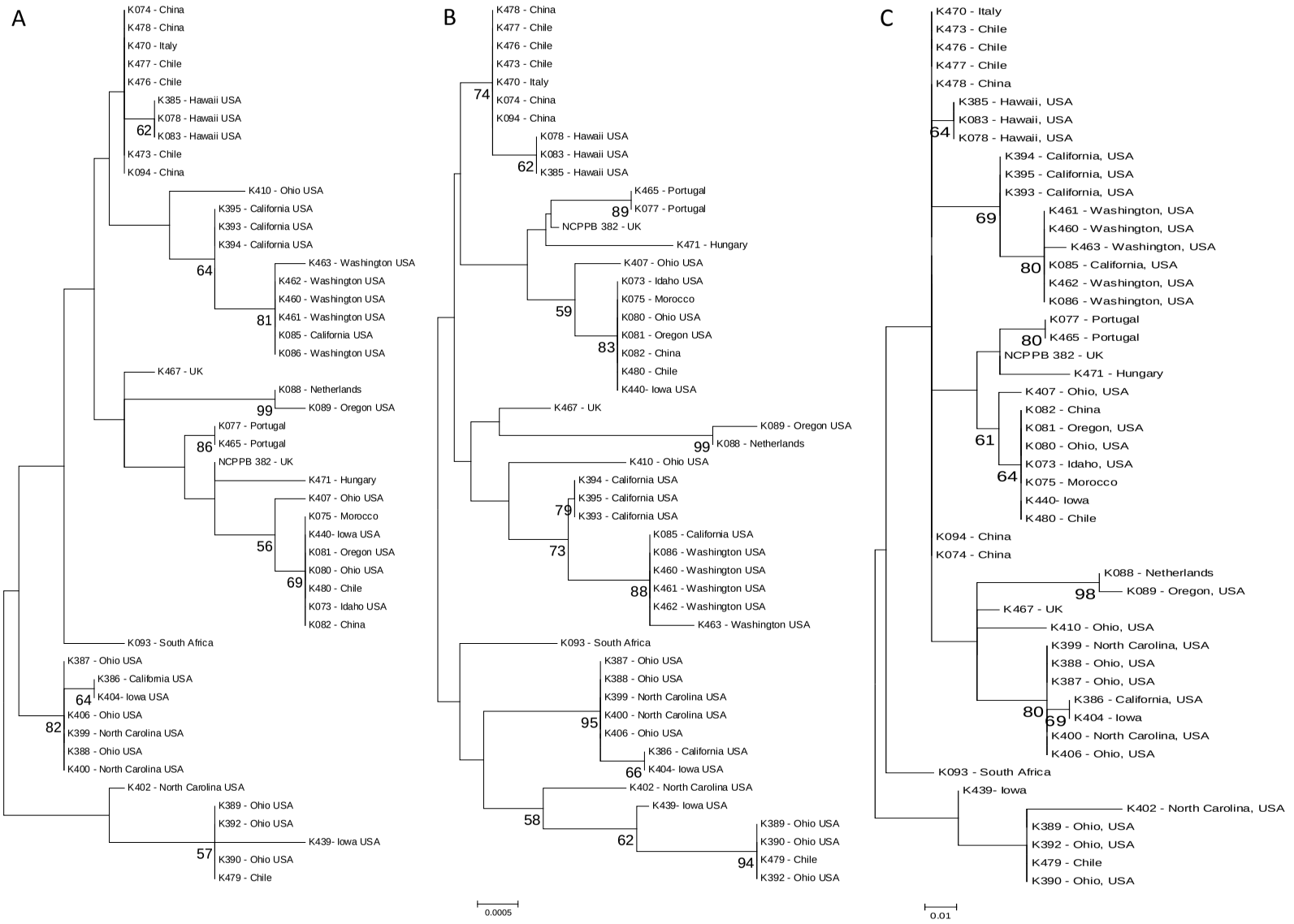
the data and phylogenetic distances were very small, suggesting individual gene sequences were highly conserved among the methods of analysis used (Appendix B, Figures B.2-B.4). MLSA was performed using different combinations of concatenated sequences. The ILD test revealed that the only compatible partitions for concatenation were *clvA* and *clvG* (p-value of 0.3800) and *clvF* and *clvG* (p-value of 0.5200). There was no significant congruence between *clvA* and *clvF* ($P < 0.05$). We opted to concatenate these genes despite the possible presence of incongruent phylogenetic signals in a modified total evidence analysis (de Queiroz, Donoghue, & Kim, 1995). Four different MLSA analyses were performed using the concatenated sequences of *clvA-clvF*, *clvA-clvG*, *clvF-clvG*, and *clvA-clvF-clvG* (data only shown for *clvA-clvF-clvG*). Once again, MP,

Table 3.3. *clvA*, *clvF* and *clvG* gene sequence alignment data[*].

Gene	Position	Transition	Transversion	Amino Acid Change	Position	# Strains[$]
clvA	11	T→C	-	I→T	4	10
	60	-	G→C	-	-	9
	61	-	C→G	L→V	21	9
	100	-	C→A	Q→K	34	2
	177	A→G	-	-	-	3
clvF	63	C→T	-	-	-	33
	90	-	G→C	-	-	13
	228	-	C→G	-	-	2
	346	G→A	-	A→T	116	1
	354	-	G→C	-	-	10
	405	-	C→A	-	-	10
	450	C→T	-	-	-	9
	698	-	C→G	A→G	233	1
clvG	58	-	C→A	L→M	20	1
	96	-	C→G	S→R	32	1
	126	T→C	-	-	-	2
	152	A→G	-	H→R	51	7
	277	G→A	-	A→T	93	2
	376	A→G	-	I→V	126	12
	399	-	G→C	-	-	14
	447	-	G→C	-	-	6
	450	-	C→G	-	-	5
	531	-	G→C	-	-	12
	591	-	G→C	-	-	32
	653	G→A	-	G→D	218	6
	666	-	G→C	-	-	37
	693	C→T	-	-	-	8
	730	-	C→A	-	-	5

[*] - implies not applicable.
[$] Number of strains that contained the respective change.

Figure 3.1. MSLA of concatenated *clvA*, *clvF* and *clvG* gene sequences. Phylogenetic trees were constructed using A) MP B) NJ and C) ML analyses with mid-point rooting. Confidence intervals were assessed using the bootstrap method with 1000 replications. Bootstrap values of 50% or more are shown to the left of corresponding nodes. Bars indicate 0.05% and 1% sequence divergence for B and C, respectively. →


A
K074 - China
K478 - China
K470 - Italy
K477 - Chile
K476 - Chile
K385 - Hawaii USA
K078 - Hawaii USA
62
K083 - Hawaii USA
K473 - Chile
K094 - China
K410 - Ohio USA
K395 - California USA
K393 - California USA
K394 - California USA
64
K463 - Washington USA
K462 - Washington USA
K460 - Washington USA
81
K461 - Washington USA
K085 - California USA
K086 - Washington USA
K467 - UK
K088 - Netherlands
99
K089 - Oregon USA
K077 - Portugal
86
K465 - Portugal
NCPPB 382 - UK
K471 - Hungary
K407 - Ohio USA
K075 - Morocco
K440- Iowa USA
56
K081 - Oregon USA
K080 - Ohio USA
69
K480 - Chile
K073 - Idaho USA
K082 - China
K093 - South Africa
K387 - Ohio USA
K386 - California USA
64
K404- Iowa USA
K406 - Ohio USA
82
K399 - North Carolina USA
K388 - Ohio USA
K400 - North Carolina USA
K402 - North Carolina USA
K389 - Ohio USA
K392 - Ohio USA
K439- Iowa USA
57
K390 - Ohio USA
K479 - Chile
B
K478 - China
K477 - Chile
K476 - Chile
K473 - Chile
K470 - Italy
74
K074 - China
K094 - China
K078 - Hawaii USA
K083 - Hawaii USA
62
K385 - Hawaii USA
K465 - Portugal
89
K077 - Portugal
NCPPB 382 - UK
K471 - Hungary
K407 - Ohio USA
K073 - Idaho USA
K075 - Morocco
59
K080 - Ohio USA
K081 - Oregon USA
83
K082 - China
K480 - Chile
K440- Iowa USA
K467 - UK
K089 - Oregon USA
99
K088 - Netherlands
K410 - Ohio USA
K394 - California USA
79
K395 - California USA
K393 - California USA
K085 - California USA
73
K086 - Washington USA
K460 - Washington USA
88
K461 - Washington USA
K462 - Washington USA
K463 - Washington USA
K093 - South Africa
K387 - Ohio USA
K388 - Ohio USA
K399 - North Carolina USA
95
K400 - North Carolina USA
K406 - Ohio USA
K386 - California USA
66
K404- Iowa USA
K402 - North Carolina USA
K439- Iowa USA
58
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K389 - Ohio USA
K390 - Ohio USA
94
K479 - Chile
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K470 - Italy
K473 - Chile
K476 - Chile
K477 - Chile
K478 - China
K385 - Hawaii, USA
K083 - Hawaii, USA
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K078 - Hawaii, USA
K394 - California, USA
K395 - California, USA
K393 - California, USA
69
K461 - Washington, USA
K460 - Washington, USA
K463 - Washington, USA
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K085 - California, USA
K462 - Washington, USA
K086 - Washington, USA
K077 - Portugal
80
K465 - Portugal
NCPPB 382 - UK
K471 - Hungary
K407 - Ohio, USA
K082 - China
K081 - Oregon, USA
61
K080 - Ohio, USA
K073 - Idaho, USA
64
K075 - Morocco
K440- Iowa
K480 - Chile
K094 - China
K074 - China
K088 - Netherlands
98
K089 - Oregon, USA
K467 - UK
K410 - Ohio, USA
K399 - North Carolina, USA
K388 - Ohio, USA
K387 - Ohio, USA
80
K386 - California, USA
69
K404 - Iowa
K400 - North Carolina, USA
K406 - Ohio, USA
K093 - South Africa
K439- Iowa
K402 - North Carolina, USA
K389 - Ohio, USA
K392 - Ohio, USA
K479 - Chile
K390 - Ohio, USA
0.01

NJ and ML all showed varying separations of strains (Figure 3.1), but no outgroup was available in GenBank to polarize the data and phylogenetic distances were very small, suggesting no significant sequence variation. Therefore, concatenated datasets of these genes produced congruent results with individual gene analyses, further demonstrating that these sequences are conserved.

5. DISCUSSION

Clavibacter michiganensis subsp. *michiganensis* is an agroeconomic bacterium that can cause devastating losses to tomato production during disease outbreaks. Tomato seed production is a global industry, and contaminated seed is considered to be the principal inoculum source leading to new outbreaks of bacterial canker in agriculture (Gartemann, et al., 2003). The severity of the disease and the problems in controlling pathogen spread have resulted in classification of *Cmm* as a quarantine organism under the European Union Plant Health Legislation, which affects import of tomato seed from many other countries (Eichenlaub & Gartemann, 2011). EPPO standards for *Cmm* testing have been published (EPPO, 2013). Extensive control measures have been taken to limit the number of outbreaks, including constant monitoring and testing of production fields. Small, portable, molecular-based detection devices enhance the ability of the field inspectors to identify and exclude contaminated plants before seed is harvested. Isothermal amplification of target genes using LAMP is particularly useful for this purpose and hand-held field devices have already been developed (Jenkins, et al., 2011; R. Kubota, et al., 2008).

In a recent study, we showed that a LAMP reaction aimed at the *clvA* gene was a useful diagnostic tool that specifically detects *Cmm* (Yasuhara-Bell & Alvarez, 2012; Yasuhara-Bell, et al., 2013). This gene was chosen based on the observation that it resides in a stable portion of the chromosome, and therefore should be present in all *Cmm* strains. The lantibiotic acts against closely related species of *Clavibacter*, so the presence of this gene should discriminate *C. michiganensis* at the subspecies level. Numerous strains were tested and characterized using PCR of seven genes, including one marker gene (*dnaA*), two plasmid-borne pathogenicity-associated genes (*ppaJ, pat*-1) and four genes located on the putative pathogenicity island (*chpC, tomA, ppaA* and *ppaC*), to better understand the *Cmm* population types existing in nature (Yasuhara-Bell & Alvarez, 2012; Yasuhara-Bell, et al., 2013).

Many bacteria produce antibiotic compounds that are either nonribosomally synthesized antibiotics or ribosomally synthesized proteins, also termed bacteriocins. These compounds are thought to be involved in the competition among bacteria for nutrients and specific habitats

(Eijsink, et al., 2002; Riley & Gordon, 1999). Bacteria located in different geographic regions are exposed to varying environmental conditions and microorganism populations. It was thought that niche competition with different bacteria may cause geographically separated *Cmm* strains to evolve unique versions of clavicidin, which would cause the previously established *clvA* LAMP to yield false negatives. Sequences of all three (*clvA, clvF* and *clvG*) showed that sequence variation was unrelated to strain origin and that these genes were sufficiently conserved to serve as general diagnostic markers.

The possibility that *Cmm* strains of different geographical origins could have evolved independently and adapted to their unique environments has brought focus on comparative genomics in order to correlate genetic variation with geographical origin. Many genes, including 16S rRNA (Lee, et al., 1997; Yim et al., 2012), *dnaA* (Schneider, et al., 2011; Yasuhara-Bell, et al., 2013; Zaluga, Stragier, Van Vaerenbergh, Maes, & De Vos, 2013; Zaluga, Van Vaerenbergh, et al., 2013), several housekeeping genes such as *gyrB* (Jacques, et al., 2012; Milijaevšić-Marčić, et al., 2012; Richert, et al., 2005; Zaluga, et al., 2011; Zaluga, Stragier, et al., 2013; Zaluga, Van Vaerenbergh, et al., 2013), *atpD*, *dnaK*, *ppK* (Jacques, et al., 2012), *recA* (Jacques, et al., 2012; Waleron, Waleron, Kamasa, Przewodowski, & E., 2011), *rpoB* (Jacques, et al., 2012; Waleron, et al., 2011), *rpoD* (Waleron, et al., 2011), *kdpA*, *sdhA*, and *ligA* (Milijaevšić-Marčić, et al., 2012), as well as the ITS region (Yim, et al., 2012), have been used individually or in multi-locus sequence analysis (MLSA). While replication initiation factor (RIF) marker analysis of *dnaA* showed no correlation between the RIF sequence of *Cmm* strains and their geographic origins (Schneider, et al., 2011), MLSA has proven more successful in attempting to correlate genetic differences with geographical origin. However, in such studies, it is often hard to correlate sequence variations with the geographical origins of strains, as only the most recent isolation site is known and not the origin of primary inoculum. This makes any study of this nature extremely difficult, especially when disease has already spread over long distances, possibly via international seed trade.

Nevertheless, genetic variations within *Cmm* populations have been reported from various countries, and these results have been used to speculate as to the most probable origins of primary inoculum (Baysal et al., 2011; Jacques, et al., 2012; Kaneshiro, et al., 2006; Kawaguchi, et al., 2010; Kleitman, et al., 2008; Milijaevšić-Marčić, et al., 2012; Quesada-Ocampo, Landers, Lebeis, Fulbright, & Hausbeck, 2012; Waleron, et al., 2011; Yim, et al., 2012). However, the majority of these studies have been focused on identifying and tracking strains within a given production area to deduce possible contamination sources within the same region (Kawaguchi, et al., 2010; Kleitman, et al., 2008; Milijaevšić-Marčić, et al., 2012; Quesada-Ocampo, et al., 2012).

DNA-based typing procedures, pulse-field gel electrophoresis (PFGE) (Kleitman, et al., 2008), Rep-PCR using spatial pattern by Morisita-index (Kawaguchi, et al., 2010) and ISSR-PCR fingerprints (Baysal, et al., 2011) are suitable for epidemiological studies such as strain tracking, discovering sources of contamination and transmission, and monitoring distribution and spread of *Cmm* (Milijaevšić-Marčić, et al., 2012). These methods show varying degrees of resolution of the tested populations, but most have limitations such as poor inter-laboratory portability, or limited exchangeability of results generated from a specific machine or compared to an in-house database (Zaluga, Stragier, et al., 2013). Recently, multi-locus variable-number-tandem-repeat analysis (MLVA) was developed to discriminate between *Cmm* strains and proved to be a promising typing technique for local surveillance and outbreak investigations in epidemiological studies (Zaluga, Stragier, et al., 2013).

In contrast, our study focused on identifying highly specific and conserved genes for general detection and identification of *Cmm* rather than genes showing the diversity needed for epidemiological studies. Currently, A. Fessehaie of Nunhems (Bayer CropScience) reported using comparative genomics to develop a PCR-based diagnostic test under the International Seed Health Initiative (ISHI) and confirmed that the *clvA* gene is specific to *Cmm* and can serve as a useful target for molecular diagnostics (personal communication from A. Fessehaie). Studies performed at Nunhems further support the use of the *clvA* (*micA*) gene for specific detection of *Clavibacter michiganensis* subsp. *michiganensis*, via a previously established LAMP assay (Yasuhara-Bell & Alvarez, 2012, 2014a; Yasuhara-Bell, et al., 2013), or PCR of the *clvA*, as well as PCR of the *clvF* or *clvG* genes, using the primers developed in the current study.

Other members of the *clv* gene cluster (*clvE, clvK, clvM* and *clvR*) encode for a putative membrane protein involved in lantibiotic immunity, a signal transduction histidine kinase (putative two-component sensor kinase involved in the biosynthesis of lantibiotic), a lantibiotic modifying enzyme, and a response regulator containing a CheY-like receiver domain and an HTH DNA-binding domain (putative two-component response regulator involved in the biosynthesis of lantibiotic), respectively. NCBI BLAST of the nucleotide sequences for these four additional genes showed matches only to the *Cmm* NCPPB 382 genomic sequence (Accession: AM711867.1), suggesting that these genes may also be specific. Results from our previous study (Yasuhara-Bell, et al., 2013), along with the observation that additional *clv* genes are in the same gene cluster as *clvA, clvF* and *clvG*, suggest it is reasonable that PCR and/or LAMP primers could be developed for any or all of the remaining *clv* genes. The *clv*-gene cluster could become the new focus for developing *Cmm*-detection systems, having application in numerous molecular detection platforms.

CHAPTER 4

BIO-LAMP FOR DETECTION OF *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS* IN SEED

1. ABSTRACT

The goal of successful diagnostic test development is to identify specific markers that give positive reactions with all target strains and negative reactions with non-target contaminants. Therefore, a rational compromise between sensitivity and specificity, based on a better understanding of population diversity, is needed. Immunodiagnostic tests based on poly- or monoclonal antibodies are known to produce false positive reactions to two major groups of plant- and seed-borne bacteria. The first group consists of non-pathogenic *Clavibacter* spp., which have been isolated from many geographical locations and are culturally identical to *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*). Standard biochemical testing has shown that these *Cmm*-like strains are in fact *Clavibacter*, however they show unique profiles when compared at the subspecies level. The role and/or importance of these strains is unknown. The second group consists of *Ochrobactrum* and *Microbacterium* spp., which also are associated with tomato plants and seeds and react positively with a commercial Immunostrip® assay, but are culturally distinct from *Cmm*. Genetic analyses of 351 *Cmm* and 58 non-*Cmm* strains revealed varying combinations of six genes, four in the *Cmm* pathogenicity island and one on each pathogenicity plasmid, explaining the failure of current molecular diagnostic tools to correctly identify *Cmm.* A Loop-mediated Amplification (LAMP) assay was developed that can discriminate *Cmm* from these *Cmm*-like strains, as well as other Immunostrip®-positive contaminants. Additionally, three new PCR primer sets have been designed to amplify three distinct genes, each of which can specifically detect *Cmm* without producing false positives. The LAMP and new PCR assays are based upon conserved markers, distinguishing them from assays that detect the presence of mobile/transmissible genetic elements and have varying results. This emphasizes a need to shift current assay development towards detection of stable/conserved genes that will result in development of more reliable assays for seed health and environmental testing. In this study, the LAMP assay was applied to seed testing, using an enrichment step on selective media. The LAMP assay detected *Cmm* in a 13-yr old naturally infected seed lot, as well as in an artificially infested seed lot, thus proving to be a useful tool for specific detection of *Cmm* from seed samples. Its utility for other types of plant and environmental sampling will be investigated.

2. INTRODUCTION

Detection and identification methods for seed-borne bacterial plant pathogens have been improved significantly, as molecular assays have become available. Nevertheless, culturing from seed lots and plant materials continues to be the most sensitive method, which assures that the sample contains viable cells. Thus, selective media continue to be used and modified for better recovery of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), a seed-borne pathogen of tomato that causes bacterial canker disease outbreaks worldwide (Gartemann, et al., 2003). A new semiselective medium for identification of *Cmm* has recently been published (Ftayeh, von Tiedemann, & Rudolph, 2011). Nevertheless, identification of *Cmm* colonies from contaminated samples continues to be a challenge without subsequent identification assays. ELISA and Immunostrips® developed for specific identification of *Cmm* in seed and plant assays yield positive results for two types of contaminants, causing concern about the specificity of the monoclonal antibody used in the assays (Kaneshiro, et al., 2006). Recently, several Immunostrip®-positive colonies were identified as *Ochrobactrum*, and a common yellow saprophyte, *Microbacterium* sp. (Yasuhara-Bell & Alvarez, 2012; Yasuhara-Bell, et al., 2013). In addition, *Clavibacter* spp. that have all the cultural characteristics of *Cmm*, but are non-pathogenic on tomato, have been found frequently in the past (Alvarez & Kaneshiro, 1999; Alvarez, et al., 2005; Kaneshiro, 2003; Kleitman, et al., 2008). Primer sets used for specific identification of *Cmm* (Dreier, et al., 1995; Sousa Santos, et al., 1997) reacted with some non-pathogenic *Clavibacter* strains and also failed to react with some pathogenic strains (Alvarez & Kaneshiro, 1999). Clearly, pathogenicity cannot be used as a means to confirm identity of *Cmm* (Kaneshiro, et al., 2006), and the need for rapid identification assays persists. Numerous studies using rep-PCR and AFLP have demonstrated diversity among *Cmm* strains, emphasizing the difficulty of developing a single test that will react positively with all *Cmm* strains and negatively with saprophytes (Kaneshiro, et al., 2006; Louws, et al., 1998; Rijlaarsdam, et al., 2004).

In past efforts to develop a specific assay for *Cmm*, the Replication Initiation Factor (RIF) on *dnaA* was examined to identify and characterize a large collection of *Cmm* strains (Schneider, et al., 2011). As a single-step PCR diagnostic test, it was specific for *Clavibacter*, however it did not distinguish *Cmm* from other *Cm* subspecies, such as *sepedonicus*, *tesselarius*, *insidiosus*, or *nebraskensis* (Schneider, et al., 2011). To provide resolution at the subspecies level, RIF DNA sequence analysis was required. The genome of *Cmm* was then examined to locate potential conserved genes on the chromosome that were not associated with pathogenicity. The *clvA* gene was selected because it encodes clavicidin, a type II lantibiotic, reasoning that such a

bacteriocin-like compound should distinguish *Cmm* from closely-related subspecies (Yasuhara-Bell & Alvarez, 2012). An isothermal Loop-mediated Amplification (LAMP) method was developed and tested for specificity against 351 *Cmm* strains from a world-wide collection (Yasuhara-Bell, et al., 2013). We also tested 58 non-*Cmm* strains, including five *Ochrobactrum* and 13 and *Microbacterium* strains previously found associated with tomato seed (kindly provided by C. Kurowski). Additionally, PCR was used to amplify six pathogenicity genes, two of which were plasmid borne (*ppaJ* and *pat-1*), and four of which were chromosomally encoded (*chpC*, *tomA*, *ppaA* and *ppaC*). The 351 *Cmm* strains were tested by PCR to determine the presence or absence of these genes and compared to the reactions obtained by LAMP (Yasuhara-Bell, et al., 2013).

The purpose of the current work was to use this rapid isothermal DNA-based assay, which can specifically identify *Cmm* strains irrespective of virulence, to test its effectiveness on tomato seed that had been previously found positive for *Cmm* and subsequently stored for 13 additional years. This naturally infected seed, alongside artificially infested seed, was tested using both a direct LAMP and a bio-LAMP assay on seed extracts to determine their ability to detect and identify *Cmm* colonies.

3. MATERIALS AND METHODS

3.1 Preparation and assay of seed samples

Artificially infested seed samples, to be used as controls, were produced in the lab and compared with a naturally infected seed lot. A pathogenic *Cmm* strain A2058 (H-160, Idaho, Azad) was removed from -80°C, plated onto TZC-S medium (17 g/L agar, 10 g/L peptone, 5 g/L sucrose and 0.001% 2,3,5-triphenyl-tetrazolium chloride (TZC)) and incubated at 26°C (± 2°C). Isolated colonies were streaked onto YSC medium (17 g/L agar, 10 g/L yeast extract, 20 g/L sucrose and 20 g/L $CaCO_3$) and incubated at 26°C (± 2°C) for inoculum production. Ten grams of pathogen-free tomato seed were soaked in 30 ml of a bacterial suspension (10^9 CFU/ml), placed in a desiccator, infiltrated under vacuum for 15 min, and then dried between layers of sterile filter paper. Individual tomato seeds were crushed in extraction buffer (0.01 M PBS, pH 7.4, containing 100 µg cyclohexamide and 0.02% Tween-20) using a sterile mortar and pestle. The crushed seed was incubated in the extraction buffer for 10 min to allow release of bacteria into solution. Bacterial suspensions were subjected to 10-fold serial dilutions. For each test, two culture plates were divided into four quadrants, each receiving three well-spaced 33.3 µl aliquots of the bacterial dilution. Cultures were incubated and colony-forming units were enumerated. A naturally infected seed lot from tomato harvests in Oregon in 2000 was used for comparison.

Assays of the naturally infected and lab-infested seed (1 infested seed per 10 g seed [~3,000 seeds]) were performed according to a previously described protocol (Alvarez & Kaneshiro, 1999; Kaneshiro, 2003). Briefly, 30 g of seed was mixed with 90 ml extraction buffer (1:3 g seed to ml buffer ratio) and placed into a strainer bag (Seward Limited, London, UK, Cat. No. BA6041/STR). Samples were incubated for 4 h then gently crushed for 15 min in a Stomacher® 400 laboratory blender (Seward Limited, London, UK, Cat. No. BA7021). Three 0.5 ml samples of extract for infected and infested seed were collected in 1.5 ml microfuge tubes for loop-mediated amplification, following Chelex® DNA extraction.

3.2 Enrichment

Extracts of naturally and artificially infested seed were spread-plated in 100 µl aliquots onto six plates each of BCT medium (Ftayeh, et al., 2011) (15 g/L agar, 2.5 g/L mannitol, 2 g/L yeast extract, 2 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 0.5 g/L NaCl, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.015 g/L $MnSO_4 \cdot H_2O$, 0.015 g/L $FeSO_4 \cdot 7H_2O$, 0.6 g/L H_3BO_3, 20 mg/L nalidixic acid, 100 mg/L trimethoprim, 20 mg/L polymyxin B sulfate and 1 ml/L 5% Opus Top® [BASF, Research Triangle Park, NC]), CNS medium (Gross & Vidaver, 1979) (15 g/L agar, 8 g/L dehydrated nutrient broth [DIFCO, Lawrence, KS, Cat. No. 231000], 2 g/L yeast extract, 5 g/L glucose, 2 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 247 mg/L $MgSO_4 \cdot 7H_2O$, 25 mg/L nalidixic acid, 32 mg/L polymyxin B sulfate and 200 mg/L cycloheximide), and CMM1 medium (Alvarez & Kaneshiro, 1999; Alvarez, et al., 2005; Kaneshiro, et al., 2006; Koenraadt, van Vliet, Neijndorff, & Woudt, 2009) (15 g/L agar, 2 g/L yeast extract, 10 g/L sucrose, 1.2 g/L Tris Base [Tris (hydroxymethyl) amino methane], 250 mg/L $MgSO_4 \cdot 7H_2O$, 5 g/L lithium chloride, 1 g/L NH_4Cl, 4 g/L casein acid hydrolysate, 28 mg/L nalidixic acid, 10 mg/L polymyxin B sulfate and 200 mg/L cycloheximide), and incubated at 26°C (± 2°C). After a 3- to 5-day incubation, when non-differentiable growth was observed, 1 ml of TE buffer was used to wash half of the plates and each wash was collected in 1.5 ml microfuge tubes for LAMP. The remaining plates were allowed to grow for an additional 2-4 d to allow distinct *Cmm* colony formation. Suspected *Cmm* colonies were picked from each plate and added to microfuge tubes containing TE buffer for LAMP.

3.3 DNA extraction

A Chelex® DNA extraction was performed on samples contained in the 1.5 ml microfuge tubes. Briefly, 40% Chelex® 100 resin (Bio-Rad, Hercules, CA) in 1X TE buffer (10 mM Tris HCl and 1 mM EDTA at pH 8) with 10% Triton™ X-100 (Sigma-Aldrich, St. Louis, MO) was added to each tube at 1:1 v/v. Samples were mixed with vigorous pipeting and vortexing and then

heated to 95°C for 10 min on a digital heat block. Samples were stored at 4°C for at least 24 h to allow for separation of the DNA.

3.4 Loop-mediated amplification

Loop-mediated amplification **(**LAMP) reactions were performed in triplicate, along with both positive and negative controls, according to a previously established protocol (Yasuhara-Bell, et al., 2013). Distilled water was used for the negative control while pathogenic *Cmm* strain A2058 DNA was used as the positive control.

4. RESULTS

4.1 Recovery of *Cmm* by different selective media

In an attempt to determine whether the *clvA*-LAMP is suitable for seed testing, both a known naturally infested seed lot and an artificially infested seed lot were assayed. Samples were taken directly from extracts of both the naturally infected and lab-infested seed lots and tested with LAMP. Seed extract was also spread-plated onto three selective media as an enrichment phase to allow *Cmm* to propagate before performing LAMP, thus the term bio-LAMP. Growth of numerous seed saprophytes occurred 2 d.p.i. and a few *Cmm*-like colonies appeared at 3 d.p.i. By 5 d.p.i. the CMM1 plates were overgrown with saprophytes and it was not feasible to run assays on the entire plate wash (Figure 4.1). Therefore, any suspected colonies (only 1-2 *Cmm*-like colonies per plate) were picked from CMM1 plates at 5 d.p.i and tested with LAMP. The BCT and CNS plates for both the infected and infested seed showed no apparent growth at 5 d.p.i. (Figure 4.1). However, these plates were nevertheless washed according to the prescribed protocol and washes were assayed with LAMP. In another set of tests, plates containing the three different selective media were spread-plated with seed extract and incubated for ~8 d to allow distinct colony formation. Again, the CMM1 plates inoculated with naturally infected seed extract were overrun with saprophytes so any observed yellow *Cmm*-like colonies (approximately 1-2 per plate) were picked from the plates at 7 d.p.i. (Figure 4.2) and tested with LAMP. The BCT and CNS plates inoculated with infected seed extract showed no colony formation at 8 d.p.i. (Figure 4.2). Nevertheless, these plates were washed according to the protocol and washes were assayed with LAMP. Samples from the lab-infested seed extract developed *Cmm*-like colonies on all plates (Figure 4.2), but the CMM1 plates had approximately 5-fold more colonies than observed on BCT and CNS, most likely due to the fact that CMM1 contains fewer antibiotics and is less inhibitory to bacterial growth.

4.2 Detection of *Cmm* in seed extracts using LAMP

LAMP was performed on Chelex®-extracted DNA from all of the samples taken at various stages and time-points (Table 4.1). Three replicates of each sample were tested with LAMP, using water as the negative control and Chelex®-extracted DNA from known *Cmm* as the positive control. The positive controls for both the infected and infested seed started amplified at ~25 min. The LAMP assay detected *Cmm* in infested-seed extract prior to plate enrichment, with amplification occurring at ~40 min. Additionally, the LAMP assay of infested-seed extract detected *Cmm* in all plate-derived samples, both from plate washes and colony picks. Wash samples from all CMM1, CNS and BCT plates started amplifying at 30 min, 35-40 min and 40-45 min, respectively. The difference in amplification start times most likely parallels the amount of growth on each plate. As CMM1, CNS and BCT have increased selectivity and growth inhibition with respect to each other, it stands to reason that each plate would have a corresponding decrease in the amount of *Cmm* growth. This corresponding decrease in growth is represented by the increasing lag time before initial amplification between CMM1, CNS and BCT plate samples, respectively. Colony samples from all CMM1, CNS and BCT plates started amplifying at 20-25 min, 25 min and 25-30, respectively. Again, the same trend of increasing lag time before the initial amplification was observed. The LAMP assay also detected *Cmm* in all plate-derived samples of naturally infected seed, with amplification occurring at 45-50 min. All samples produced a positive reaction in at least two out of the three replicate wells used for the LAMP assay, with the BCT washes and colony picks from CMM1 having amplification in all three replicates. The LAMP assay did not detect *Cmm* in infected-seed extract prior to enrichment.

5. DISCUSSION

This study represents the first attempt to use the *clvA* LAMP assay for detection of *Cmm* in seed extracts. The bio-LAMP assay detected *Cmm* in seed at least 10^4 CFU/10 g of seed following primary extraction. *Cmm* was detected as early as 3-4 d following plating on CMM1, as well as 5 d following plating on CNS and BCT, before any significant growth of the target bacterium was observed. The assay also demonstrated that *Cmm* can survive on seed for at least 10 years, and to date, this is the longest known recovery of *Cmm* from seed. The failure to observe bacterial growth when the plates were washed for sample collection could lead to the assumption that the positive results were merely a reaction with dead cells/DNA still associated with seed. However, the fact that 1-2 *Cmm*-like colonies were observed on the CMM1 plates and that these colonies gave a positive reaction with the LAMP indicates that the *Cmm* cells in the

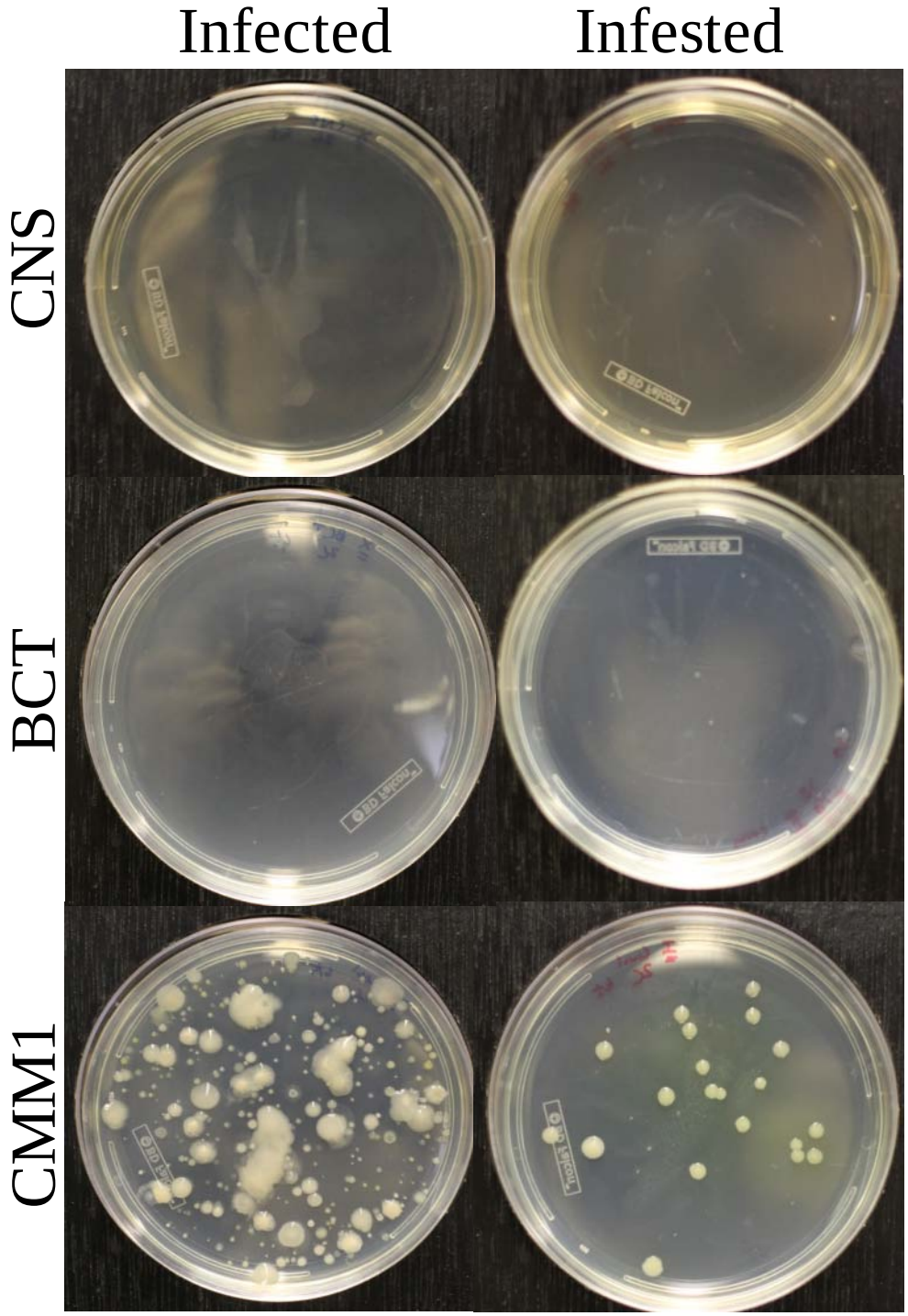
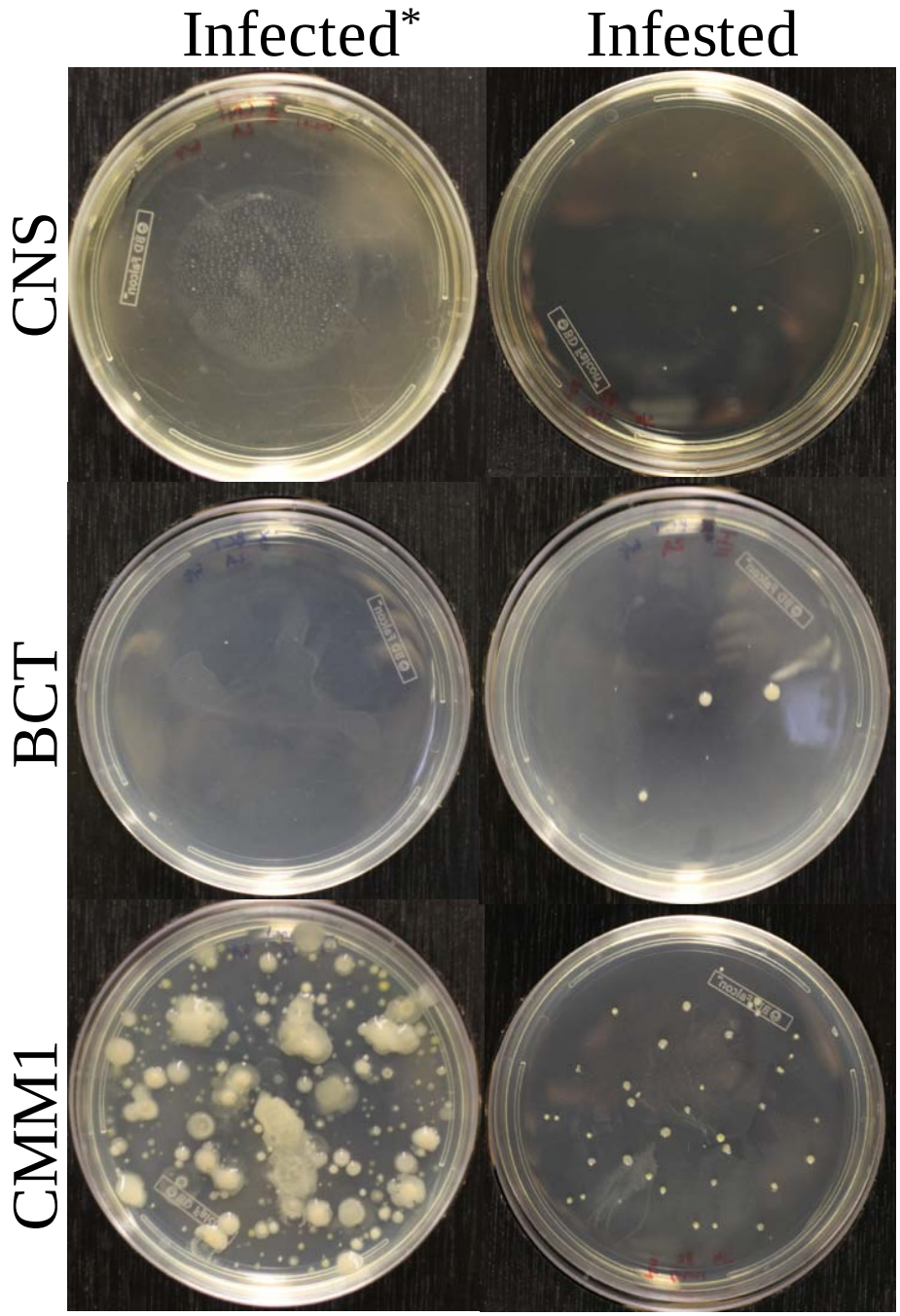


Figure 4.1. Growth on selective medium 5 days post-inoculation. Seed extract samples were taken from naturally infected and lab-infested seed lots and spread-plated onto three selective media (CNS, BCT and CMM1) and incubated for five days. CNS and BCT plates were washed before observable/differentiable growth and tested further with LAMP. *Cmm*-like colonies were picked from CMM1 plates due to overgrowth of saprophytes and/or because colonies were obvious and only needed confirmation.



*Pictures were taken and colonies of *Cmm* were picked from CMM1 plates at 7 d.p.i. due to overgrowth of saprophytes.

Figure 4.2. Growth on selective medium 7-8 days post-inoculation. Seed extract samples were taken from naturally infected and lab-infested seed lots and spread-plated onto three selective media (CNS, BCT and CMM1) and incubated for eight days. CNS and BCT plates inoculated with infected-seed extract were washed before observable/differentiable growth and tested further with LAMP. *Cmm*-like colonies were picked from all other plates due to overgrowth of saprophytes and/or because colonies were obvious and only needed confirmation.

Table 4.1. Results for LAMP assay of seed.

Sample		LAMP d.p.i.	LAMP Result	LAMP Time (min)
Infested Seed:	Extract	0	+	40
	CMM1 (colony)	5	+	30
	CNS (wash)	5	+	35-40
	BCT (wash)	5	+	40-45
	CMM1 (colony)	8	+	20-25
	CNS (colony)	8	+	25
	BCT (colony)	8	+	25-30
Infected Seed:	Extract	0	-	na
	CMM1 (colony)	5	+	45-50
	CNS (wash)	5	+	45-50
	BCT (wash)	5	+	45-50
	CMM1 (colony)	7	+	45-50
	CNS (wash)	8	+	45-50
	BCT (wash)	8	+	45-50

na, not applicable.

naturally infected seed lot were in fact alive. The small amount of growth on the CMM1 plates and the lack of any observed growth on both the CNS and BCT plates can be attributed to the age of the seed sample and reduced viability after 10 years. Moreover, the bacteria associated with the seed were most likely in a quiescent state and plating onto selective media containing inhibitory antibiotics hindered reconstitution of the population. Regardless, the LAMP was still able to detect and identify *Cmm*. Depending on the inoculum titer in an infected sample, the LAMP assay may or may not require enrichment. The time for the enrichment phase of the bio-LAMP will vary depending on the inhibitory nature of the selective medium chosen, as well as the bacterial titer and age of the seed sample. The *clvA* bio-LAMP provides a useful tool for specific detection of *Cmm* from seed samples and its utility for other types of plant and environmental sampling will be investigated.

CHAPTER 5

DETECTION OF *CLAVIBACTER MICHIGANENSIS* SUBSP. *MISHIGANENSIS* IN TOMATO SEED AND PLANT TISSUE BY LOOP-MEDIATED AMPLIFICATION

1. ABSTRACT

Loop-mediated amplification (LAMP) was used previously to specifically identify *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), which causes bacterial canker of tomato. The results of this study showed that this LAMP reaction can detect *Cmm* in plant and seed tissues. Results were compared to the ImmunoStrip® and the LAMP reaction had equal efficacy in detecting positive samples. These results, combined with the specificity established previously, suggest that this LAMP reaction can be a successful alternative to current diagnostic tests designed to detect *Cmm*. This study also included *Clavibacter michiganensis* subsp. *phaseoli* subsp. nov., a newly describe subspecies of *Clavibacter*, and the specificity of the LAMP was uncompromised. A handheld real-time fluorescence monitoring device was tested with LAMP and compared to in-lab equipment. This device performed equally to the in-lab real-time machine, thus demonstrating the potential applicability of this LAMP detection system for field detection. This LAMP reaction can be seamlessly integrated into the European Plant Protection Organization's standardized testing regimen as the definitive *Cmm* confirmatory test, which would follow isolation of colonies by plating. Perhaps one day this LAMP reaction will be the gold standard for *Cmm* diagnosis.

2. INTRODUCTION

Bacterial canker of tomato (*Solanum lycopersicum*) (Davis, et al., 1984; Strider, 1969) and pepper (*Capsicum annuum* and *Capsicum frutescens*) (Lai, 1976; Zutra & Cohn, 1970) is caused by *Clavibacter michiganensis* subspecies *michiganensis* (*Cmm*). Natural infections occur through wounds of the root or stem, hydathodes, or via contaminated seed (Gartemann, et al., 2003). During infections, *Cmm* colonizes the xylem vessels of the host plants, where it then spreads systemically through the whole plant. Systemic infection with high populations of $>10^8$ CFU/g lead to the characteristic wilting, stem canker, and vascular discoloration (Bryan, 1930; Gartemann, et al., 2003). Additionally, the pathogen can ooze from cankers and hydathodes, and in combination with rain and wind, spread to distal leaves, fruit, and surrounding plants (Bryan, 1930; Sharabani, Manulis-Sasson, et al., 2013).

What makes *Cmm* an especially dangerous pathogen is the frequent occurrence of latent infections (Franc, 1999; Gitaitis, et al., 1991) and its ability to invade seeds (Biddle, et al., 1990; Bugbee & Gudmestad, 1988; Franken, et al., 1993; McBeath & Adelman, 1986; Nemeth, et al., 1991; Samac, et al., 1998; Tsiantos, 1987), which allows long-distance spread of the pathogen. Small bacterial populations often spread rapidly during commercial transplant production by water splash and/or equipment (Carlton, Braun, & Gleason, 1998). One infected seed in 10,000 can cause outbreaks (Gitaitis, et al., 1991), and no resistant plant cultivars are known. However, on non-host plants, a hypersensitive response (HR) prevents the multiplication and spread of the pathogen (Gitaitis, 1990). The severity of the diseases and the problems in controlling the spread of these pathogens have resulted in their classification as quarantine organisms under the European Union Plant Health Legislation, as well as in many other countries (Eichenlaub & Gartemann, 2011; Jahr, et al., 1999), with cuttings and seeds of tomato requiring certification as *Cmm*-free. The European Plant Protection Organization (EPPO) has released minimal standards for the identification of *Cmm* in plants and seeds (EPPO, 2013).

Immunodiagnostic and molecular methods, based on primers developed from ribosomal RNA genes, repetitive sequences or known virulence genes, are available to confirm identity of *Cmm*, following isolation of suspect bacteria by growth on semiselective media (Dreier, et al., 1995; Lee, et al., 1997; Louws, et al., 1998; Pastrik & Rainey, 1999; Sousa Santos, et al., 1997). Pathogenicity tests are time-consuming and unreliable because avirulent populations occur frequently in natural *Cmm* populations. Additionally, PCR assays based solely on virulence genes may produce false results (Alvarez & Kaneshiro, 1999; Alvarez, et al., 2005; Kaneshiro, 2003; Kleitman, et al., 2008). Recently, a loop-mediated amplification (LAMP) assay was developed that can specifically detect all *Cmm* strains, both virulent and avirulent (Yasuhara-Bell, et al., 2013). This assay was also shown to detect *Cmm* from tomato seed (Yasuhara-Bell & Alvarez, 2014a). In order to further establish the utility the LAMP protocol for detecting *Cmm* in the field, the assay's ability to detect *Cmm* in a variety of samples, simulating potential test samples during routine practices, were tested in this study. Additionally, tests were compared to a commercially available field test, ImmunoStrip® (Agdia® Inc.). Immunodiagnostics assays have known cross reactions with non-*Cmm* bacteria, including *Microbacterium* spp., *Ochrobactrum* spp., and other *Clavibacter* subsp. However, because the ImmunoStrip® is a rapid and efficient test designed for field application, it was the primary focus for comparison with the LAMP assay.

3. MATERIALS AND METHODS

3.1 Bacterial strains and culture conditions

The bacterial strain used in this study (K78; also called A1749 and A 518-5) was from the Pacific Bacterial Collection at the University of Hawai ʻi at Mānoa. This strain represents pattern 2 from the genetic pathogenicity profiles established previously (Yasuhara-Bell, et al., 2013). Included in this study was the type strain of a newly characterized subspecies, *Clavibacter michiganensis* subsp. *phaseoli* subsp. nov. (A6135; also called LPPA 982^T, CECT 8144^T and LMG 27667^T), in order to determine any possible LAMP cross-reactions with this new strain. This strain was provided by Ana González of Servicio Regional de Investigacio´n y Desarrollo Agroalimentario (SERIDA), Spain. Bacteria were removed from -80°C, plated onto TZC-S medium (17 g/L agar, 10 g/L peptone, 5 g/L sucrose and 0.001% 2,3,5-triphenyl-tetrazolium chloride (TZC)) and then incubated at 26°C (± 2°C). Isolated colonies were streaked onto YSC medium (17 g/L agar, 10 g/L yeast extract, 20 g/L sucrose and 20 g/L $CaCO_3$) and incubated at 26°C (± 2°C) for large quantity production. Bacterial cells were harvested from YSC plates (approximately 50-250 µl worth) and put into 1.5 ml microfuge tubes for DNA extraction.

3.2 Seed inoculation

Seed inoculation was performed according to methods reported previously (Yasuhara-Bell & Alvarez, 2014a). Briefly, 10 g of tomato seed (*Lycopersicon esculentum* cv. 'Kewalo') were inoculated with 30 ml of bacterial suspension (10^9 CFU/ml) inside a desiccator, under vacuum for 15 min. Infested seed concentrations were ~10^4 CFU/seed, as determined by dilution plating.

3.3 Dilution plating

Dilution plating was performed according to methods reported previously (Yasuhara-Bell & Alvarez, 2014a). Briefly, bacterial suspensions were subjected to 10-fold serial dilutions. Stock solutions were diluted to 10^{-7}. Two BCT (Ftayeh, et al., 2011) (15 g/L agar, 2.5 g/L mannitol, 2 g/L yeast extract, 2 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 0.5 g/L NaCl, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.015 g/L $MnSO_4 \cdot H_2O$, 0.015 g/L $FeSO_4 \cdot 7H_2O$, 0.6 g/L H_3BO_3, 20 mg/L nalidixic acid, 100 mg/L trimethoprim, 20 mg/L polymyxin B sulfate and 1 ml/L 5% Opus Top® [BASF, Research Triangle Park, NC]) culture plates were divided into four quadrants, each of which was used to spot-plate a particular dilution, including the stock. Each quadrant contained three

well-spaced bacterial spots, using 33.3 µl of bacterial dilution. Cultures were incubated, colonies counted, and bacterial titer determined.

3.4 Seed assay

Laboratory-infested seeds (100 seeds) were pressed into BCT medium and observed for bacterial growth, at a density of 10-12 seeds per plate. Seeds were incubated for 4 d at 26°C (± 2°C), removed from plates and then heated to 95°C for 10 min in 100 µl 1X TE buffer (10 mM Tris HCl and 1 mM EDTA at pH 8). BCT plates were allowed to incubate for an additional 3 d, at 26°C (± 2°C), to allow further colony growth. Colonies were picked from plates and then heated to 95°C for 10 min in 100 µl 1X TE buffer. Seed and colony samples were tested with LAMP and the ImmunoStrip® (Agdia Inc., Elkhart, IN, Cat. No. ISK 44001). Additionally, LAMP and ImmunoStrip® were used to test seed directly, without plate enrichment, after heating to 95°C for 10 min in 100 µl 1X TE buffer. All tests were performed in triplicate alongside positive and negative controls, using *Cmm* strain A1749 DNA and non-infected seed and ddH_2O, respectively.

3.5 Plant assay

Juvenile tomato plants (n=5) were inoculated as described previously (Yasuhara-Bell, et al., 2013). Briefly, a sterile scalpel was used to make a vertical incision (~1 mm) on the stem, between the cotyledons, of juvenile tomato plants (*Lycopersicon esculentum* cv. 'Kewalo') that were approximately 3-4 weeks old. Bacteria (~10^9 CFU/ml) were applied to the wound, using the scalpel tip. Negative controls were made by applying 50 µl ddH_2O to the wound. Plants were allowed to grow for an additional 30 d post inoculation.

To sample plants, a 0.5 cm stem sections were removed from 5 cm above the inoculation site, using a sterile scalpel. Stem samples were pressed onto BCT medium and plates were incubated for 5-7 d at 26°C (± 2°C) to allow colony growth, and then soaked for 20 min and heated to 95°C for 10 min in 500 µl 1X TE buffer. After the plate incubation, any visible colonies were picked from plates and then heated to 95°C for 10 min in 100 µl 1X TE buffer. Plant and colony samples were tested with LAMP and the ImmunoStrip® (Agdia Inc., Elkhart, IN). All tests were performed in triplicate alongside positive and negative controls, using *Cmm* strain A1749 DNA and ddH_2O, respectively.

3.6 DNA extraction

Briefly, bacteria were suspended in 0.75-1.0 ml of 20% Chelex® 100 resin (Bio-Rad, Hercules, CA) in 1X TE buffer (10 mM Tris HCl and 1 mM EDTA at pH 8) with 10% Triton™ X-100 (Sigma-Aldrich, St. Louis, MO). Samples were mixed with vigorous pipeting and vortexing and then heated to 95°C for 10 min on a digital heat block. Samples were stored at 4°C for at least 24 h to allow for separation of the DNA.

3.7 Loop-mediated amplification

LAMP primers used in this study were reported previously (Yasuhara-Bell, et al., 2013). LAMP reactions were performed in triplicate and contained 5 µl sample, 5 µl primer master mix [micALAMP2-F3 (0.2 µM), micALAMP2-B3 (0.2 µM), micALAMP2-FIP (1.6 µM), micALAMP2-BIP (1.6 µM), micALAMP2-LoopF (0.8 µM), micALAMP2-LoopF Probe (0.08 µM), and Quencher probe (0.16 µM)] and 15 µl Isothermal Mastermix (OptiGene, West Sussex, UK, Cat. No. ISO-001nd). Negative controls used 5 µl ddH_2O and/or pathogen-free sample. LAMP reactions were run and analyzed using the iQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and a hand-held real-time assessment device (SMART-DART) (DiaGenetix Inc., Honolulu, HI), under the following conditions: 65°C for 30 min, with fluorescence readings being taken at 1-min or 30-s intervals, respectively, and a final 2 min at 85°C.

3.8 Sensitivity assay

Bacterial DNA was isolated using the Wizard® Genome DNA Purification Kit (Promega, Madison, WI, Cat. No. A1125). DNA was quantified using the NanoDrop™ 2000 spectrophotometer (Thermo Scientific, Waltham, MA), diluted to a starting concentration of 1 ng/µl and subsequent 10-fold serial dilutions were made to reach a final concentration of 1 fg/µl. LAMP reactions were performed in triplicate, using ddH_2O as the negative control.

4. RESULTS

4.1 Seed assay

Cmm was artificially inoculated onto seed to be used for plant studies. This lab-infested seed was tested to determine parameters that could possibly affect plant studies. Seeds were pressed into BCT medium and incubated for 4 d as an enrichment step, which would allow bacteria to propagate and produce visible colonies for comparisons between molecular (LAMP)

and immunodiagnostic (ImmunoStrip®) tests. Following the 4-d enrichment, 64±12% of seed had germinated due to the moisture in the medium and discernible colony formation was observed only from 59±19% of the tested seed (Appendix E). The inability to identify colony formation was due to the accumulated moisture on and around the seed. Seeds were removed from the plates and tested with LAMP and ImmunoStrip®, with both tests producing identical results, showing 100% positive reactions for all seed tested (Appendix E). All colonies were positive with LAMP and ImmunoStrip® (Appendix E). Interestingly, three seeds did not showed colony growth on the plate, following the additional 3-d incubation (Appendix E). The reason for a few seeds not producing colonies can be attributed to human error, due to inconsistencies in the plate thickness and varying depth of seed embedded in the medium. This resulted in there not being enough media between the seed and the petri plate, and when combined with the lack of surrounding moisture due to seed absorption, bacteria were denied enough nutrients to propagate. This human error also factors in to the varying germination rate of seed on the agar, which is in addition to variation found to occur naturally (Sueno, Ingram, & Alvarez, 2014). Additionally, artificially infected seed was tested directly, without enrichment on BCT. The LAMP and ImmunoStrip® showed positive results for all infected seed tested, while all negative controls, including clean seed, were negative with both tests. These tests, along with results published previously (Yasuhara-Bell & Alvarez, 2014a), further support the use of the *Cmm*-LAMP for use during seed testing, as well as support the use of this seed for plant studies describe here, as the infection rate is 100% and therefore was not a factor when interpreting plant study results.

4.2 Plant assay

Plant samples were taken from stem-inoculated plants, touched to BCT medium to check for colony growth, and then tested with LAMP and Immunostrips®. All samples showed colony growth on BCT plates after 5 d, with colonies yielding positive results for LAMP and Immunostrips®. The plant samples were also tested directly, and again, both test showed positive reactions.

5. DISCUSSION

The specificity of the *Cmm*-LAMP reaction has been demonstrated previously (Yasuhara-Bell & Alvarez, 2014b; Yasuhara-Bell, et al., 2013; Yasuhara-Bell, Marrero, & Alvarez, 2014), and was maintained when tested on the newly describe *Clavibacter michiganensis* subsp. *phaseoli* subsp. nov. (data not shown). In this study, the LAMP reaction was sensitive down to 500 pg of DNA. The LAMP was successful in detecting *Cmm* directly on seed, as well as on seed

and colonies following a 5 to 7-d enrichment step. The LAMP reaction was also able to detect *Cmm* in stem-inoculated plant samples, both directly and following colony enrichment on solid media. The LAMP reaction presented here performed at least as well as the Immunostrip® in terms of its ability to detect true positives and eliminate false negatives. Additionally, the SMART-DART performed equally to the in-lab real-time fluorescence monitoring device.

Once an effective assay is developed, sample processing remains the underlying factor. Various types of sample extraction were tried during these tests, including maceration in 1X TE buffer and boiling at 95°C for 10 min, diluting the maceration 2-fold and boiling at 95°C for 10 min, and soaking the stem in 1X TE buffer and then boiling at 95°C for 10 min. As with any molecular-based assay, plant inhibitors are always a concern, as taking samples directly from plant did produce some false negatives, under certain extraction protocols. Soaking samples in buffer resulted in no inhibition, and diluting macerations removed enough inhibitors to allow proper detection directly from the plant samples, as shown previously (Yasuhara-Bell, Ayin, Hatada, & Alvarez, 2014).

Ultimately, to avoid any problems with plant inhibitors, it is recommended to simply enrich samples on solid media and test recovered colonies to eliminate all false results. This would also eliminate any problems of sensitivity when testing plant samples of low bacterial titer. Since the EPPO's minimal standards for the identification of *Cmm* in plants and seeds (EPPO, 2013) involves such a step, it seems reasonable that this LAMP assay could fit seamlessly into the testing regimen, as the confirmatory test of recovered colonies for accurate *Cmm* identification. The specificity of the LAMP reaction will disregard presence and non-*Cmm* bacteria, including non-pathogenic seed-associated *Clavibacter* spp., recently named *Clavibacter michiganensis* subsp. *chilensis* subsp. nov. and *Clavibactermichiganensis* subsp. *californiensis* subsp. nov. (Yasuhara-Bell & Alvarez, 2014b). Perhaps one day this LAMP test will become the gold standard for detection and identification of *Clavibacter michiganensis* subsp. *michiganensis.*

CHAPTER 6

SEED-ASSOCIATED *CLAVIBACTER* SPP. ARE CLEARLY DISTINGUISHABLE FROM *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS*

1. ABSTRACT

The genus *Clavibacter* contains one recognized species, *Clavibacter michiganensis*. *Clavibacter michiganensis* is subdivided into subspecies based on host specificity and bacteriological characteristics, with *Clavibacter michiganensis* subspecies *michiganensis* (*Cmm*) causing bacterial canker of tomato. *Cmm* is often spread through contaminated seed leading to outbreaks of bacterial canker in tomato production areas worldwide. The frequent occurrence of non-pathogenic *Cmm*-like bacteria is a concern for seed producers because *Cmm* is a quarantine organism and detection of a non-pathogenic variant may result in destruction of an otherwise healthy seed lot. A thorough biological and genetic characterization of these seed-associated *Cmm*-like strains was performed using standard biochemical tests, cell wall analyses, metabolic profiling using BIOLOG, single-gene and multilocus sequence analyses. Combined, these tests revealed two distinct populations of seed-associated *Clavibacter* that differed from each other, as well as all other described *Clavibacter michiganensis* subspecies. DNA-DNA hybridization values are 70% or higher, justifying placement into the single recognized species, *C. michiganensis*, but other analyses justify separate subspecies designations. Additionally, *Clavibacter* strains isolated from pepper also represent a distinct population and warrant separate subspecies designation. On the basis of these data we propose subspecies designations for separate nonpathogenic subpopulations of *Clavibacter michiganensis*: *Clavibacter michiganensis* subsp. *californiensis* subsp. nov. and *Clavibacter michiganensis* subsp. *chilensis* subsp. nov. for seed-associated strains represented by C55^T (=CFBP 8216^T) and ZUM3936^T (=CFBP 8217^T), respectively. Recognition of separate subspecies is essential for improved international seed testing operations.

2. INTRODUCTION

The genus *Clavibacter*, which belongs to the plant pathogenic actinomycetes (family *Microbacteriaceae*) (Stackebrandt, et al., 1997), was first defined by Davis et al. (1984). The *Clavibacter* species are Gram-positive, aerobic, non-spore forming, coryneform bacteria that were previously grouped within the *Corynebacterium* genus (Eichenlaub, et al., 2006). The production of exopolysaccharides often causes them to display mucoid colony morphology

(Evtushenko & Takeuchi, 2006). There exists only one recognized species of *Clavibacter*, *C. michiganensis*, which comprises five subspecies that are based on host specificity and other characteristics (Burger & Eichenlaub, 2003; Davis, et al., 1984; Eichenlaub & Gartemann, 2011; Gartemann, et al., 2003). *Clavibacter* subspecies are generally pigmented yellow to orange, with the exception of *C. michiganensis* subsp. *sepedonicus*, which is non-pigmented. The known subspecies of *Clavibacter* are plant pathogens that cause disease in agriculturally important plants (Eichenlaub, et al., 2006; Evtushenko & Takeuchi, 2006; Gartemann, et al., 2003), usually infecting one primary host and perhaps some closely related species (Eichenlaub & Gartemann, 2011; Eichenlaub, et al., 2006). Non-pathogenic *Clavibacter* strains are isolated frequently isolated from the environment (Nazina, et al., 2002; Zaluga, et al., 2014; Zinniel, et al., 2002), though these strains are often referred to simply as *Clavibacter* sp.

Bacterial canker of tomato (*Solanum lycopersicum*) is caused by *Clavibacter michiganensis* subspecies *michiganensis* (*Cmm*) (Davis, et al., 1984; Strider, 1969) and is considered one of the most significant bacterial diseasess of tomato (Strider, 1969). *Cms* is the causal agent of ring rot of potato (*Solanum tuberosum*) (Manzer & Genereux, 1981). *Clavibacter michiganensis* subspecies *insidiosus* (*Cmi*) causes wilting and stunting in alfalfa (*Medicago sativa*) (McCulloch, 1925). The remaining two subspecies infect monocotyledonous plants, with *Clavibacter michiganensis* subspecies *nebraskensis* (*Cmn*) causing wilt and blight of maize (*Zea mays*) (Schuster, 1975; Vidaver & Mandel, 1974) and *Clavibacter michiganensis* subspecies *tessellarius* (*Cmt*) causing leaf freckles and leaf spots in wheat (*Triticum aestivum*) (R.R. Carlson & A.K. Vidaver, 1982; R.R. Carlson & A.K. Vidaver, 1982). Recently, a new subspecies was described (Gonáslez & Trapiello, 2012, 2014), *Clavibacter michiganensis* subsp. *phaseoli*, that infects bean (*Phaseolus vulgaris* L.) and causes bacterial bean leaf yellowing. In addition to the six described subspecies, *Clavibacter* strains were isolated as epiphytes or endophytes on several asymptomatic plant species (Alvarez & Kaneshiro, 1999; Alvarez, et al., 2005; Chun, 1982; Eichenlaub & Gartemann, 2011; Kaneshiro, 2003; Kaneshiro & Alvarez, 2001; Kaneshiro, et al., 2006), but these strains are only classified as *Clavibacter* sp.

Contaminated seed is considered the major inoculum source leading to outbreaks of bacterial canker (Gartemann, et al., 2003). As a result, *Cmm* is classified as a quarantine organism in many countries, with cuttings and seeds of tomato requiring certification as *Cmm*-free. The European Plant Protection Organization (EPPO) has released minimal standards for the identification of *Cmm* in plants and seeds (EPPO, 2013). Immunodiagnostic and molecular methods based on primers developed from ribosomal RNA genes, repetitive sequences or known virulence genes are available to confirm identity of *Cmm,* following isolation of suspect bacteria

by growth on semiselective media (Alvarez, et al., 1993; Chun, 1982; Dreier, et al., 1995; Kaneshiro & Alvarez, 2001; Lee, et al., 1997; Louws, et al., 1998; Pastrik & Rainey, 1999; Rijlaarsdam, et al., 2004; Sousa Santos, et al., 1997). However, these diagnostic tests can produce false results.

A major concern when testing tomato seed is the frequent occurrence of *Cmm*-like bacteria. These bacteria are Gram-positive, coryneform and pigmented, but nonpathogenic on tomato (Jacques, et al., 2012; Kaneshiro, et al., 2006; Nazina, et al., 2002; Yasuhara-Bell, et al., 2013; Zaluga, et al., 2011; Zaluga, Van Vaerenbergh, et al., 2013; Zinniel, et al., 2002). *Cmm*-like bacteria do not appear to colonize vascular tissues of tomato (Zaluga, Van Vaerenbergh, et al., 2013) and do not induce a hypersensitivity reaction in *Nicotiana tabacum* and *N. benthamiana* (Jacques, et al., 2012), as well as *Mirabilis jalapa* (Zaluga, et al., 2014). These bacteria react to tests designed to detect *Cmm* such as the standard field test, the Agdia Inc. ImmunoStrip, leading to false diagnosis of *Cmm* infection (Jacques, et al., 2012; Kaneshiro & Alvarez, 2001; Kaneshiro, et al., 2006; Yasuhara-Bell, et al., 2013; Zaluga, et al., 2011). Subsequently, a LAMP reaction was designed to specifically detect *Cmm*, and distinguish them from these seed-associated *Cmm*-like bacteria (Yasuhara-Bell & Alvarez, 2012, 2014a; Yasuhara-Bell, et al., 2013). Additonally, PCR primers were designed to react only with *Cmm* and not with the seed-associated *Cmm*-like bacteria (Yasuhara-Bell, Marrero, et al., 2014).

PCR primers designed for specific pathogenicity-associated genes of *Cmm* revealed that the Cmm-like bacteria lacked the *ppaJ*, *pat*-1, *chpC*, *tomA*, *ppaA* and *ppaC* genes (Jacques, et al., 2012; Yasuhara-Bell, et al., 2013). In previous studies utilizing RIF marker sequence analysis of *dnaA* (Schneider, et al., 2011), these nonpathogenic *Cmm*-like strains formed a clade separate from all other associated *Clavibacter* subspecies (Yasuhara-Bell, et al., 2013; Zaluga, Van Vaerenbergh, et al., 2013). Similar findings were reported in studies using *gyrB* analysis (Zaluga, et al., 2011; Zaluga, Van Vaerenbergh, et al., 2013), while the another used multilocus sequence analysis of six house-keeping genes (*atpD*, *dnaK*, *gyrB*, *ppK*, *recA*, and *rpoB***)** (Jacques, et al., 2012). Together, these data suggest that the seed-associated *Clavibacter* potentially warrant at least a new subspecies designation.

Reports concerning these *Cmm*-like bacteria are lacking and biological information regarding these strains is limited. A recent draft genome of *Cmm*-like bacteria strain LMG 26808 (Zaluga, et al., 2014) may provide informative insights into the differences in virulence determinants, genetic content and adaptation to a lifestyle in their natural habitat. The current study provides a thorough biological characterization of these seed-associated *Cmm*-like strains.

3. MATERIALS AND METHODS

3.1 Bacterial strains and culture conditions

A virulent strain of *Cmm* (K73, aka H-160) previously determined to contain all genes necessary for pathogenicity (Yasuhara-Bell, et al., 2013), *Cmi* (K0091, aka A1149 and ATCC10253), *Cms* (A2041, aka R8) and the seed-associated *Cmm*-like strains C55^T, C59-A, C63, C6-A, C74A and C91-A were from the Pacific Bacterial Collection at the University of Hawai‘i at Mānoa. *Cmn* strains NCPPB 2579 (aka LMG 3698), 20037 and 200800460 were from Anne Vidaver at the University of Nebraska, Lincoln. A strain of *Cmt* (LMG 7294, aka ATCC 33566) and the seed-associated *Cmm*-like strains ZUM3064, ZUM3065, ZUM3936^T, ZUM4206, ZUM4207, ZUM4209, ZUM4210 and ZUM4211 were provided by Bert Woudt at Syngenta Seeds B.V. (Enkhuizen, NL). *Clavibacter* pepper strains PS003, PS005^T, PS006, PF007 and PF008 were provided by Jae-Soon Cha at Chungbuk National University, Korea. Strains were removed from -80°C, plated onto TZC-S medium (17 g/L agar, 10 g/L peptone, 5 g/L sucrose and 0.001% 2,3,5-triphenyl-tetrazolium chloride (TZC)) and then incubated at 26°C (± 2°C). Isolated colonies were streaked onto YSC medium (17 g/L agar, 10 g/L yeast extract, 20 g/L sucrose and 20 g/L $CaCO_3$) and incubated at 26°C (± 2°C).

Medium 6 (15 g/L agar, 5 g/L peptone, 5 g/L yeast extract, 10 g/L glucose), CNS medium (Gross & Vidaver, 1979) (15 g/L agar, 8 g/L dehydrated nutrient broth [DIFCO, Lawrence, KS], 2 g/L yeast extract, 5 g/L glucose, 2 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 247 mg/L $MgSO_4 \cdot 7H_2O$, 25 mg/L nalidixic acid, 32 mg/L polymyxin B sulfate and 200 mg/L cycloheximide), CMM1 medium (Alvarez & Kaneshiro, 1999; Alvarez, et al., 2005; Kaneshiro, et al., 2006; Koenraadt, et al., 2009) (15 g/L agar, 2 g/L yeast extract, 10 g/L sucrose, 1.2 g/L Tris Base [Tris (hydroxymethyl) amino methane], 250 mg/L $MgSO_4 \cdot 7H_2O$, 5 g/L lithium chloride, 1 g/L NH_4Cl, 4 g/L casein acid hydrolysate, 28 mg/L nalidixic acid, 10 mg/L polymyxin B sulfate and 200 mg/L cycloheximide) and selective BCT medium (Ftayeh, et al., 2011) (15 g/L agar, 2.5 g/L mannitol, 2 g/L yeast extract, 2 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 0.5 g/L NaCl, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.015 g/L $MnSO_4 \cdot H_2O$, 0.015 g/L $FeSO_4 \cdot 7H_2O$, 0.6 g/L H_3BO_3, 20 mg/L nalidixic acid, 100 mg/L trimethoprim, 20 mg/L polymyxin B sulfate and 1 ml/L 5% Opus Top [BASF, Research Triangle Park, NC]) were used for strain characterization based on their ability to grow on these media. Growth on media was assessed by spot-plating ~10^4 cells, in triplicate, of each strain on each medium, with incubation at 26°C (± 2°C) for 7 d.

3.2 Colony color and morphology

Strains were streaked onto peptone-sucrose agar (17 g/L agar, 10 g/L peptone, 5 g/L sucrose) and then incubated at 26°C (± 2°C). Individual colonies were viewed under an Olympus® SZ40 Zoom Stereo Microscope with an SZ-STS arm and SZH-ILLD brightfield/darkfield transmitted light illumination base (Olympus®, Center Valley, PA) to assess colony color and morphology.

3.3 Gram stain and cell morphology

A Gram stain (Gephardt et al., 1981; Gram, 1884) was performed to determine the nature of the bacterial cell wall and to allow visualization of cells under a microscope. Briefly, cells were suspended in ddH_2O at a low density and then 100 µl of suspension was applied to a clean microscope slide. Slides were allowed to air-dry completely and then bacteria were heat-fixed to the slides by passing the slide over an open flame 3-4 times. The heat-fixed bacteria were then flooded with crystal violet staining reagent for 1 min. Slides were washed with tap water, blotted dry with absorbent paper, and then flooded for 1 min with Gram's iodine. Slides were washed again with water, blotted dry with absorbent paper, and then washed with decolorizing agent until the decolorizing agent ran clear. Slides were blotted dry and then flooded with the safranin counterstain for 1 min. Slides were blotted dry and results were observed under oil immersion using an Olympus® BX41 Laboratory Microscope (Olympus®, Center Valley, PA) at 100X objective.

3.4 Cell-wall analyses

Analyses of cellular fatty acids, respiratory quinones, polar lipids, whole-cell sugars and peptidoglycan structure were performed by the Identification Service of the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) (Braunschweig, Germany). Cellular fatty acid, respiratory quinine and polar lipid analyses were performed by Dr. Susanne Verbarg according to protocols by Miller (1982) and Kuykendall et al. (1988) with minor modifications, Tindall (1990a, 1990b), and Bligh and Dyer (1959) (modified) and Tindall et al. (2007), respectively. Whole cell sugar and peptidoglycan analyses were performed by Dr. Peter Schumann according to protocols by Staneck and Roberts (1974) and Schumann (2011), respectively.

3.5 Oxidase test

Oxidase activity was assayed using oxidase reagent droppers (DIFCO, Lawrence, KS, Cat. No. 261181), according to the manufacturer's instructions. Briefly, a few drops of oxidase reagent (N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride) was added to a strip of filter paper. Bacteria were then streaked onto the filter paper. Reaction results were read at ~10-15 s. Positive reactions result in a color change of the bacteria (violet to purple).

3.6 Starch, casein and esculin hydrolysis

Starch and casein hydrolysis were tested by growth of bacteria on respective media and observed zones of clearing. For starch hydrolysis analyses, bacteria were spotted onto FS medium without antibiotics/antifungals (15 g/L agar, 10 g/L starch [soluble potato], 0.8 g/L KH_2PO_4, 0.8 g/L K_2HPO_4, 0.1 g/L $MgSO_4$, 15 mg/L methyl green, 3 mg/L D-methionine). A strain of *Xanthomonas citri* pv. *mangiferaeindicae* isolated from mango in Honolulu, Hawai'i was used as a positive control. For casein hydrolysis analyses, bacteria were streaked onto skim milk agar (10 g/L agar, 50g/L skim milk powder). For esculin hydrolysis analyses, bacteria were spotted onto ET medium without antibiotics/antifungals (1 g/L K_2HPO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 5 g/L NaCl, 0.3 g/L $FeCl_3$, 1 g/L esculin, 0.5 g/L trehalose, 15 g/L agar and 3 mg/L D-methionine). Esculin hydrolysis was determined by appearance of dark brown pigment within the medium.

3.7 H_2S production from peptone

H_2S production from peptone was assessed by bacterial growth on PIA medium (15 g/L peptone, 5 g/L proteose peptone, 0.5 g/L ferric ammonium citrate, 1 g/L sodium glycerophosphate, 0.08 g/L sodium thiosulfate, 15 g/L agar) (DIFCO, Lawrence, KS, Cat. No.289100). Bacteria were stab-inoculated into 3 ml of media in culture tube. Positive results were evident by the presence of black precipitate in the medium.

3.8 Acid production

Acid production from various carbohydrates was assessed using the mineral medium of Ayers et al. (1919), supplemented with peptone, agar and a pH indicator. Briefly, media was made (1.0 g/L $NH_4H_2PO_4$, 0.2 g/L KCl, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 1.0 g/L peptone, 3.0 g/L agar, 80 mg/L bromomethyl blue, 1% carbohydrate) and adjusted to pH 7.0-7.1, giving the medium an olive-green color. The carbohydrates used in this study can be seen in Tables 1 and 2. Bacteria

were inoculated into the media (20 µl of ~10^8 cells/ml) and observed for growth and a color change in the medium, with acid production indicated by a change to yellow.

3.9 Methyl red test

The methyl red test was performed in order to test for the ability to perform mixed-acid fermentation. Bacteria were suspended in 1 ml MR-VP broth (7 g/L peptone, 5 g/L glucose, 5 g/L K_2HPO_4; pH 6.9) and incubated for 7 d at 26°C (± 2°C). A drop of methyl red was added to the culture and observed for a color change. A positive result was indicated by a red color, while negative results were observed as an orange or yellow color change. Uninoculuated broth was used as the negative control and an *E. coli* strain isolated from river water in Honolulu, Hawai ʻi was used as the positive control.

3.10 BIOLOG

The MicroLog™ 3 MicroStation™ microbial identification system (BIOLOG, Hayward, CA, Cat. No. 62401A) was used to determine the carbon source utilization and chemical sensitivity. The GEN III MicroPlate™ (BIOLOG, Hayward, CA, Cat. No. 1030) was used according to the manufacturer's instructions (Protocol A). Briefly, cultures were removed from YSC plates and streaked onto BUG™ agar (BIOLOG, Hayward, CA, Cat. No. 70101) and incubated at 26°C (± 2°C) for 24 h. Cultures were removed from the BUG™ agar and suspended in IF-A (BIOLOG, Hayward, CA, Cat. No. 72401) to turbidity of approximately 90-98% T. The GEN III MicroPlates™ were inoculated with 100 µl/well of bacterial suspension. Plates were incubated at 26°C (± 2°C) and analyzed using MicroLog™ 3 version 5.2.01 at 12, 24 and 48 h. Data for reference *Clavibacter* subsp. were obtained from GEN III Database version 1.1.21 (BIOLOG, Hayward, CA, Cat. No. 22730D). Plates were run in triplicate to assess test consistency, and an additional plate was run on a separate occasion to assess reproducibility.

3.11 API® tests

The API® Coryne test strip (bioMérieux, Marcy l'Etiole, France, Cat. No. 20900) was used to determine enzymatic activity and the fermentation of carbohydrates by coryneform bacteria, according to the manufacturer's instructions. Briefly, the incubation box was filled with ~5 ml of ddH_2O, and then the API® Coryne test strip was removed from its casing and placed into the incubation box. Bacterial cultures were removed from YSC plates and suspended in an ampule of API Suspension Medium (bioMérieux, Marcy l'Etiole, France, Cat. No.70700), with

turbidity greater than 6 McFarlands. Approximately 100-150 µl of this suspension was distributed into each of the first eleven tests of the strip. For the remaining nine tests, the bacterial suspension was mixed into API GP Medium (0.5 g/L L-cystine, 20 g/L Tryptone [bovine/ porcine origin], 5 g/L NaCl, 0.5 g/L Na_2SO_3, and 0.17 g/L phenol red) and approximately 100-150 µl of this suspension was distributed into each of the remaining nine tests of the strip. Mineral oil was used to cover the urease test and the last nine tests. Strips were incubated at 26°C (± 2°C) for 24 h. Results were assessed according to the manufacturer's instructions. Briefly, one drop each of NIT 1 and NIT 2 (bioMérieux, Marcy l'Etiole, France, Cat. No.70442) were added to the nitrate test. One drop of PYZ (bioMérieux, Marcy l'Etiole, France, Cat. No.70492) was added to the pyrazinamidase test. For enzymatic tests (3-8), one drop of ZYM A (bioMérieux, Marcy l'Etiole, France, Cat. No.70494) and ZYM B (bioMérieux, Marcy l'Etiole, France, Cat. No.70493) were added. To test for catalase activity, 1 drop of H_2O_2 was added to the gelatin test. Results were based on color changes as designated by the manufacturer.

The API® ZYM test strip (bioMérieux, Marcy l'Etiole, France, Cat. No. 25200) was used to determine various enzymatic activities, according to the manufacturer's instructions. Briefly, the incubation box was filled with ~5 ml of ddH_2O, and then the API® ZYM test strip was removed from its casing and placed into the incubation box. Bacterial cultures were removed from YSC plates, suspended in an ampule of API Suspension Medium with turbidity of 5-6 McFarlands, and then 65 µl of this suspension was dispensed into each test cupule. Strips were incubated at 26°C (± 2°C) for 4 h. Results were assessed according to the manufacturer's instructions. Briefly, one drop of ZYM A and ZYM B were added to each test. Results were based on color changes as designated by the manufacturer.

3.12 G+C content

The molar G+C content of each strain's genomic DNA was determined by a lab technician, under the supervision of Dr. Ilse Cleenwerck, of the Identification Service of the BCCM/LMG (Belgian Coordinated Collections of Microorganisms/Laboratorium voor Microbiologie - Universiteit Gent) (Gent, Belgium), using the high-performance liquid chromatography (HPLC) technique (Mesbah, Premachandran, & Whitman, 1989).

3.13 DNA-DNA hybridization

DNA-DNA hybridization analyses were performed by a lab technician, under the supervision of Dr. Ilse Cleenwerck, of the Identification Service of the BCCM/LMG (Gent, Belgium). DNA was isolated according to a modification of the procedure of Gevers et al. (2001). Hybridizations were performed in the presence of 50% formamide at 54°C according to a modification (Cleenwerck, Vandemeulebroecke, Janssens, & Swings, 2002; Goris, Suzuki, De Vos, Nakase, & Kersters, 1998) of the method described by Ezaki et al. (1989). Reciprocal reactions were performed (A x B and B x A) were performed. The DNA relatedness percentages reported are the mean of minimum 7 hybridizations.

3.14 DNA extraction

A Chelex® DNA extraction was performed on samples contained in the 1.5 ml microfuge tubes. Briefly, 0.75-1.0 ml of 40% Chelex® 100 resin (Bio-Rad, Hercules, CA) in 1X TE buffer (10 mM Tris HCl and 1 mM EDTA at pH 8) with 10% Triton™ X-100 (Sigma-Aldrich, St. Louis, MO) was added to each tube. Samples were mixed with vigorous pipeting and vortexing and then heated to 95°C for 10 min on a digital heat block. Samples were stored at 4°C for at least 24 h to allow for separation of the DNA.

3.15 Loop-mediated amplification

LAMP reactions were performed in triplicate, along with both positive and negative controls, using previously reported primers (Yasuhara-Bell, et al., 2013). Lamp reactions were 25 µl and contained 5 µl bacterial DNA, 15 µl ISO-001nd master mix (OptiGene Ltd., West Sussex), and 5 µl primer mix so that final primer concentrations were as reported previously (Yasuhara-Bell, et al., 2013). Distilled water was used for the negative control while pathogenic *Cmm* strain K73 DNA was used as the positive control.

3.16 PCR

PCR amplifications were carried out using primer pairs designed to amplify *dnaA* (Schneider, et al., 2011), partial 16S rRNA (Weisburg, Barns, Pelleteir, & Lane, 1991), *atpD* (Jacques, et al., 2012), *dnaK* (Jacques, et al., 2012), *gyrB* (Jacques, et al., 2012), *ppK* (Jacques, et al., 2012), *recA* (Jacques, et al., 2012), *rpoB, clvA* (Yasuhara-Bell, Marrero, et al., 2014), *clvF* (Yasuhara-Bell, Marrero, et al., 2014) and *clvG* (Yasuhara-Bell, Marrero, et al., 2014) gene sequences (Table 6.1). The *rpoB* primers used in this study were the reverse compliments to those

previously published by Jacques et al. (2012). PCR reactions for all primers were performed in a 10 µl reaction volume containing 1 µl bacterial DNA and 9 µl PCR reaction master mix [0.5 µl of each primer (10 µM), 5.0 µl JumpStart™ REDTaq® ReadyMix™ (Sigma-Aldrich, St. Louis, MO, Cat. No. P1107), and 3 µl ddH_2O]. PCR reaction conditions for all primers used are listed in Table 6.1 PCR products were resolved using 1.5% agarose gel electrophoresis. Gels were analyzed using the Foto/Analyst® Express System (Fotodyne Inc., Hartland, WI).

Table 6.1. PCR primers used in this study.

Gene	Primers	Cycle Conditions (35 cycles of)	Size (bp)	Source/Reference
16S	fD1/rP2	94°C for 5 min, (94°C for 30 s, 61°C for 1 min, 72°C for 2 min), 72°C for 5 min	1500	Weisburg et al. 1991
dnaA	dnaAF/R	94°C for 5 min, (94°C for 30 s, 61°C for 1 min, 72°C for 30 s), 72°C for 10 min	933	Schneider et al. 2011
atpD	atpdF/R	94°C for 5 min, (94°C for 30 s, 60°C for 30 s, 72°C for 1 min), 72°C for 10 min	697	Jacques et al. 2012
dnaK	dnakF/R		704	
gyrB	gyrbF/R		909	
ppK	ppkF/R		604	
recA	recaF/R		724	
*rpoB**	rpobF/R		662	
clvA	clvA-F/R	95°C for 5 min, (95°C for 30 s, 58°C for 30 s, 72°C for 1 min), 72°C for 5 min	338	Yasuhara-Bell et al. 2013
clvF	clvF-F/R		1166	
clvG	clvG-F/R		1178	

*Primers were made in this study from the reverse compliment sequences published by Jacques et al. (2012).

3.17 DNA sequencing

PCR products were cleaned for sequencing using ExoSAP-IT® (Affymetrix®, Santa Clara, CA, Cat. No. 78200), according to the manufacturer's instructions. Cleaned PCR products were sequenced at the University of Hawai'i sequencing facilities, using each forward and reverse primer, according to their specifications.

3.18 Phylogenetic analyses

Sequence alignment and phylogenetic analyses were performed using MEGA5 version 5.05 (Tamura, et al., 2011). The *dnaA* sequence data for *Cmm* strains NCPPB 382, K0074, K0079, K84, K87, K0428, K0448, K0449, K0465 and K0469 (Accession: AM711867.1, HM181168.1, HM181170.1, HM181247.1, HM181274.1, HM181201.1, HM181213.1, HM181216.1, HM181227.1 and HM181230.1, respectively), *Cmi* strain K0091 (Accession: HM469685.1), *Cms* strain K0090 (Accession: HM181287.1), *Cmm*-like strains C6-A, C55^{T}, C59-A, C63, C74A, C91-A, X01 (ZUM3064), X02 (ZUM3065), X05 (ZUM3936^{T}), X06 (ZUM4209), X07 (ZUM4210), X09 (ZUM4211), X11 (ZUM4206) and X12 (ZUM4207) (Accession: KC416011-KC416025), *Cmm*-like strains AFLP 50, AFLP 121, AFLP 173, NCB 4040, NCB 4041, NCB 4042, NCB 4043, NCB 4228 and NCB 4229 (Accession: KC594335, KC594330, KC594335, KC594322- KC594327, respectively), and out-group *Aquifex aeolicus* (Accession: AE000657.1) were obtained from NCBI GenBank. Sequences were trimmed to 661 nt and multiple alignments were made using ClustalW, taking into account the corresponding amino acid alignments for protein-coding genes. Maximum-parsimony (MP) analysis was used to construct trees with the closest neighbor interchange at search level 3 (Nei & Kumar, 2000). Confidence intervals were assessed using the bootstrap method with 1000 replications (Felsenstein, 1985).

The *atpD*, *dnaK*, *gyrB*, *ppK*, *recA* and *rpoB* sequence data for *Cmm*, *Cmi*, C*mn*, *Cms*, *Cmt* and *Cmm*-like strains, and out-group *Rathayibacter iranicus* were obtained from NCBI GenBank (Table 6.2). Sequences were trimmed to 561 nt, 576 nt, 744 nt, 564 nt, 594 nt and 516 nt for *atpD*, *dnaK*, *gyrB*, *ppK*, *recA* and *rpoB*, respectively, and multiple alignments were made using ClustalW, taking into account the corresponding amino acid alignments for protein-coding genes.

MLSA of housekeeping genes was performed according to a previous publish methods (Jacques, et al., 2012). Briefly, sequences were concatenated and neighbor-joining (NJ) trees (Saitou & Nei, 1987) were constructed using the Jukes-Cantor method (Jukes & Cantor, 1969) to compute evolutionary distances. Maximum-parsimony (MP) analysis was used to construct trees with the closest neighbor interchange at search level 3 (Nei & Kumar, 2000). The model of evolution for maximum likelihood (ML) analysis was determined using Modeltest 3.7 in PAUP* (Zaluga, Stragier, et al., 2013). Both the hierarchical likelihood ratio test (hLRT) and the Akaike Information Criterion (AIC) were used to evaluate model scores. Phylogenetic trees and bootstrap values for the nucleotide sequences of each gene fragment and of concatenated sequences were obtained with PhyML (Guindon, et al., 2010) using an online bioinformatics platform, available

Table 6.2. GenBank accession numbers for the previously published gene sequences used in this study.

Organism	Strain	*atpD*	*dkaK*	*gyrB*	*ppk*	*recA*	*rpoB*
Cmm	CFBP1940	JX889740	JX889918	JX890007	JX890096	JX890185	JX889829
Cmm	CFBP2500	JX889753	JX889931	JX890020	JX890109	JX890198	JX889842
Cmm	CFBP4999	JX889761	JX889939	JX890028	JX890117	JX890206	JX889850
Cmm	CFBP5842	JX889763	JX889941	JX890030	JX890119	JX890208	JX889852
Cmm	CFBP7158	JX889768	JX889946	JX890035	JX890124	JX890213	JX889857
Cmm	CFBP7311	JX889771	JX889949	JX890038	JX890127	JX890216	JX889860
Cmm	CFBP7312	JX889772	JX889950	JX890039	JX890128	JX890217	JX889861
Cmm	CFBP7313	JX889773	JX889951	JX890040	JX890129	JX890218	JX889862
Cmm	CFBP7478	JX889784	JX889962	JX890051	JX890140	JX890229	JX889873
Cmm	CFBP7555	JX889797	JX889975	JX890064	JX890153	JX890242	JX889886
Cmm	CFBP7562	JX889799	JX889977	JX890066	JX890155	JX890244	JX889888
Cmm	CFBP7584	JX889809	JX889987	JX890076	JX890165	JX890254	JX889898
Cmm	CFBP7589	JX889811	JX889989	JX890078	JX890167	JX890256	JX889900
Cmm	CFBP7590	JX889812	JX889990	JX890079	JX890168	JX890257	JX889901
Cmm	CFBP7599	JX889815	JX889993	JX890082	JX890171	JX890260	JX889904
Cmm	NCPPB382	JX889820	JX889998	JX890087	JX890176	JX890265	JX889909
Cmm	NCPPB1064	JX889818	JX889996	JX890085	JX890174	JX890263	JX889907
Cmm	NCPPB2034	JX889819	JX889997	JX890086	JX890175	JX890264	JX889908
Cmi	CFBP2404	JX889743	JX889921	JX890010	JX890099	JX890188	JX889832
Cmi	CFBP6488	JX889765	JX889943	JX890032	JX890121	JX890210	JX889854
Cmi	CFBP6492	JX889766	JX889944	JX890033	JX890122	JX890211	JX889855
Cmn	CFBP2405	JX889744	JX889922	JX890011	JX890100	JX890189	JX889833
Cmn	CFBP3521	JX889758	JX889936	JX890025	JX890114	JX890203	JX889847
Cmn	CFBP7553	JX889796	JX889974	JX890063	JX890152	JX890241	JX889885
Cmn	CFBP7577	JX889807	JX889985	JX890074	JX890163	JX890252	JX889896
Cms	CFBP2049	JX889741	JX889919	JX890008	JX890097	JX890186	JX889830
Cms	CFBP3559	JX889759	JX889937	JX890026	JX890115	JX890204	JX889848
Cms	CFBP3560	JX889760	JX889938	JX890027	JX890116	JX890205	JX889849
Cmt	CFBP3494	JX889755	JX889933	JX890022	JX890111	JX890200	JX889844
Cmt	CFBP3496	JX889756	JX889934	JX890023	JX890112	JX890201	JX889845
Cmt	CFBP3499	JX889757	JX889935	JX890024	JX890113	JX890202	JX889846
Clavibacter-like	CFBP7492	JX889787	JX889965	JX890054	JX890143	JX890232	JX889876
Clavibacter-like	CFBP7495	JX889788	JX889966	JX890055	JX890144	JX890233	JX889877
Clavibacter-like	CFBP7500	JX889789	JX889967	JX890056	JX890145	JX890234	JX889878
Clavibacter-like	CFBP7505	JX889791	JX889969	JX890058	JX890147	JX890236	JX889880
Clavibacter-like	CFBP7575	JX889805	JX889983	JX890072	JX890161	JX890250	JX889894
Clavibacter-like	CFBP7576	JX889806	JX889984	JX890073	JX890162	JX890251	JX889895
Rathayibacter iranicus	CFBP807	JX889817	JX889995	JX890084	JX890173	JX890262	JX889906

at http://www.atgc-montpellier.fr/phyml/. ML trees were edited using TreeGraph 2 graphical editor for phylogenetic trees (Stöver & Müller, 2010). Final ML trees were produced using MEGA5. Confidence intervals were assessed using the bootstrap method with 1,000 replications (Felsenstein, 1985).

3.19 Nucleotide accession numbers

The gene sequences for *Cmm*-like strains C55^T, C59-A, C63, C6-A, C74A, C91-A, *Cmi* strain A1149, *Cmm* strain K73, *Cmn* strain NCPPB 2579, *Cms* strain A2041, and *Cmt* strain LMG 7294, *Clavibacter* strains PF007, PF008, PS003, PS005^T and PS006 and *Cmm*-like strains ZUM3064, ZUM3065, ZUM3936^T, ZUM4206, ZUM4207, ZUM4209, ZUM4210, ZUM4211 have been deposited into the GenBank Database with accession numbers KF663873-KF663896 for *atpD*, KF663907-KF663930 for *dnaK*, KF663931-KF663954 for *gyrB*, KF663955-KF663978 for *ppK*, KF663979-KF664002 for *recA*, and KF664003-KF664026 for *rpoB*, respectively. The 16S rDNA sequences for *Cmm*-like strains C55 and ZUM3936 have been deposited into the GenBank Database with accession numbers KF663871-KF663872. The *dnaA* gene sequence data for *Cmn* strains 20037, 200800460 and NCPPB 2579, *Cms* strain 2041, *Cmt* strain LMG 7294, and *Clavibacter* strains PF007, PF008, PS003, PS005^T and PS006 have been deposited into the GenBank Database with accession numbers KF663897-KF663906, respectively.

3.20 Pathogenicity testing

Pathogenicity tests were performed according to a previously reported method (Yasuhara-Bell, et al., 2013). Briefly, a sterile scalpel was used to make a vertical incision (ca 1 mm) on the stem of juvenile tomato (*S. lycopersicum* cv. 'Kewalo'), chili pepper (*Capsicum annuum* cv. 'Jalapeños' and 'Pasilla Bajio'), and hybrid cabbage (*Brassica oleracea* [capitata group] cv. 'Early Green farao f_1') plants that were approximately 3-4 weeks old. Bacteria (ca ~10^9 CFU/ml) were applied to the wound, using the scalpel tip. Negative controls were made by applying ca 500 µl ddH_2O to the wound. Inoculated plants and controls were then carefully placed into plastic bags, closed using twist-ties, and incubated at room temperature for 24 h, After the initial incubation, plants were removed from the bags and allowed to grow for 3-4 wks in a growth room (30°C) under Philips F40/AGRO Agro-Lite^{TM} fluorescent lamps (Philips, NL). Plants were checked periodically for symptoms and pathogenicity was determined at 3-4 wks post-inoculation, relative to controls. Two plants were used per test for each strain, with the entire set of tests being repeated three times.

4. RESULTS

4.1 Characteristics as determined by conventional bacteriological tests

Conventional bacteriological tests were performed on two different *Cmm*-like bacteria ($C55^T$ and $ZUM3936^T$) that were isolated from tomato seed (California and the Netherlands, respectively). A summary of these results can be found in Tables 6.3 and 6.4. Both of these strains have mucoid colony morphology similar to *Cmm.* Colonies of $C55^T$ appeared to be yellow-orange in color, and produced larger amounts of exopolysaccharide (EPS), making them thick and gummy. Colonies of $ZUM3936^T$ were light to dark yellow and consistency was similar to *Cmm*. Both strains were Gram-positive and had coryneform cell morphology. Cell-wall analysis revealed that both strains possessed peptidoglycan, menaquinone, and major fatty acid and polar lipid profiles indicative of *Clavibacter.* Acid production from various carbohydrates, utilization of nitrate/nitrite, hydrolysis of casein and esculin, and activity of certain enzymes have been used to differentiate between genera of the family *Microbaceriaceae* (Table 6.3). These results in combination with previous reports that these strains reacted with *Clavibacter*-specific *dnaA* primers (Yasuhara-Bell, et al., 2013) provide strong support for inclusion of these strains in the genus *Clavibacter.*

Analysis of the primary cell-wall sugars provided interesting results. According to data presented by Saddler and Kerr (2012), the primary cell-wall sugars of *Clavibacter* are rhamnose, galactose and mannose. Strain $C55^T$ had the characteristic rhamnose, galactose and mannose, but also possessed fucose. In contrast, strain $ZUM3936^T$ had galactose, mannose and ribose, but no rhamnose. These data together show that these two strains not only differ from known *Clavibacter* species, but also differ from each other. Further examination of the fatty acid profiles of both strains also provided interesting, and contrasting results. According to data presented by Saddler and Kerr (2012), the major fatty acids possessed by *Clavibacter* are 14-methylhexadecanoic ($C_{17:0}$ anteiso), 12-methyltetradecanoic ($C_{15:0}$ anteiso) and 14-methylpentadecanoic ($C_{16:0}$ iso) acids. Significant amounts of octadecenoic acid ($C_{18:1}$) are also found (Saddler & Kerr, 2012). Both $C55^T$ and $ZUM3936^T$ possessed the same major fatty acids, suggesting inclusion into the genus *Clavibacter*, however they did not possess $C_{18:1}$. Additionally, *Cmm* possesses saturated, straight-chain fatty acids dodecanoic ($C_{12:0}$) and hexadecanoic ($C_{16:0}$) acids in significant amounts (Saddler & Kerr, 2012). Both $C55^T$ and $ZUM3936^T$ possessed $C_{16:0}$, however they did not possess $C_{12:0}$. Saddler and Kerr (2012) reported that the presence of the unsaturated, branched-chain acid, 12-methyltetradecenoic acid ($C_{15:1}$ anteiso), was considered diagnostic for *Cmm*. Interestingly, strain $ZUM3936^T$ had $C_{15:1}$ anteiso in significant amounts, while strain $C55^T$ did not. This would suggest that strain

ZUM3936^T is more closely related to *Cmm* than is C55^T, which is also reinforced by ZUM3936^T's more *Cmm*-like colony color and morphology. However, as stated previously, primary cell-wall sugar analyses showed that strain C55^T has all the cell-wall sugars found in *Clavibacter*, though it does contain an extra sugar not found in *Clavibacter,* while strain ZUM3936^T lacked one of the *Clavibacter* cell-wall sugars and contained one sugar not found in *Clavibacter* (ribose). Additionally, phylogenetic analysis of *Clavibacter* based on *dnaA* sequence analysis showed that C55^T grouped closer to *Cmm* strains (Yasuhara-Bell, et al., 2013), again contradicting an identification based on the presence of $C_{15:1}$ anteiso in strain ZUM3936^T. Nevertheless, all results presented to this point justify inclusion of strains C55^T and ZUM3936^T within the genus *Clavibacter,* and establish that these strains are different from *Cmm,* as well as each other.

Strains C55^T and ZUM3936^T strains are representatives of two separate populations of *Clavibacter* associated with tomato seed. Thus, further bacteriological tests were performed in an attempt to either place them within a known subspecies, or demonstrate that these strains represent novel species/subspecies. A summary of these results can be found in Table 6.4. These tests, along with all remaining tests in this study, also include a "*Cmm*" strain (PS005) isolated from pepper (*Capsicum annum*) and characterized as a phenotypic variant of *Cmm* by Yim et al. (2012). Rep-PCR and phylogenetic analyses based on 16S rRNA and ITS sequences all showed that the pepper "*Cmm*" formed a clade separate from all known subspecies of *Clavibacter michiganensis*. Since their work clearly demonstrated that the pepper strains were not typical *Cmm,* but perhaps another unknown subspecies, the pepper strain PS005^T was included in the current study.

Growth of all tested strains was assessed on two complex media and three selective media (Appendix F), under the conditions of this study. All strains grew on both YSC and Medium-6 (Table 6.4 and Appendix F). On YSC, strains C55^T and ZUM3936^T and *Cmm* (K73) produced a yellow pigment, whereas strains PS005^T, *Cmn* (NCPPB 2579) and *Cmt* (LMG 7294) were orange. *Cmi* (A1149) was yellow-orange with indigoidine production that made it appear greenish and *Cms* (A2041) lacked any pigment. Medium-6 is a growth medium recommended by BCCM/LMG. On Medium-6, all strains were varying shades of yellow, except for *Cmi* (greenish) when enough indigoidine was produced (5-7 d.p.i.), as well as strain PS005^T (orange) at ≥5 d.p.i. The fact that all strains were of similar color when grown on Medium-6 makes subspecies differentiation difficult based on appearance. Additionally, growth on this media was not as robust as compared with YSC, with very weak growth observed for *Cms*. This confirms previous

Table 6.3: Characteristics differentiating plant-associated genera of the family *Microbacteriaceae*.*

Characteristic	Strain C55	Strain ZUM3936	*Agreia*$	*Clavibacter*$	*Curtobacterium*$	*Frigoribacterium*$	*Leifsonia*$	*Okibacterium*$	*Plantibacter*$	*Rathayibacter*$
Colony Color[a]	OY	Y	O / Y	O / W / Y	I / OY	Y	I / R / Y / W	Y	Y	Y
Gram Stain	+	+	+	+	+	+	+	+	+	+
Morphology	Coryneform	Coryneform	Coryneform	Coryneform	Coryneform	Coryneform	Coryneform	Coryneform	Coryneform	Coryneform
Motility	-	-	+	-	v	+	v	-	-	-
Spores	-	-	-	-	-	-	-	-	-	-
Peptidoglycan type	B2γ	B2γ	B	B2γ	B2β	B2β	B2γ	B	B2γ	B2γ
Peptidoglycan diamino acid[b]	DL-DAB	DL-DAB	L-DAB; D-Orn; Hyg	DL-DAB	D-Orn	L-Lys	DL-DAB	Lys	L-DAB	L-DAB
Mycolic acids	nd	nd	-	-	-	-	-	-	-	-
Major fatty acids	14-methylhexadecanoic; 12-methyltetradecanoic; 14-methylpentadecanoic	14-methylhexadecanoic; 12-methyltetradecanoic; 14-methylpentadecanoic	14-methylhexadecanoic; 12-methyltetradecanoic; 14-methylpentadecanoic	14-methylhexadecanoic; 12-methyltetradecanoic; 14-methylpentadecanoic	14-methylhexadecanoic; 12-methyltetradecanoic; 14-methylpentadecanoic	14-methylpentadecanoic; 12-methyltetradecanoic	14-methylhexadecanoic; 12-methyltetradecanoic; 14-methylpentadecanoic	14-methylhexadecanoic; 12-methyltetradecanoic	14-methylhexadecanoic; 12-methyltetradecanoic; 14-methylpentadecanoic; hexadecanoic	14-methylhexadecanoic; 12-methyltetradecanoic; 14-methylpentadecanoic
Major polar lipids[c]	PG, DPG, GL	PG, DPG, GL	PG, DPG, GL	PG, DPG, unknown glycosyldiacylglycerols	PG, DPG, di-mannosyl glyceride, tri-mannosyl glyceride	PG, DPG, GL	PG, DPG, glycosyldiacylglycerols G_2 and G_1	PG, DPG, GL	PG, DPG, GL	PG, DPG, glycosyldiacylglycerols
Polyamines[d]	nd	nd	SPM	SPD; SPM; (PUT; CAD)	SPD; SPM	SPD; SPM	nd	nd	nd	SPD; SPM; (PUT; CAD)
Major menaquinones	MK-9	MK-9	MK-10	MK-9	MK-9	MK-9	MK-11	MK-10, 11	MK-9, 10	MK-10
Primary cell-wall sugars	rhamnose; galactose; mannose; fucose	ribose; galactose; mannose	rhamnose; mannose; fucose	rhamnose; galactose; mannose	nd	nd	rhamnose	rhamnose; glucose	rhamnose	rhamnose; glucose; mannose
Acid produced from:										
Adonitol	-	-	-	-	nd	- / w	- / v	-	v	-
Glucose	w	w	nd	+	+	- / w	+ / -	+	+	+
Glycerol	w	w	nd	+	nd	nd	+ / v	+	nd	-
Melezitose	nd	nd	nd	-	v	nd	- / v	-	+	nd
meso-Inositol	nd	nd	nd	-	nd	nd	- / v	nd	v	-
Raffinose	-	-	nd	-	v	nd	- / v	-	v	-
Rhamnose	-	-	nd	-	v	nd	- / v	+	+	-
Ribose	-	-	nd	-	v	nd	- / v	-	v	-
Utilization of:										
Nitrate/Nitrite	-	-	nd	-	-	nd	- / +	nd	-	-
Hydrolysis of:										
Casein	-	-	-	-	v	nd	- / w	-	-	-
Esculin	+	+	+	+	+	+	v	+	+	+
Activity of:										
Catalase	+	+	+	+	+	+	+ / -	+	+	+
Oxidase	-	-	v	-	-	-	v	+	-	- / w
Lipase	-	-	nd	-	nd	nd	nd	nd	nd	nd
Tyrosinase	nd	nd	nd	-	nd	nd	- / v	nd	nd	nd
Urease	-	-	nd	-	-	nd	- / v	nd	nd	-

*Symbols: +, positive; -, negative; /, separates the majority and minority; nd, not determined; v, varies depending on species; w, weak positive.

$Data obtained from Saddler and Kerr (2012).

[a] I, ivory; O, orange; R, Red; Y, yellow; W, white.

[b] DAB, 2,4-diaminobutyric acid; Orn, ornithine; Lys, lysine.

[c] PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; GL, glycolipids.

[d] SPD, spermidine; SPM, spermine; CAD, cadaverine; PUT, putrescine. Polyamines found in parentheses are only found in small amounts.

φ Data obtained in this study.

reports that *Clavibacter* subspecies grow better on sucrose than glucose (Chun, 1982; Kaneshiro, 2003; Kaneshiro, et al., 2006). BCT inhibited growth of both *Cmi* and *Cms*, while all other strains showed various levels of growth. Only growth of *Cms* was inhibited on CNS. Inhibition of *Cms* by CNS and BCT confirms data presented by Saddler and Kerr (2012) and Ftayeh et al. (2011), respectively. Growth of *Cmi* on CNS and inhibition of *Cmi* by BCT contradicts data reported by Saddler and Kerr (2012) and Ftayeh et al. (2011), repectively. *Cmn* was pigmented orange on BCT, in contrast to data reported by Ftayeh et al. (2011). Strains $C55^T$ and $ZUM3936^T$ were yellow on BCT, while strain $PS005^T$ was orange. At an inoculum of 10^4 cells/spot, only *Cmi*, *Cmt*, $C55^T$ and $ZUM3936^T$ showed growth on CMM1 plates at 7 and 9 d.p.i., with *Cmi* and $C55^T$ showing very little growth, just enough to be seen. At a higher inoculum (~5 x 10^5 CFU/spot), more robust growth was seen on all media (Appendix F, Figure F.4)). Again, on CNS, *Cmi* growth was apparent, though very slow and far less than that observed for all other strains. *Cms* still showed no growth. BCT medium again inhibited growth of both *Cmi* and *Cms* when inoculated at the higher titer. All strains, except *Cms*, grew on CMM1 media when applied at higher titer and produced pigments with varying shades of yellow to yellow-orange. Strain $ZUM3936^T$ showed the most robust growth on CMM1, followed by *Cmn.* All other tested strains grew slower (Appendix F, Figure F.4).

To compare the *Clavibacter* from tomato seed and from pepper to data presented by Saddler and Kerr (2012), strains were tested for their ability to produce acid aerobically from various carbohydrates, hydrolyze gelatin and starch, produce mixed acid from glucose (methyl red test), and produce H_2S from peptone. As seen in Table 6.4, strains $C55^T$, $ZUM3936^T$ and $PS005^T$ were unable to produce acid aerobically from inulin, mannitol, mannose, and sorbitol. Only strain $PS005^T$ hydrolyzed gelatin. All three strains were negative for the methyl red test. In our study, *Cmi* was negative for methyl red, in contrast to data presented by Saddler and Kerr (2012). All three strains were negative for the H_2S production. Additionally, *Cmm* was negative for H_2S production, again in contrast to data presented by Saddler and Kerr (2012). When tested on FS medium without any antibiotics, strains $C55^T$, $ZUM3936^T$ and $PS005^T$ showed weak starch hydrolysis compared to the control. Of the known *Clavibacter michiganensis* subsp., only *Cmt* hydrolyzed starch, albeit weakly. These results are consistent with data presented by Saddler and Kerr (2012), where *Cmt* is positive, *Cmi* is negative, and all other subspecies are variable in their ability to hydrolyze starch.

4.2 BIOLOG

The BIOLOG system was used to determine the utilization of vast array of carbon sources and chemical sensitivity (Table 6.5 and Appendix G). The metabolic profiles obtained from BIOLOG system are compared to a database (Appendix H) in order to provide a bacterial identification. The BIOLOG program returned bacterial IDs of *Cmt* for the *Cmm*, *Cmn*, *Cmt*, tomato-seed *Clavibacter* strains C55^T and ZUM3936^T, and the pepper *Clavibacter* strain PS005^T used in this study. These bacterial IDs had an average similarity and probability index of 0.748 and 0.898, 0.564 and 0.686, 0.812 and 0.912, 0.605 and 0.698, 0.728 and 0.896 and 0.740 and 0.899 for *Cmm*, *Cmn*, *Cmt*, tomato seed *Clavibacter* strains C55^T and ZUM3936^T, and the pepper *Clavibacter* strain PS005^T, respectively. These results are similar to that found previously (Harris-Baldwin & Gudmestad, 1996; Kaneshiro, 2003; Kaneshiro, et al., 2006). BIOLOG gave an ID of *Cms*/*Cmi* for *Cmi* and *Cms* for *Cms*. These Bacterial IDs had an average similarity and probability index of 0.379 for *Cmi* (could not provide probability) and 0.559 and 0.697 for *Cms*, respectively. Only the *Cms* and *Cmt* strains were properly identified and *Cmi* was partially identified. The BIOLOG data for the known subspecies of *Clavibacter michiganensis* differed from data reported by Holt et al. (2000) with respect to utilization of acetate, lactate, citrate, propionate and succinate. It is apparent that the BIOLOG database should be reevaluated for plant pathogenic bacteria, especially for the closely related subspecies of *Clavibacter michiganensis*. Regardless, the BIOLOG system does group the two unknown tomato seed- and pepper-associated *Clavibacter* strains with the other subspecies of *Clavibacter michiganensis*.

The metabolic profiles as determined by the BIOLOG system are shown in Table 6.5 and Tables G.1-G.8. Tomato seed-associated *Clavibacter* strain C55^T differed from *Cmm* strain K73 in its utilization of L-fucose, D-glucose-6-phosphate, D-fructose-6-phosphate, L-serine, mucic acid, D-lactic acid methyl ester, citric acid, α-ketoglutaric acid, D-malic acid, bromo-succinic acid, Tween 40, and propionic acid, and differed in chemical sensitivity to guanidine HCl. These differences reflect strain C55^T's inability to utilize these carbon sources, whereas *Cmm* possesses the ability. This might reflect the loss of genetic material and/or an adaptation to a more specific niche. Tomato seed-associated *Clavibacter* strain ZUM3936^T differed from *Cmm* strain K73 in its utilization of N-acetyl D-glucosamine, N-acetyl-β-D-mannosamine, L-rhamnose, methyl pyruvate, L-lactic acid, and α-ketobutyric acid in its ability to utilize these carbon sources, whereas *Cmm* could not. Perhaps, the fact that this strain can utilize L-rhamnose correlates with the fact that rhamnose is absent as a primary cell-wall sugar. Strain ZUM3936^T differed from *Cmm* strain K73 in that it could not use D-fucose, L-fucose, D-glucose-6-phosphate, D-fructose-6-phosphate, glucuronamide, mucic acid, quinic acid, D-lactic acid methyl ester,

Table 6.4: Characteristics differentiating *Clavibacter* species and subspecies.*

Characteristic	Strain C55Ψ	Strain ZUM 3936Ψ	Strain PS005Ψ	*Cm.* subsp. *insidiosus*$	*Cm.* subsp. *michiganensis*$	*Cm.* subsp. *nebraskensis*$	*Cm.* subsp. *sepedonicus*$	*Cm.* subsp. *tesselarius*$	*C. iranicus*$	*C. rathayi*$	*C. tritici*$	*C. xyli* subsp. *cynodontis*$	*C. xyli* subsp. *xyli*$
Yellow or Orange Pigment	+	+	+	+	+	+	-	+	+	+	+	+	-
Growth on YSCΨ	+	+	+	+	+	+	+	+	nd	nd	nd	nd	nd
Growth on Medium-6Ψ	+	+	+	+	+	+	+	+	nd	nd	nd	nd	nd
Growth on CNS‡	+	+	+	-	+	+	-	+	nd	nd	nd	nd	nd
Growth on CMM1Ψ	+	+	+	+	+	+	-	+	nd	nd	nd	nd	nd
Growth on BCT€	+	+	+	+	+	+	-	+	nd	nd	nd	nd	nd
Colony Color on BCT€	yellow	yellow	orange	pink w/ internal violet flecks	yellow	yellow	na	light pink	nd	nd	nd	nd	nd
Acid produced aerobically from:													
Inulin	-	-	-	-	-	-	-	-	-	-	+	-	-
Mannitol	-	-	-	-	-	-	+	+	-	+	+	+	+
Mannose	-	-	-	+	+	+	v	nd	+	-	+	+	w
Melezitose	nd	nd	nd	-	-	-	-	-	+	-	-	-	-
Sorbitol	-	-	-	-	-	+	+	+	-	-	-	-	-
Utilization of:													
Acetate	w	-	+	-	+	+	+	+	-	-	+	-	-
Citrate	-	+	+	-	+	+	+	+	+	+	+	+	-
Lactate	-	w	-	-	v	+	-	nd	-	-	-	-	-
Propionate	-	-	-	-	-	w	-	-	-	-	-	-	-
Succinate	-	+	+	-	+	+	+	+‡	+	+	+	-	-
Hydrolysis of:													
Gelatin	-	-	+	-	w	-	-	-	-	+	-	-	-
Starch	w	w	w	-	v	v	v	+	-	v	-	+	-
Methyl red	-	-	-	+	-	v	-	-	-	v	-	-	-
H_2S produced from peptone	-	-	-	-	+	v	-	-	+	+	+	-	-

*Symbols: +, positive; -, negative; nd, not determined; v, varies depending on species; w, weak; na, not applicable.

ΨData obtained in this study.

$Data obtained from Holt et al. (2000).

‡ Data obtained from Saddler and Kerr (2012).

€Data obtained from Ftayeh et al. (2011).

D-malic acid, propionic acid and acetic acid. Strain ZUM3936^T differed from *Cmm* in chemical sensitivity to 8% NaCl, guanidine HCl and sodium butyrate. Again, these differences might reflect the loss or gain of genetic material and/or an adaptation to a more specific niche. Pepper strain PS005^T differed from *Cmm* strain K73 in that it could not use D-fucose, L-fucose, glucuronamide, quinic acid, D-lactic acid methyl ester, D-malic acid and propionic acid. Pepper strain PS005^T differed from *Cmm* in that it was able to use methyl pyruvate, gelatin and glycyl-L-proline. Strain PS005 also differed from *Cmm* in chemical sensitivity to 8% NaCl, guanidine HCl and sodium butyrate. Strain C55^T, ZUM3936^T and PS005^T all differed from *Cmm* as well as each other. It should be noted that *Cmm* strain K73 is a fully pathogenic strain that was previously shown to possess all six pathogenicity-associated genes (pattern 1) (Yasuhara-Bell, et al., 2013). Strains from other patterns were missing one or more of these genes (Yasuhara-Bell, et al., 2013) and therefore could potentially produce a different BIOLOG profile.

The PS005^T pepper strain was most similar to *Cmt* on YSC medium. Pepper strain PS005^T differed from *Cmt* strain LMG 7294 in that it could not use α-hydroxy-butyric acid, β-hydroxy-D,L-butyric acid, α-ketobutyric acid and propionic acid, and that it utilized D-glucose-6-phosphate, D-fructose-6-phosphate, glycyl-L-proline, L-aspartic acid, mucic acid, methyl pyruvate and α-ketoglutaric acid. Strain PS005^T did not differ from *Cmt* in its chemical sensitivity.

4.3 API® test

The API® Coryne and API® ZYM test strips were used to determine enzymatic activities and fermentation of carbohydrates. All strains tested showed similar profiles by both the API® Coryne (Table 6.6) and API® ZYM tests (Table 6.7). All strains showed alkaline phosphatase, β-galactosidase, α-glucosidase, β-glucosidase and catalase activity on the API® Coryne test strip (Table 6.6). Only strain PS005^T hydrolyzed gelatin (Table 6.6). Strain PS005^T's ability to hydrolyze gelatin confirms BIOLOG data (Table 6.5). *Cmt* showed weak reactions with gelatin for BIOLOG (Table 6.5) but was negative for gelatin hydrolysis via API® (Table 6.6), which is supported by data from Holt et al. (Holt, 2000). Both BIOLOG and API® suggest that that *Cmm* cannot hydrolyze gelatin, which contradicts data reported by Holt et al. (Holt, 2000). The API® ZYM test also contained alkaline phosphatase, β-galactosidase, α-glucosidase, β-glucosidase and β-glucuronidase and results were consistent with API® Coryne (Table 6.7). All strains had reactions for esterase (C4), esterase lipase (C8), leucine arylamidase and acid phosphatase. *Cmi* and *Cms* were the only strains negative for α-galactosidase while *Cmi* was the only strain positive for α-mannosidase. Strain ZUM3936^T, *Cmi* and *Cmm* were negative for

Table 6.5: Carbon source utilization and chemical sensitivity according to the BIOLOG test system.*

Test	Strain C55	Strain ZUM3936	Strain PS005	*Cmi* strain A1149	*Cmm* strain K73	*Cmn* strain NCPPB 2579	*Cms* strain A2041	*Cmt* strain LMG 7294
Carbon Source Utilization:								
Dextrin	+	+	+	+	+	+	-	+
D-Maltose	+	+	+	w/v	+	+	-	+
D-Trehalose	+	+	+	-	+	+	-	+
D-Cellobiose	+	+	+	+	+	+	+	+
Gentiobiose	+	+	+	-	+	+	-	+
Sucrose	+	+	+	+	+	+	-	+
D-Turanose	+	+	+	+	+	+	-	+
Stachyose	+	+	+	-	+	+	-	+
D-Raffinose	+	w	w/-	-	+	-	+	w/-
α-D-Lactose	+	+	+	+	+	+	-	+
D-Melebiose	+	+	+	-	+	+	-	+
β-Methyl-D-Glucoside	+	+	+	-	+	+	-	+
D-Salicin	+	+	+	-	+	+	-	+
N-Acetyl-D-Glucosamine	-	+	-	-	-	-	-	-
N-Acetyl-β-D-Mannosamine	-	w/-	-	-	-	-	-	-
N-Acetyl-D-Galactosamine	-	-	-	-	-	-	-	-
N-Acetyl-Neuraminic Acid	-	-	-	-	-	-	-	-
α-D-Glucose	+	+	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	-	+
3-Methyl Glucose	-	-	-	+/v	-	-	-	-
D-Fucose	w	-	-	w/v	w/v	-	-	-
L-Fucose	-	-	-	+/v	w	-	-	-
L-Rhamnose	-	+	-	w/v	-	-	-	-
Inosine	w/v	+	+	-	+/w	-	-	+
D-Sorbitol	+/w	+	+	w	+	+	-	+
D-Mannitol	+	+	+	+	+	+	+	+
D-Arabitol	-	-	-	-	-	-	-	-
myo-Inositol	+	+	+	+	+	+	-	+
Glycerol	+	+	+	+	+	+	-	+
D-Glucose-6-Phosphate	-	-	w	-	w/-	-	-	-
D-Fructose-6-Phosphate	-	-	w	-	w	-	-	-
D-Aspartic Acid	-	-	-	-	-	-	-	-
D-Serine	-	-	-	-	-	-	-	-
Gelatin	-	-	+	-	-	-	-	w/v
Glycyl-L-Proline	-	-	+/w	-	-	-	-	-
L-Alanine	v	+	+	-	w/v	+	-	+
L-Arginine	-	-	-	-	-	-	-	-
L-Aspartic Acid	+	w	w	+	+	w	-	-
L-Glutamic Acid	+	+	+	-	+	w	-	+/w
L-Histidine	-	-	-	-	-	-	-	-
L-Pyroglutamic Acid	-	-	-	-	-	-	-	-
L-Serine	-	+	+	-	+	+	-	+
Pectin	+	+	+	+	+	+	+	+
D-Galacturonic Acid	-	-	-	-	-	-	-	-
L-Galactonic Acid Lactone	-	-	-	-	-	-	-	-
D-Gluconic Acid	+	+	+	-	+	+	+	+

Table 6.5 cont: Carbon source utilization and chemical sensitivity according to the BIOLOG test system.*

Test	Strain C55	Strain ZUM3936	Strain PS005	*Cmi* strain A1149	*Cmm* strain K73	*Cmn* strain NCPPB 2579	*Cms* strain A2041	*Cmt* strain LMG 7294
D-Glucuronic Acid	-	-	-	-	-	-	-	-
Glucuronamide	w	-	-	+/w	+/w	-	-	-
Mucic Acid	-	-	w	-	w	-	-	-
Quinic Acid	+	-	-	+	+	-	-	-
D-Saccharic Acid	-	-	-	-	-	-	-	-
p-Hydroxy-Phenylacetc Acid	-	-	-	-	-	-	-	-
Methyl Pyruvate	-	w	+	-	-	-	-	-
D-Lactic Acid Methyl Ester	-	-	-	-	w/-	-	-	-
L-Lactic Acid	-	w	-	-	-	-	-	-
Cirtic Acid	-	+	+	-	+	w	-	+
α-Ketoglutaric Acid	-	w	w	-	w	-	-	-
D-Malic Acid	-	-	-	-	w	-	-	-
L-Malic Acid	+	+	+	+	+	-	-	+
Bromo-Succinic Acid	-	+	+	-	+	-	-	+
Tween 40	-	+	+	+	+	+	-	+
γ-Amino-Butyric Acid	-	-	-	-	-	-	-	-
α-Hydroxy-Butyric Acid	-	-	-	-	-	-	-	+
β-Hydroxy-D,L-Butyric Acid	-	-	-	-	-	-	-	+
α-Keto-Butyric Acid	-	w	-	-	-	-	-	w/v
Acetoacetic Acid	+	+	+	+	+	+	+	+
Propionic Acid	-	-	-	-	w	w/-	-	w/-
Acetic Acid	w	-	+	+	+	w/-	-	+/w
Formic Acid	-	-	-	-	-	-	-	-
Chemical Sensitivity:								
pH6	+	+	+	+	+	+	+	+
pH5	+/w	+	+	-	+	w	-	+
1% NaCl	+	+	+	+	+	+	w	+
4% NaCl	+/w	+	+	-	+	v	-	+
8% NaCl	-	+/w	w	-	-	-	-	+/v
1% Sodium Lactate	+	+	+	-	+/v	+	-	+
Fusidic Acid	-	-	-	-	-	-	-	-
D-Serine	-	-	-	-	-	-	-	-
Troleandomycin	-	-	-	-	-	-	-	-
Rifamycin SV	-	-	-	-	-	-	-	-
Minocycline	-	-	-	-	-	-	-	-
Lincomycin	-	-	-	-	-	-	-	-
Guanidine HCl	-	-	-	-	+	-	-	-
Niaproof 4	-	-	-	-	-	-	-	-
Vancomycin	-	-	-	-	-	-	-	-
Tetrazolium Violet	-	-	-	-	-	-	w	-
Tetrazolium Blue	-	-	-	-	-	-	-	-
Nalidixic Acid	+	+	+	+	+	+	+	+
Lithium Chloride	+	+	+	w	+	w	-	+
Potassium Tellurite	+	+	+	+	+	+	+	+
Aztreonam	+	+	+	+	+	+	+	+
Sodium Butyrate	-	+	w/-	w	-	-	-	+
Sodium Bromate	w	+	+	-	+	+	-	+

* Symbols: +, positive; -, negative; w, weak positive; v, variable; /, separates the majority and minority.

naphthol-AS-BI-phosphohydrolase, while all other strains had weak reactions. These API® tests have a reference database for strain identification; however one does not exist for plant-associated *Clavibacter*. The results of the API® ZYM tests in this study are highly similar to that presented previously by Palomo et al. (Palomo, López, Garcia-Benevides, Velázquez, & Martinez-Molina, 2006). These tests, like BIOLOG, are aimed at identification of medically relevant bacteria. Results show that *Clavibacter michiganensis* subspecies produce highly similar profiles that may pose a problem if trying to use these tests for subspecies differentiation. The results presented here, in conjunction with those presented by Palomo et al. (Palomo, et al., 2006), could be used to create a reference database that could allow for identification of plant-associated *Clavibacter*. As *Clavibacter* strains generally grow slowly, an alternative protocol most likely will be needed for identification of *Clavibacter* using the API® test.

Table 6.6: Enzymatic activities and fermentation of carbohydrates according to the API® Coryne test strip.*

Characteristic	Strain C55	Strain ZUM3936	Strain PS005	*Cmi* strain A1149	*Cmm* strain K73	*Cmn* strain NCPPB 2579	*Cms* strain A2041	*Cmt* strain LMG 7294
Nitrate Reduction	-	-	-	-	-	-	-	-
Enzymatic activity:								
Pyrazinamidase	-	-	-	-	-	-	-	-
Pyrrolidonyl Arylamidase	-	-	-	-	-	-	-	-
Alkaline Phosphatase	+	+	+	w	+	+	w	+
β-glucuronidase	-	-	-	-	-	-	-	-
β-galactosidase	+	+	w	w	+	+	w	+
α-glucosidase	+	+	w	+	+	+	w	+
N-acetyl-β-glucosaminidase	-	-	-	-	-	-	-	-
β-glucosidase	+	+	+	w	+	+	w	+
Urease	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+
Hydrolysis of:								
Gelatin	-	-	w	-	-	-	-	-
Fermentation of:								
D-glucose	-	-	-	-	-	-	-	-
D-ribose	-	-	-	-	-	-	-	-
D-xylose	-	-	-	-	-	-	-	-
D-mannitol	-	-	-	-	-	-	-	-
D-maltose	-	-	-	-	-	-	-	-
D-lactose	-	-	-	-	-	-	-	-
D-saccharose (sucrose)	-	-	-	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-	-

* Symbols: +, positive; -, negative; w, weak positive.

Table 6.7: Enzymatic activities according to the API® ZYM test strip.*

Enzymatic Activity	Strain C55	Strain ZUM3936	Strain PS005	*Cmi* strain A1149	*Cmm* strain K73	*Cmn* strain NCPPB 2579	*Cms* strain A2041	*Cmt* strain LMG 7294
Alkaline phosphatase	+	+	+	+	+	+	+	+
Esterase (C 4)	w	w	w	w	w	w	w	w
Esterase Lipase (C 8)	w	w	w	w	w	w	w	w
Lipase (C 14)	-	-	-	-	-	-	-	-
Leucine arylamidase	+	+	+	+	+	+	+	+
Valine arylamidase	-	-	-	-	-	-	-	-
Cystine arylamidase	-	-	-	-	-	-	-	-
Trypsin	-	-	-	-	-	-	-	-
α-chemotrypsin	-	-	-	-	-	-	-	-
Acid phosphatase	+	+	+	w	w	+	+	+
Naphthol-AS-BI-phosphohydrolase	w	-	w	-	-	w	w	w
α-galactosidase	+	+	+	-	+	+	-	+
β-galactosidase	+	+	+	+	+	+	+	+
β-glucuronidase	-	-	-	-	-	-	-	-
α-glucosidase	+	+	+	+	+	+	+	+
β-glucosidase	+	+	+	w	w	w	w	+
N-acetyl-β-glucosaminidase	-	-	-	-	-	-	-	-
α-mannosidase	-	-	-	+	-	-	-	+
α-fucosidase	-	-	-	-	-	-	-	-

* Symbols: +, positive; -, negative; w, weak positive.

4.4 G+C content

DNA G+C content is a useful parameter and its relationship to codon usage is clearly illustrated in genome analysis. It is also an important prerequisite for determining the conditions used in DNA-DNA hybridizations (Tindall, Rosselló-Móra, Busse, Ludwig, & Kampfer, 2010). The G+C content for strains C55^T and ZUM3936^T were 72.6% and 73.6%, respectively. The G+C content for these strains is similar to those of other subspecies *C. michiganensis* (Table 6.8). The G+C content for strains C55^T and ZUM3936^T show less than 3% difference, the generally accepted G+C content range within a well-defined species (P. Vandamme et al., 1996), from that of the type strain of *C. michiganensis* (73%) reported by Sasaki et al. (1998).

4.5 DNA-DNA hybridization

DNA-DNA hybridization was performed on strains C55^T and ZUM3936^T because they share more than 97% 16S rRNA gene sequence similarity (Tindall, et al., 2010) with each other and with other subspecies of *Clavibacter michiganensis*. DNA preparations isolated from strains C55^T and ZUM3936^T were hybridized to each other, as well as to *Cmm* type strain LMG 7333^T.

Table 6.8: Genome comparison.[*]

Characteristic	Strain C55	Strain ZUM3936	Cm. strain LMG 26808[£]	*Cm.* subsp. *michiganensis*[$]	*Cm.* subsp. *nebraskensis*[ǂ]	*Cm.* subsp. *sepedonicus*[¥]
Chromosome size (Mb)	nd	nd	3.47	3.30	3.06	3.26
G-C Content (%)	72.6	73.6	72.0	72.7	73.0	72.6
Plasmids[ɣ]	nd	nd	pC1 (≤70 kb)	pCM1 (27 kb) pCM2 (70 kb)	-	pCS1 (50 kb) pCSL1 (95 kb)

[*] Symbols: -, none; nd, not determined.
[ɣ] Plasmid content varies depending on the strain.
[£] Data obtained from Zaluga et al. (2014).
[$] Data obtained from Gartemann et al. (2008).
[ǂ] Data obtained from Eichenlaub et al. (2011).
[¥] Data obtained from Bentley et al. (2008).

The hybridization values for C55^T:ZUM3936^T, C55^T:LMG 7333^T and ZUM3936^T:LMG 7333^T pairings were 69%, 73% and 70%, respectively. From these results, it can be concluded that strains C55^T and ZUM3936^T belong to the species *C. michiganensis*, as 70% DNA-DNA related~ness is generally accepted as the limit for species delineation (Tindall, et al., 2010; Wayne et al., 1987). DNA-DNA hybridization is not suitable for identification to the subspecies level within *C. michiganensis*, as high hybridization values are obtained amongst the different subspecies (unpublished data, personal communication from Dr. Van Trappen, BCCM/LMG, Belgium). Therefore, these strains were not tested against type strains of the other four known subspecies of *C. michiganensis*. Thus, while hybridization values suggest that these strains are not a new species of *Clavibacter*, it is still likely that these two strains comprise two new subspecies.

4.6 PCR, LAMP and phylogenetic analyses

PCR amplifications of the *clvA*, *clvF* and *clvG* genes produced bands of corresponding product size only for the tested *Cmm* strain (K73). All other strains tested were negative by PCR for all three genes. These results confirm previous findings (Yasuhara-Bell & Alvarez, 2012; Yasuhara-Bell, et al., 2013; Yasuhara-Bell, Marrero, et al., 2014) that the *clvA* (formerly *micA*) gene, and associated *clvF* and *clvG* genes are highly specific for *Cmm*. A LAMP assay was performed using previously reported primers (Yasuhara-Bell, et al., 2013) and a master mix containing a novel polymerase. Again, only *Cmm* reacted with the LAMP reaction. This also further reinforces the utility of the *clvA* LAMP (Yasuhara-Bell & Alvarez, 2012, 2014a; Yasuhara-Bell, et al., 2013) to discriminate all non-*Cmm*, including the most recently described "*Cmm*" from pepper that was isolated by Yim et al. (2012).

PCR amplifications of the *dnaA*, 16S rRNA, *atpD*, *dnaK*, *gyrB*, *ppK*, *recA* and *rpoB* genes of all tested strains produced bands corresponding to the correct product size for each respective primer pair. The products were sequenced and subjected to various forms of analysis. The 16S rRNA sequences from test strains C55^T and ZUM3936^T were input into NCBI BLAST and had 100% maximum identity to *Clavibacter michiganensis* strain LPPA 982 (Accession: HE608962.1). Even though 16S rRNA comparisons are only useful to the genus level, this genetic analysis confirms traditional characterization methods that suggested *Cmm*-like strains C55^T and ZUM3936^T do in fact belong to the *Clavibacter* genus.

Analyses of the *dnaA* gene combined data from data from previously published results by Yasuhara-Bell et al. (2013) and Zaluga et al. (2013) with new data acquired for strains tested in this study. The resulting MP tree (Figure 6.1) shows congruent topology to trees presented by Yasuhara-Bell et al. (2013) and Zaluga et al. (2013). *Cmm*-like strain AFLP 50 grouped with tomato seed-associated *Clavibacter* from California. These strains formed a separate clade that was closest to *Cmm.* Note that strain LMG 26808 grouped more closely with the tomato seed-associated *Clavibacter* from California than from the Netherlands. *Clavibacter* from pepper formed a separate clade and *Cmm*-like strain AFLP 121 was grouped closely. The pepper strain clade was close to another clade formed by tomato seed-associated *Clavibacter* from the Netherlands and the majority of the *Cmm*-like strains presented by Zaluga et al. (2013). NJ analysis produced consensus trees that were topologically congruent with that produced by MP analysis (Appendix B, Figure B.5).

MLSA analyses of concatenated housekeeping genes *atpD*, *dnaK*, *gyrB*, *ppK*, *recA* and *rpoB* combined data previously reported by Jacques et al. (2012) with data acquired in this study. MP analysis produced a tree with congruent topology to that produced previously (Jacques, et al., 2012) (Figure 6.2). NJ and ML analysis of concatenated sequences produced consensus trees with congruent topology to that produced by MP analysis, as well as to each other (Appendix B, Figure B.6) and to ML trees produced previously (Jacques, et al., 2012). Again, tomato seed-associated *Clavibacter* from California and from the Netherlands, as well as *Clavibacter* from pepper, formed clades separate from all known subspecies of *Clavibacter* and each other. The clade formed by tomato seed-associated *Clavibacter* from California was closest to *Cmm.* Tomato seed-associated *Clavibacter* from the Netherlands grouped with the majority of the *Cmm*-like strains presented by Jacques et al. (2012) to form its clade. *Clavibacter* from pepper formed a separate clade and *Cmm*-like strain CFBP7576 was grouped closely. Results of phylogenetic analyses show that strains C55^T, ZUM3936^T and PS005^T are not *Cmm* and are all individually unique subspecies.

4.7 Pathogenicity testing

The positive control strain of *Cmm* (K73) was the only strain tested that produced symptoms on tomato. None of the strains tested produced symptoms on any non-tomato host, even the pepper strain when inoculated into the two chili pepper cultivars. These results may simply reflect the mode of inoculation used in this study, as different pathogens vary in modes of invasion and infection. The lack of symptoms produced by the pepper strain on pepper may result from this, or perhaps the pepper strains are cultivar specific, thus defining a race. These pathogenicity tests show that these *Cmm*-like strains appear to be non-pathogens on the hosts tested, especially on tomato which they are found associated with in nature. More pathogenicity tests across a broader range of host plants would be helpful in determining if these strains are true non-pathogens, however such tests would be laborious as many different plant genera/species would need to be tested, with different routes of inoculation tested for each.

5. DISCUSSION

The results of this study, in combination with results from previous studies (Jacques, et al., 2012; Yasuhara-Bell, et al., 2013; Yim, et al., 2012; Zaluga, Van Vaerenbergh, et al., 2013), make it apparent that the *Clavibacter* strains from tomato seed ($C55^T$ and $ZUM3936^T$) and pepper are three separate subspecies that differ from the five well-described subspecies of *Clavibacter michiganensis*, and thus should be given individual taxonomic designations. Strains represented by strain $C55^T$ were all isolated from seed produced in California, therefore we propose *Clavibacter michiganensis* subsp. *californiensis* subsp. nov. for strains represented by strain $C55^T$ (=CFBP 8216^T). Strain $ZUM3936^T$ was isolated from seed produced in Chile, and strains represented by $ZUM3936^T$ were isolated from seed produced in Chile and India. As seed production areas moved from Chile to China and then India, it is likely that strains found in India originated from Chile. Therefore we propose *Clavibacter michiganensis* subsp. *chilensis* subsp. nov. for strains represented by $ZUM3936^T$ (=CFBP 8217^T). Strains of *Clavibacter* isolated from pepper, and represented by strain PS005, should also be given a separate subspecies designation, as evidence suggests that they are a new pathogenic subspecies. Further studies on these strains are currently underway in Korea (personal communication from Kyu-Ock Yim and Jae-Soon Cha). Strains $C55^T$ and $ZUM3936^T$ were isolated from tomato seed and may have arisen from *Cmm* (or vice versa) by loss or gain of genetic material and/or an adaptation to a more specific niche. On the other hand, pepper strain PS005 appears to be more closely related to *Cmt*. Further pathogenicity testing on a broader range of hosts, as well as whole-genome comparisons,

Figure 6.1. Maximum-parsimony (MP) tree based on *dnaA*. *dnaA* sequences were trimmed to 661 nt and multiple alignments were made using ClustalW, taking into account the corresponding amino acid alignments for protein-coding genes. MP analysis was used to construct trees with the closest neighbor interchange at search level 3. Confidence intervals were assessed using the bootstrap method with 1000 replications.

Figure 6.2. Multi-locus Sequence Analysis (MLSA) of concatenated *atpD, dnaK*, *gyrB, ppK*, *recA* and *rpoB* gene sequences. MP analysis was used to construct trees with the closest neighbor interchange at search level 3. Confidence intervals were assessed using the bootstrap method with 1000 replications. Bootstrap values of 50% or more are shown to the left of corresponding nodes.

will elucidate the lineage of these strains. Additionally, due to the fact that the tomato seed-associated strains look like *Cmm* in culture and react with immunological tests, further investigation of their role in nature is needed to determine whether these strains could cause disease in combination with other bacteria. This line of investigation is currently underway.

CHAPTER 7

NON-PATHOGENIC *CLAVIBACTER MICHIGANENSIS* STRAINS ASSOCIATED WITH TOMATO PLANTS AND SEED DO NOT ELICIT DISEASE DURING CO-INFECTION

1. ABSTRACT

Clavibacter michiganensis subsp. *michiganensis* causes bacterial wilt and canker of tomato (*Solanum lycopersicum*). Non-pathogenic *C. michiganensis* subsp. *michiganensis* and seed-associated *Clavibacter* are often isolated from tomato seed and plant tissues and react to the standard immunoassay. The role of these bacteria in nature is not well understood. In this study, co-inoculation studies were performed, using rifampicin-resistant mutants of two specific non-pathogenic *C. michiganensis* subsp. *michiganensis* and two different seed-associated *Clavibacter* strains, to determine the effects of these strains on colonization and symptom production during a co-infection, either by gene transfer (reversion to virulence) or synergy through complementation of secreted enzymes. Results revealed no genetic exchange of six pathogenicity-related genes (*ppaJ*, *pat*-1, *chpC*, *tomA*, *ppaA*, and *ppaC*) during co-infection. No significant changes in movement or colonization occurred for any of the strains tested when co-inoculated with any of the other three test strains. No wilt or canker symptoms were produced by any of the nonpathogenic strains, alone or in combination with the other test strains. Although further vigilance is always required, results of the current study suggest that these non-pathogenic *Clavibacter* populations, which represent four larger groups of non-pathogenic strains recovered in nature, pose no threat to plant and seed health.

2. INTRODUCTION

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*) is the causal agent of bacterial canker of tomato (*Solanum lycopersicum*) (Davis, et al., 1984; Strider, 1969) and pepper (*Capsicum annuum* and *Capsicum frutescens*) (Lai, 1976; Zutra & Cohn, 1970). It is one of the most important bacterial pathogens of tomato (Strider, 1969), causing substantial economic losses worldwide (EPPO, 2013; Strider, 1969). Contaminated seed is considered the major inoculum source leading to outbreaks of bacterial canker (EPPO, 2013; Strider, 1969). As a result, *C. michiganensis* subsp. *michiganensis* is classified as a quarantine organism in many countries, with cuttings and seeds of tomato requiring certification as *C. michiganensis* subsp. *michiganensis*-free.

The European Plant Protection Organization (EPPO) has released minimal standards for the identification of *C. michiganensis* subsp. *michiganensis* in plants and seeds (EPPO, 2013). Current testing regimens for this known quarantine pathogen mainly focus on detecting pathogenic species in an attempt to avoid outbreaks. However, multiple factors can create non-pathogenic strains. Most pathogenic *C. michiganensis* subsp. *michiganensis* strains harbor two circular plasmids pCM1 (27 kb) and pCM2 (70 kb), but plasmid content varies among *C. michiganensis* subsp. *michiganensis* isolates (Alvarez & Kaneshiro, 1999; Alvarez, et al., 2005; Kaneshiro, 2003; Kaneshiro & Alvarez, 2001; Kleitman, et al., 2008; Yasuhara-Bell, et al., 2013). Virulence plasmids can be lost by stress (e.g., growth at temperatures above 30°C), resulting in a reduced virulence phenotype, and curing of both plasmids results in a non-pathogenic phenotype that may attain *in planta* titers as high as virulent wild-type strains without eliciting symptoms (Meletzus, et al., 1993). Since any strain of *C. michiganensis* subsp. *michiganensis* can potentially become non-pathogenic through curing of the pathogenicity-associated plasmids, loss of plasmids in a natural *C. michiganensis* subsp. *michiganensis* population during high temperatures associated with tomato harvest has resulted in isolation of avirulent strains from tomato stems and seed (Alvarez, et al., 2005; Kaneshiro, 2003; Kleitman, et al., 2008; Yasuhara-Bell, et al., 2013).

Non-pathogenic strains of *C. michiganensis* subsp. *michiganensis* may possess the two plasmids containing virulence genes and yet be incapable of producing colonization titers sufficient to produce a pathogenic phenotype because they lack one or more genes located on a chromosomal putative pathogenicity island (PI) (Gartemann, et al., 2008; Kleitman, et al., 2008; Stork, et al., 2008; Yasuhara-Bell, et al., 2013). Such strains may provide a reservoir for virulence plasmids. Coexistence of these two populations with plasmid-free populations within the environment could theoretically produce one fully pathogenic strain through plasmid exchange. Kaneshiro (2003) looked for gene transfer between a pathogenic and non-pathogenic strain and found no exchange of pathogenicity-related genes (Kaneshiro, 2003). Currently, no other studies have been performed to determine whether two naturally occurring non-pathogenic strains can complement each other *in planta* without gene exchange.

Non-pathogenic strains that are culturally indistinguishable from *C. michiganensis* subsp. *michiganensis* have been isolated from tomato (Alvarez & Kaneshiro, 1999; Alvarez, et al., 2005; Jacques, et al., 2012; Kaneshiro, 2003; Zaluga, et al., 2014; Zaluga, Van Vaerenbergh, et al., 2013). These bacteria were Gram-positive, coryneform, pigmented yellow, and reacted with the Cmm-ImmunoStrip®, (Agdia® Inc.) (Jacques, et al., 2012; Yasuhara-Bell, et al., 2013; Zaluga, et al., 2011; Zaluga, Van Vaerenbergh, et al., 2013; Zinniel, et al., 2002; Zybailov et al., 2006). Two

phylogenetically distinct groups of non-pathogenic strains, which were isolated from tomato plants and seed, were recently compared with other *Clavibacter michiganensis* subspecies and classified as two new *Clavibacter* subspecies (Yasuhara-Bell & Alvarez, 2014b). A draft genome of a strain from one of these new taxa has been published (Zaluga, et al., 2014) and revealed the presence of some putative virulence factors that are present in *C. michiganensis* subsp. *michiganensis*; however, the role of these new non-pathogenic taxa within the environment has not yet been investigated.

The prevalence of non-pathogenic *C. michiganensis* subsp. *michiganensis*, as well as the *C. michiganensis*-like bacteria, on tomato seed and plants is of concern to the tomato seed industry because critical decisions with serious economic consequences must be made as to whether seed/plants harboring such strains should be sold or destroyed. In this study, non-pathogenic *C. michiganensis* subsp. *michiganensis* and *C. michiganensis*-like strains were tested for their potential to cause disease symptoms during co-infection. Rifampicin-resistance was used as a selectable marker for strain differentiation during co-inoculation experiments with one Rif^r strain and a different Rif^s strain.

3. MATERIALS AND METHODS

3.1 Bacterial strains and culture conditions

Two non-pathogenic *C. michiganensis*-like strains (C55 and ZUM3936) isolated from tomato seed and two non-pathogenic *Clavibacter michiganensis* subsp. *michiganensis* strains, each representing a distinct subpopulation lacking one or more virulence genes (A4775 [also called strain F293; pattern 6 = $chpC^-/tomA^-$] and A4818 [pattern 8 = $chpC^-/tomA^-/ppaA^-/ppaC^-$]), were described previously (Yasuhara-Bell, et al., 2013). A known pathogenic *C. michiganensis* subsp. *michiganensis* strain (A2058; also called strain K73 or H-160) was used as control. Bacteria were removed from -80°C, plated onto TZC-S medium (17 g/L agar, 10 g/L peptone, 5 g/L sucrose and 0.001% 2,3,5-triphenyl-tetrazolium chloride (TZC)) and then incubated at 26°C (± 2°C). Isolated colonies were streaked onto YSC medium (17 g/L agar, 10 g/L yeast extract, 20 g/L sucrose and 20 g/L $CaCO_3$) and incubated at 26°C (± 2°C) for large quantity production.

Rifampicin-resistant (Rif^r) mutants were created using previously established methods (Ruiz, Mensa, Pons, Vila, & Gascon, 2008; Weller & Saettler, 1978) as a guideline. Briefly, 10^9 cells were plated onto PS-Rif medium (17 g/L agar, 10 g/L peptone, 5 g/L sucrose, and 50 µg/ml rifampicin) and allowed to grow. Colonies were subcultured on PS-Rif with increasing concentrations of rifampicin (75 µg/ml, 100 µg/ml, 125 µg/ml and 150 µg/ml). Rif^r mutants were

restreaked onto YSC and subcultured at least 10 times before replating onto PS-Rif, in order to ensure a stable Rif^r mutation.

3.2 Characterization of Rif^r mutants

The *rpoB* gene was amplified and sequenced from both wild-type (WT; Rif^s) and Rif^r mutant strains. Sequences were compared to identify the position of the mutation that conferred Rif^r. Growth of Rif^r strains was compared to the wild-type strains (in vitro and *in planta*) to assess any changes in growth rate due to the Rif^r mutation. For in vitro growth, 10 µl of overnight cultures grown to approximately 0.1 OD_{A600} were inoculated into 200 µl of PS broth (10 g/L peptone, 5 g/L sucrose), in a 96-well plate. Rif^r strains were also grown in PS-Rif-Opus broth (10 g/L peptone, 5 g/L sucrose, 50 µg/ml rifampicin and 1 ml/L 5% Opus Top® [BASF, Research Triangle Park, NC]). Strains were allowed to grow for 72 h at 26°C (± 2°C) and OD_{A600} readings were taken every 2 h, following a one-min linear shake before reading, using a Synergy™ H1 Hybrid Multi-Mode Microplate Reader (BioTek®, Winooski, VT) with Gen5™ data analysis software (BioTek®, Winooski, VT). In vitro growth experiments were performed in replicates of four.

For *in planta* growth, plants were inoculated as described for the pathogenicity tests. Nine plants were used per strain, with three plants being sampled at 7 d intervals, for 21 d. Briefly, 1 cm sections were taken up to 7 cm above and 4 cm below the inoculation site. Samples were individually placed into separate 1.5 ml sterile microfuge tubes, weighed, dry-macerated using a sterile pestle, and then mixed with 1 ml sterile dH_2O. Plant debris was allowed to settle and the supernatant was removed to a new sterile microfuge tube for further testing. Extracts containing wild-type strains were dilution-plated onto selective BCT medium (Ftayeh, et al., 2011) (15 g/L agar, 2.5 g/L mannitol, 2 g/L yeast extract, 2 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 0.5 g/L NaCl, 0.1 g/L $MgSO_4 \cdot 7H_2O$, 0.015 g/L $MnSO_4 \cdot H_2O$, 0.015 g/L $FeSO_4 \cdot 7H_2O$, 0.6 g/L H_3BO_3, 20 mg/L nalidixic acid, 100 mg/L trimethoprim, 20 mg/L polymyxin B sulfate and 1 ml/L 5% Opus Top®), while extracts containing Rif^r strains were dilution-plated onto BCT and PS-Rif-Opus medium, using a Spiral Biotech Autoplate® 4000 (Advanced Instruments Inc., Norwood, MA), according to the manufacturer's instructions. Colonies were counted and used to calculate the CFU/g tissue. *In planta* growth experiments were performed three times.

3.3 DNA extraction

A Chelex® DNA extraction was performed on bacterial suspensions. Briefly, 0.75-1.0 ml of 40% Chelex® 100 resin (Bio-Rad, Hercules, CA) in 1X TE buffer (10 mM Tris HCl and 1 mM EDTA at pH 8) with 10% Triton™ X-100 (Sigma-Aldrich, St. Louis, MO) was added to each

tube. Samples were mixed by pipeting vigorously and vortexing and then heated to 95°C for 10 min on a digital heat block. Samples were stored at 4°C for at least 24 h to allow for separation of the DNA.

3.4 PCR

Genetic profiles, as described previously (Yasuhara-Bell, et al., 2013), were reestablished through PCR amplifications, using six primer pairs designed to amplify six different regions of the *C. michiganensis* subsp. *michiganensis* genome (1 region on each plasmid and 4 chromosomal regions). Two of the commercially available PCR primer pairs were designed to detect pathogenicity plasmids pCM1 (CM_3/CM_4 - *ppaJ*) (Sousa Santos, et al., 1997) and pCM2 (Cmm5/Cmm6 - *pat*-1) (Dreier, et al., 1995). Four primers sets were designed to amplify pathogenicity-related genes *tomA* (tomatinase A) and *chpC* (chromosomal homology of *pat*-1) (Kleitman, et al., 2008) and *ppaA* and *ppaC* (Yasuhara-Bell, et al., 2013), which are all contained within the putative PI of the chromosome. Primers were designed to amplify the RNA polymerase β subunit (*rpoB*) gene for sequence comparison. The primer sequences and PCR reaction conditions are listed in Table 7.1. PCR reactions for all primers were performed in a 10 µl reaction volume containing 1 µl bacterial DNA and 9 µl PCR reaction master mix [0.5 µl of each primer (10 µM), 5.0 µl JumpStart™ REDTaq® ReadyMix™ (Sigma-Aldrich, St. Louis, MO), and 3 µl ddH_2O].

3.5 DNA sequencing

PCR products of the *rpoB* amplifications were cleaned for sequencing using ExoSAP-IT® (Affymetrix®, Santa Clara, CA), according to the manufacturer's instructions. Cleaned PCR products were sequenced at the Greenwood Molecular Biology Facility at the University of Hawai'i, using the same forward and reverse primers (rpoB-F/R), according to specifications. New primers (rpoB-Fi/Ri) (Table 7.1) based on returned sequence data were used for primer walking in order to sequence the entire gene.

3.6 Sequence analyses

Sequence alignments were performed using MEGA5 version 5.05 (Tamura, et al., 2011). Sequence data for *Cmm* strain NCPPB 382 (Accession: AM711867.1) was obtained from NCBI GenBank. Multiple alignments were made using ClustalW, taking into account the corresponding amino acid alignments for protein-coding genes.

Table 7.1. Oligonucleotide primers and reaction conditions used in this study.

Primer	Sequence (5'-3')	Cycle Conditions (35 cycles of)[$]	Size (bp)	Source/Reference
PCR				
Cmm5*	GCGAATACGCCCATATCAA	94°C for 7 min, (94°C for 1 min, 55°C for 1 min, 72°C for 1 min), 72°C for 5 min	614	Dreier et al., 1995; Kokoskova et al., 2010
Cmm6	CGTCAGGAGGTCGCTAATA			
CM_3	CCTCGTGAGTGCCGGGAACGTATCC	94°C for 7 min, (94°C for 1 min, 60°C for 1 min, 72°C for 1 min), 72°C for 5 min	645	Sousa Santos et al., 1997
CM_4	CCACGGTGGTTGATGCTCGCGAGAT			
chpC-F	GCTCTTGGGCTAATGGCCG	94°C for 5 min, (94°C for 30 s, 55°C for 30 s, 72°C for 1 min), 72°C for 5 min	638	Kleitman et al., 2008
chpC-R	GTCAGTTGTGGAAGATGCTG			
tomA-F	CGAACTCGACCAGGTTCTCG		528	Kleitman et al., 2008
tomA-R	GGTCTCACGATCGGATCC			
ppaAF	CTGGTTCTGGTTTGCGGGC		496	Yasuhara-Bell et al., 2013
ppaAR	TGCTGCTGGTCCTCCTGGTA			
ppaCF	TGGTCGTCGTTGAGGAGGCA		140	Yasuhara-Bell et al., 2013
ppaCR	CTGTCGTCGGAGCGTCGGAT			
rpoB-F	CGTCGAGCAATTTTCCTTCTC	94°C for 5 min, (94°C for 1 min, 51°C for 45 s, 72°C for 2 min), 72°C for 10 min	3645	This Study
rpoB-R	GCGAGCAACTCCATCAGG			
Sequencing				
rpoB-FI	GATTGAGGATGACGTCGACC			This Study
rpoB-RI	CGAGCAGGTCGCCGCCGAGG			

[*]**C** indicates the change made by Kokoskova et al. (2010).
[$]Cycle conditions for chpC, tomA, ppaA and ppaC were developed in this study.

3.7 Nucleotide accession numbers

The *rpoB* sequences have been deposited into the GenBank Database with accession numbers KJ418430–KJ418439 for A2058 (K73), A2058Rif (K73Rif), A4775, A4775Rif, A4818, A4818Rif, C55, C55Rif, ZUM3936 and ZUM3936Rif, respectively.

3.8 Pathogenicity testing

A sterile scalpel was used to make a vertical incision (~1 mm) on the stem, between the cotyledons of juvenile tomato plants (*Lycopersicon esculentum* cv. 'Kewalo') that were approximately 3-4 weeks old. Twenty microliters of bacterial suspensions (~10^9 CFU/ml) were applied to the wound, using a pipette. Negative controls were made by applying 20 µl ddH_2O to the wound. Positive controls were made using the known pathogenic strain. Inoculated plants and controls were then carefully placed into plastic bags, closed using twist-ties, and incubated at 26°C (± 2°C) for 24 h, After the initial incubation, plants were removed from the bags and allowed to grow for 3-4 wks in a growth room (30°C) under Philips F40/AGRO Agro-Lite™ fluorescent lamps (Philips, NL). Plants were checked periodically for symptoms and pathogenicity was determined at 3-4 wks post-inoculation, relative to controls. Two plants were used per test for each strain, with the entire set of tests being repeated three times.

3.9 Complementation testing

For in vitro complementation, 100 µl of overnight cultures grown to approximately 0.1 OD_{A600} were mixed together in 2 ml PS medium without agar in a sterile test tube and allowed to grow for 48 h in a refrigerated incubator shaker at 26°C (± 2°C) and 160 rpm. Co-cultures were dilution-streaked onto PS-Rif medium at 24 and 48 h to select for the Rif^r strains. DNA from purified strains was tested with PCR, as described previously, to determine any changes to their genetic pathogenicity profiles.

For *in planta* complementation, a Rif^r strain was mixed 1:1 v/v with a Rif^s strain and inoculated into tomato plants, as described previously. Pathogenicity tests were performed, as described previously, to determine whether strains produced symptoms by gene exchange and/or synergy. Changes in strain colonization and movement were also monitored as described previously. Extracts were dilution-plated onto PS-Rif-Opus media. Reisolated strains were also subjected to PCR, as described previously, to determine any changes to their genetic pathogenicity profiles. The co-inoculation groups for in vitro and in vivo tests were as follows:
1) A4775Rif-A4818, 2) A4775Rif-C55, 3) 4775Rif-ZUM3936, 4) A4818Rif-A4775,
5) A4818Rif-C55, 6) A4818Rif-ZUM3936, 7) C55Rif-A4775, 8) C55Rif-A4818,
9) C55Rif-ZUM3936, 10) ZUM3936Rif-A4775, 11) ZUM3936Rif-A4818, and
12) ZUM3936Rif-C55.

4. Results

4.1 Rif^r mutant characterization

The Rif^r mutants contained varying mutations relative to the wild-type strains (Table 7.2). Full *rpoB* gene DNA and amino acid sequence alignments between rifampicin resistant strains of K73, A4775, A4818, C55 and ZUM3936 and their wild-type counterparts are presented in Appendix D, Figures D.7-D.11. To demonstrate that mutant strains were not significantly different from wild-type strains, genetic pathogenicity profiles were assessed using PCR. Results showed that the wild-type and mutants strains had identical patterns, for each respective pair of strains (Table 7.3). To further characterize the mutant strains, in vitro (Figure 7.1) and *in planta* (Table 7.4) growth studies were performed. Growth of all Rif^r mutants was comparable to wild-type strains in PS medium (Figure 7.1 A-E), with the exception of A4818Rif, which was slower in reaching log-phase growth, but reached a comparable final OD_{A600} (Figure 7.1C). When grown in PS-Rif-Opus medium, all Rif^r mutants showed a delay in achieving log-phase growth (Figure 7.1F); however once log-phase growth commenced, all strains showed similar growth patterns as when grown in PS medium, with A4818Rif showing the same phenomenon observed

in Figure 7.1C. Tomato plants were inoculated with individual strains and sampled every 7 d to follow *in planta* growth. All Rif^r mutants reached titers identical to their wild-type counterparts (Table 7.4). Plants were also observed for pathogenicity and only the known pathogenic strain, A2058 (K73), and its corresponding Rif^r mutant, A2058Rif (K73Rif), produced disease symptoms.

Table 7.2. *rpoB* gene sequence alignment data comparing rifampicin-resistant strains to wild-type strains.*

Strain	Position	Transition	Transversion	Amino Acid Change	Position
A2058Rif	1312 1313	C→T A→G	- -	H→C	438
A4775Rif	1313 2617	A→G C→T	- -	H→R -	438 -
A4818Rif	1274	-	A→T	Q→L	425
C55Rif	1312	C→T	-	H→Y	438
ZUM3936Rif	1312	C→T	-	H→Y	438

* - = not applicable.

Table 7.3. Characterization of test strains based on PCR and pathogenicity tests.*

Strain	Cmm5/6	$CM_{3/4}$	chpC	tomA	ppaA	ppaC	Pathogenicity	Pattern #
A2058 / A2058Rif	+	+	+	+	+	+	+	1
A4557 / A4775Rif	+	+	-	-	+	+	-	6
A4818 / A4818Rif	+	+	-	-	-	-	-	8
C55 / C55Rif	-	-	-	-	-	-	-	N/A
ZUM3936 / ZUM3936Rif	-	-	-	-	-	-	-	N/A

* Pattern numbering established previously (Yasuhara-Bell, et al., 2013); N/A = no assigned pattern number (not *Clavibacter michiganensis* subsp. *michiganensis*)

4.2 Strain complementation

The results of in vitro complementation studies suggest no apparent gene transfer between any strains after 48 h, as reisolated mutants had the same genetic pathogenicity profiles established previously by PCR analysis, and reconfirmed during individual mutant characterization (Table 7.3). *In planta* co-inoculation studies revealed no significant difference in movement within the plant, nor difference in titers reached when strains were inoculated alone or with another (Table 7.4). No disease symptoms were produced in co-inoculated plants by any combination of strains.

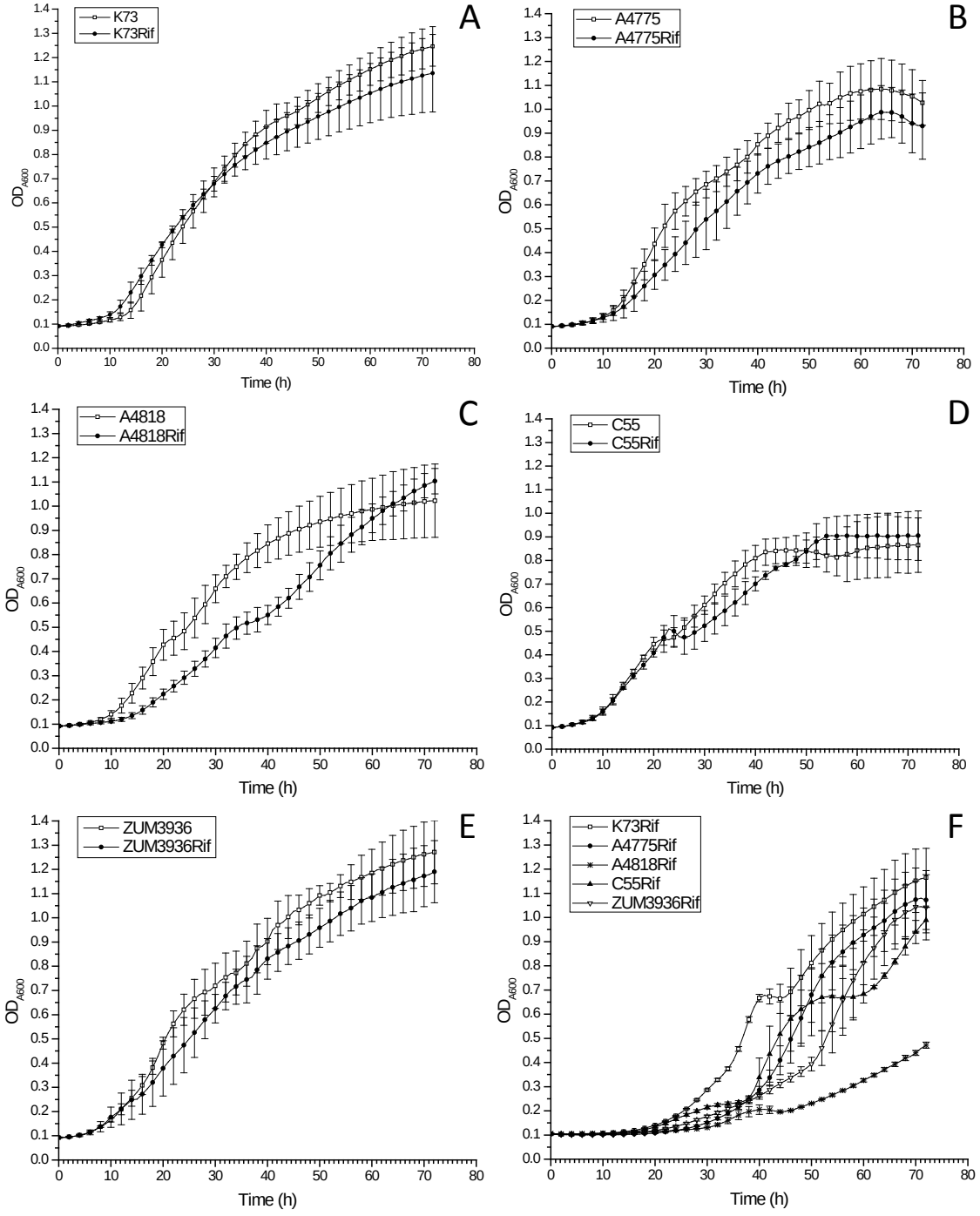


Figure. 7.1 In vitro bacterial growth comparison. Growth of wild-type strains was compared to Rif^r mutants in liquid culture. Briefly, 10 µl of overnight culture grown to OD_{A600} of 0.1 were inoculated into 200 µl of medium and OD_{A600} was monitored for 72 h, with readings taken every 15 min (only data points every 2 h were graphed for ease of view) and error bars represent the standard deviation. Graphs A-E show growth of wild-type compared to mutants of K73, A4775, A4818, C55 and ZUM3936 in PS media, respectively. Graph F shows the growth of all Rif^r mutants in PS-Rif-Opus media.

Table 7.4. *In planta* titers of individual and co-inoculated strains.*

Strain / Mixture	Max Dist* (cm)	Titer (CFU/g)
A2058 (K73)	7	10^9
A2058Rif (K73Rif)	7	10^9
A4775	7	10^3
A4775Rif	7	10^3
A4818	7	10^3
A4818Rif	7	10^3
C55	1	10^6
C55Rif	1	10^6
ZUM3936	7	10^2
ZUM3936Rif	7	10^2
A4775Rif and A4818	7	10^3
A4775Rif and C55	7	10^3
A4775Rif and ZUM3936	7	10^3
A4818Rif and A4775	7	10^3
A4818Rif and C55	7	10^3
A4818Rif and ZUM3936	7	10^3
C55Rif and A4775	1	10^5
C55Rif and A4818	1	10^5
C55Rif and ZUM3936	1	10^5
ZUM3936Rif and A4775	7	10^2
ZUM3936Rif and A4818	7	10^2
ZUM3936Rif and C55	7	10^2

* Results represent bacterial titers at the highest distance above the inoculation site that bacteria were isolated. In co-inoculations, titers are based on counts on the PS-Rif-Opus medium containing rifampicin.

5. Discussion

The role of non-pathogenic *C. michiganensis* subsp. *michiganensis* and seed-associated *C. michiganensis*-like bacteria in nature has been of concern after they were shown to be widely prevalent on tomato seed and plant tissues (Alvarez & Kaneshiro, 1999; Alvarez, et al., 2005; Kaneshiro, 2003). While the nature of the association between these strains and tomatoes remains unclear, results of the current study showed that strains representing two distinct populations of non-pathogenic *C. michiganensis* subsp. *michiganensis*, comprising patterns 6 and 8 described previously (Yasuhara-Bell, et al., 2013), did not complement each other or show increased

colonization during co-infection. Furthermore, two different seed-associated *Clavibacter* strains (ZUM3936 and C55), representing *C. michiganensis* subsp. *chilensis* subsp. nov. and *C. michiganensis* subsp. *californiensis* subsp. nov. (Yasuhara-Bell & Alvarez, 2014b), respectively, produced no symptoms during co-infection with each other or with non-pathogenic strains of *C. michiganensis* subsp. *michiganensis* (44). Previous studies demonstrated that non-pathogenic strains colonize plants without causing symptoms and co-inoculations between pathogenic and non-pathogenic strains did not result in measurable gene transfer (Kaneshiro, 2003). However, the genetic profiles needed for detailed characterization of strains were not available during these earlier studies and multiple possibilities for gene transfer could not be examined, hence the necessity for a different approach. Additionally, differentiation of co-inoculated strains was based on the presence or absence of exopolysaccharide. In the current study, rifampicin-resistance, rather than presence or absence of surface antigens, was used to distinguish between two types of bacteria as they moved up the vascular tissues in co-inoculations.

Rifampicin-resistance was used as a selectable marker for strain differentiation during co-inoculation experiments with one Rif^r strain and a different Rif^s strain. Among the antibiotics that inhibit the function of RNA polymerase (Severinov, Soushko, Goldfarb, & Nikiforov, 1993; M. Xu, Zhou, Goldstein, & Jin, 2005), rifampicin, a derivative of rifamycin (Kunin, Brandt, & Wood, 1969; Riva & Silvestri, 1972; Sensi, Timbal, & Maffii, 1960; Wehrli, Handschin, & Wunderli, 1976; Wehrli & Staehelin, 1971), it is the most important in clinical use (Riva & Silvestri, 1972). Rifampicin binds to RNA polymerase with high affinity (Wehrli, et al., 1976). Rif^r mutations have been attributed to mutations in the RNA polymerase β subunit gene (*rpoB*) (Ezekiel & Hutchins, 1968; Jin & Gross, 1988; Lisitsyn, Sverdlov, Moiseyeva, Danilevskaya, & Nikiforov, 1984; Ovchinnikov et al., 1983; Rabussay & Zillig, 1969; Severinov, et al., 1993; Singer, Jin, Walter, & Gross, 1993; M. Xu, et al., 2005). Spontaneous rifampicin-resistance has been shown to occur in many bacteria. The mode of action of rifampicin has been studied in detail, using *Escherichia coli* RNA polymerase as the model system (M. Xu, et al., 2005). Rifampicin-resistance has been used as a selectable marker to aid in recovering a variety plant pathogens from environmental samples, including *Xanthomonas phaseoli* and *Xanthomonas phaseoli* var. *fuscans* (Weller & Saettler, 1978), *Agrobacterium tumefaciens* (Anderson & Moore, 1976; Moore, 1977) and *C. michiganensis* subsp. *michiganensis* (R. S. Chang, S. M. Ries, & J. K. Pataky, 1992; Gleason, Braun, Carlton, & Peterson, 1991; Moffett & Wood, 1984).

The *rpoB* gene in *E. coli* encodes for a 1342 amino acid peptide. Except for a mutation located around the 5' end, most of the Rif^r mutations in *E. coli* are found in three clusters near the middle of the *rpoB* gene (cluster I, cluster II and cluster III, encompassing amino acids residues 505-532, 560-572 and 687, respectively) and affect a limited amino acid segment of the β subunit (Jin & Gross, 1988; Lisitsyn, et al., 1984; Ovchinnikov, et al., 1983; Severinov, et al., 1993; Singer, et al., 1993). The RNA polymerase β subunit of *Clavibacter* spp. is 1163 amino acids. In order to determine the nature of the Rif^r mutation in the strains selected for this study, the *rpoB* genes of the wild-type and mutant strains were sequenced and compared. Data suggest that a histidine at position 438 plays an important role in rifampicin susceptibility, as 4/5 rifampicin-resistant mutants showed amino acid changes at this position. One strain had an amino acid change at the glutamine residue at position 425, demonstrating this residue's importance for rifampicin susceptibility, though this mutation appears to be uncommon.

After determining the nature of the mutations conferring resistance to rifampicin in the strains selected for this study, tests were performed to show that these mutant strains were sufficiently similar to the wild-type for any data collected to be meaningful. Genetic profiles based on PCR of six pathogenicity-associated genes were identical in the wild-type and mutant strains. In vitro analyses were performed to show that Rif^r mutations did not significantly alter growth kinetics. Notably, A4818Rif, which was the only mutant to have an amino acid change at a position other than 438, was the only strain to differ from the wild-type (Figure 7.1). The mutant entered log-phase growth five hours later than the wild-type, but after 55 to 70 h, the cell yield was not significantly different from the wild type (Figure 7.1C). All mutant strains showed a 10 to 15-h extended lag phase when grown in PS-Rif-Opus (Figure 7.1F), most likely due to the presence of rifampicin in the media. However, after 40 hours, when nearly all strains had entered log phase, growth rates and final yields were not significantly different from the control A2058Rif (K73Rif) on PS-RIF-Opus, except for strain A4818, which again, grew significantly slower. The in vitro growth studies showed that the mutant strains could be used to represent the wild-type strains in plant co-inoculation studies.

To further justify the use of these strains, *in planta* growth studies were performed using plants inoculated with individual strains. Results revealed that all Rif^r mutants colonized the stems and attained similar titers at the highest point from which the strains were reisolated (Table 7.4). The three true *C. michiganensis* subsp. *michiganensis* strains, along with *Clavibacter michiganensis*-like strain ZUM3936, were detected 7 cm above the inoculation site. Strain A2058 reached titers (~10^9 CFU/g tissue) typical for pathogenic *C. michiganensis* subsp. *michiganensis* (Meletzus, et al., 1993). Both of the non-pathogenic *C. michiganensis* subsp. *michiganensis*

strains reached titers of ~10^3 CFU/g tissue, far lower than those observed for the pathogenic control. Seed-associated *Clavibacter* strain ZUM3936 moved through the plant, but reached titers (~10^2 CFU/g tissue) below even non-pathogenic strains of *C. michiganensis* subsp. *michiganensis*. Seed-associated *Clavibacter* strain C55, though reaching titers ~10^6 CFU/ml, did not colonize the tomato vasculature, being found only up to 1 cm above the inoculation site.

Non-pathogenic *C. michiganensis* subsp. *michiganensis* and seed-associated *Clavibacter* populations have been consistently isolated from widely separated global origins. The significance of these strains was examined by assessing their potential to cause disease, individually and during co-inoculations. No significant changes in movement or colonization titer between strains inoculated individually and when inoculated in any combination of the four test strains (Table 7.4). Movement and colonization of individual and co-inoculated strains were to be quantified over time; however, many factors causing inconsistencies in the data were present, including: varying plant growth rates, having to use a new plant for each sampling, ensuring that each plant retained the same amount of inoculum, macerating tissues enough to extract essentially all bacteria, having non-revertant bacteria for reisolation, and proper spiral-plating for successful colony counts. Therefore, accumulated data were thoroughly analyzed and only endpoint data was presented (Table 7.4). No differences were found at endpoint and ultimately no disease symptoms were observed in any plant, for any combination of strains. In order to properly quantify movement and colonization, future studies could utilize a *lux* (X. Xu, et al., 2010) or GFP (Chalupowicz, et al., 2012) reporter, which would allow monitoring of the same plant over time. In this study, no transfer of tested pathogenicity-associated genes was expected, and experimental results confirmed this in vitro and *in planta*. The fact that no differences were observed during co-inoculations, including lack of disease symptoms, suggests that these strains do not complement each other in a synergistic capacity, and together do not possess the complete set of secretory enzymes required to produce disease.

A recently developed LAMP assay for *C. michiganensis* subsp. *michiganensis* (Yasuhara-Bell & Alvarez, 2014a; Yasuhara-Bell, et al., 2013) provides specific detection and eliminates false positive reactions with seed-associated *Clavibacter* strains. False-positive reactions with the ImmunoStrip® and *Ochrobactrum*, *Microbacterium*, and non-pathogenic "*Clavibacter*-like" strains is due to these bacteria sharing common epitopes (or antigenic determinants) in their exopolysaccharide. The LAMP reaction eliminates these strains because they do not share conserved *clv* genes that define *C. michiganensis* subsp. *michiganensis*, as reported previously (Yasuhara-Bell, Marrero, et al., 2014). The acquisition of a pathogenicity profile by PCR, described here and previously (Yasuhara-Bell, et al., 2013), will provide critical information. As

shown in this study, strains found to belong to patterns 6 and 8 pose no potential threat, as they are incapable of producing disease. The LAMP detection assay, combined with the information provided in this study, will allow companies to make critical decisions on seed/plant health by disregarding non-*C. michiganensis* subsp. *michiganensis* strains and giving them information to use as a guide when non-pathogenic *C. michiganensis* subsp. *michiganensis* strains have been detected.

CHAPTER 8
CONCLUSIONS

Standard field tests for detecting *Cmm* is laborious and time consuming. In addition, current detection methods commonly produce both false positive and false negative results. This study determined that the clavicidin gene cluster, comprising genes *clvA, clvE, clvF, clvG, clvK, clvM* and *clvR,* was unique to and conserved in *Clavibacter michiganensis* subsp. *michiganensis.* Molecular-based assays targeting any or all of these genes will provide highly specific detection systems for accurate identification of *Cmm.* These genes have the potential to be the new focus of molecular-based detection of *Cmm*, having application to all molecular-based diagnostic platforms.

In this study, a LAMP reaction was designed to amplify the *clvA* region of *Cmm* and proved to be a useful diagnostic, being able to detect *Cmm* strains, regardless of pathogenicity, as well as discriminate between *Clavibacter* subspecies. The *clvA* LAMP was able to detect *Cmm* on tomato seed and from infected tomato tissue, with and without an enrichment step. The LAMP reaction has advantages over conventional detection methods because of its specificity, isothermal nature, and the fact that it is not affected by as many inhibitors as is polymerases used for PCR. In conjunction with portable real-time fluorescence monitoring devices, this study using SMART-DART™ technology specifically, brings the the specificity of nucleic acid-based technologies to the field.

The *clvA* LAMP described in this study has additional advantages because it detects both pathogenic and non-pathogenic *Cmm*. Non-virulent strains cannot be simply disregarded during routine field screenings because cohabitation of tomato seed or plant with two non-virulent strains, one being a plasmid-free strain and the other possessing the virulence plasmids but missing *chpC, tomA, ppaA* and/or *ppaC*, presents a potentially dangerous situation, with possible disastrous consequences. Current tests designed to detect virulence genes may miss potentially devastating populations.

This study also represents the largest study of *Cmm* populations, with strains coming from various regions throughout the world. Included in this study were seed-associated *Clavibacter* strains. Two different populations of seed-associated *Clavibacter* were characterized and found to comprise two new non-pathogenic *Clavibacter* subspecies, with proposed names of *Clavibacter michiganensis* subsp. *chilensis* subsp. nov. and *Clavibacter michiganensis* subsp. *californiensis* subsp. nov. These strains are often found associated with tomato tissue and seed, and cross react with the standard Immunostrip® test, causing false positives and confusion as to

potential seed health. Fortunately, the LAMP detection system presented here can discriminate between these *Clavibacter* subspecies. However, the role of these strains in nature has been contemplated for some time. The fact that this LAMP test would screen out these strains, as well as the fact that it detects non-pathogenic *Cmm*, could pose potential problems if these strains were capable of causing disease during co-infection, either by gene exchange or by complementation of secretory enzymes required for disease. Thus, this study performed co-inoculation studies between non-pathogenic *Cmm* strains and these seed-associated strains. Results showed that non-pathogenic strains of *Cmm* that fall into patterns 6 and 8, as established in this study, along with these two new non-pathogenic species of *Clavibacter*, are unable to produces disease in any combination with other strains, and therefore should be considered harmless.

The sum of results in this study leads to a proposal for a new testing regimen for tomato plant and seed (Figure 8.1). Both plant tissue samples and seed samples can be processed as normal. Once a liquid suspension containing potential bacterial threats is attained, performing a simple rapid test via the Immunostrip® and *Cmm*-LAMP will be the first step. Together, the Immunostrip® and *Cmm*-LAMP tests possess the necessary sensitivity and specificity to determine whether or not *Cmm* is present in a sample. A negative result from both the Immunostrip® and *Cmm*-LAMP tests would indicate that samples are *Cmm*-free and therefore safe for sale/export. If only one test is positive, it is likely to be the Immunostrip® test, as it is known to produce cross-reactions with non-target bacteria. Another possibility is the presence of inhibitors within the sample, and/or bacterial titers below the LAMP detection threshold. Samples showing only one positive test should be retested, taking into account the aforementioned possibilities during sample preparation. Positive results from both tests would indicate *Cmm* contamination and samples should be cultured in an attempt to rercover *Cmm* colonies. The *Cmm*-LAMP can be used again to identify recovered *Cmm* colonies.Once *Cmm* is identified, PCR profiling can be performed using the protocol described in this study, and strains falling into patterns 6 and 8 can be considered safe and pass through inspection. Strains found to belong to patterns 1-5 and 7 should be considered a potential threat and dealt with accordingly. If preferred, *in planta* pathogenicity testrs can be performed. Any strain showing pathogenicity should obviously be considered a threat and dealt with accordingly, and any strain found to be non-pathogenic should be screened via PCR profiling to determine any possibility of reversion to pathogenicity, again with strains falling into patterns 6 and 8 being considered safe, while strains belonging to patterns 1-5 and 7 being considered a potential threat. This new testing scheme will save both time and money. Aside from being able to directly assay samlpes on-site, the elimination of a culturing step during presumptive identification allows results to be gathered

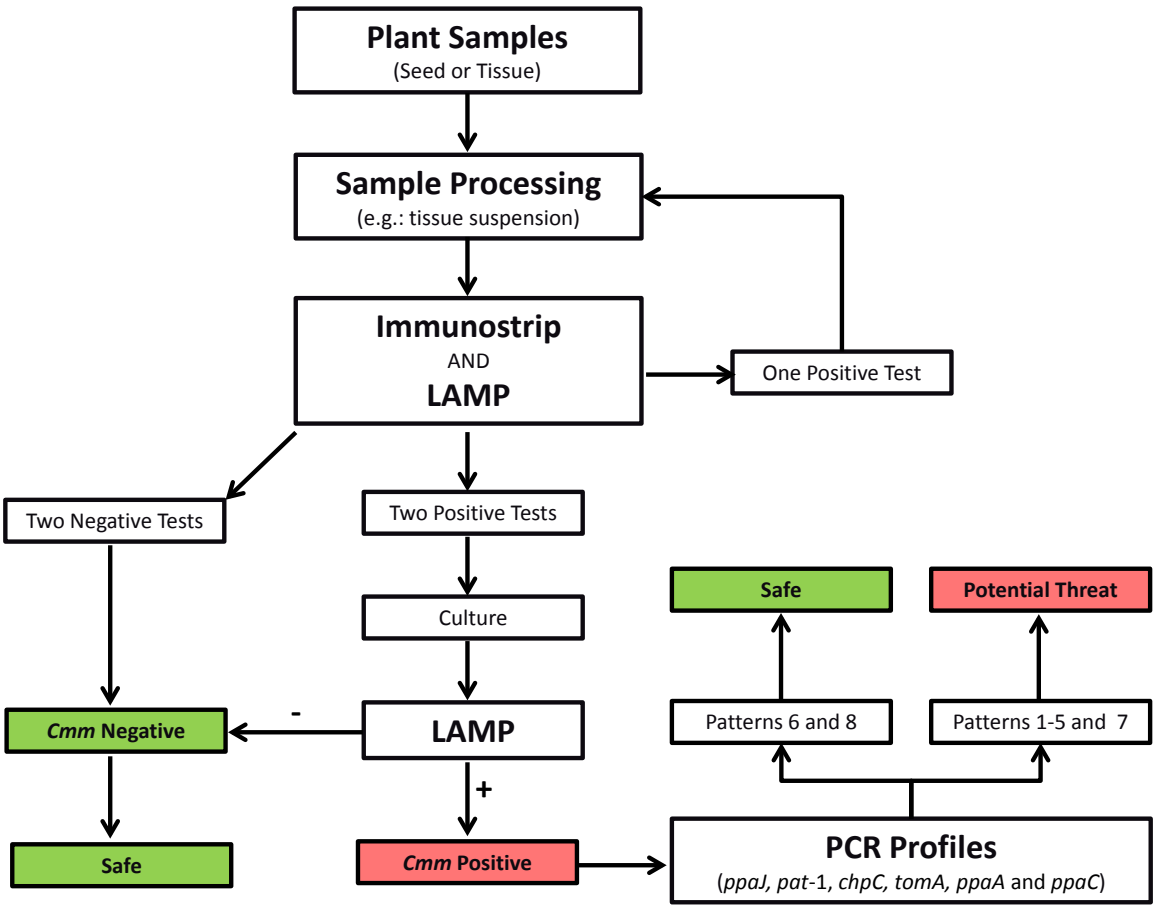


Figure 8.1. Proposed scheme for detection and identification of *Clavibacter michiganensis* subsp. m*ichiganensis* in samples from tomato plants and seed.

immediately, ensuring critical decisions regarding plant help can be made in a timely manner. Additionally, this new testing regimen circumvents the need for standard pathogenicity testing, which is both costly and time consuming.

This dissertation reveals a new diagnostic test for specifically detecting *Clavibacter michiganensis* subsp. *michiganensis*, the cause of bacterial canker and wilt of tomato, and shows that the *clv* gene cluster can be targets for all forms of molecular-based diagnostic approaches. Future studies could encorporate screening of more non-target bacteria and extensive testing in the field. Additionally, for use as a stand-alone commercial test, multiplexing with an internal control would be required to ensure accurate results. However, loss of sensitivity has been reported for multiplexed LAMP reactions (Kanagawa, 2003; Polz & Cavanaugh, 1998); therefore redesign or further optimization of primers may be required. Perhaps one day this research will be the basis leading to the production of the gold standard for detection of *Clavibacter michiganensis* subsp. *michiganensis.*

APPENDIX A: PCR and LAMP Data.

Table A.1. Data for all *Clavibacter michiganensis* subsp. *michiganensis* strains tested.

Strain	Other ID	Origin	Pattern #	dnaA	Cmm5/6	$CM_{3/4}$	chpC	tomA	ppaA	ppaC	LAMP
A4810		MI, USA	1	+	+	+	+	+	+	+	+
A4825		MI, USA	1	+	+	+	+	+	+	+	+
A4827		OH, USA	1	+	+	+	+	+	+	+	+
C117		NL	1	+	+	+	+	+	+	+	+
C131-A		NL	1	+	+	+	+	+	+	+	+
C202		OH, USA	1	+	+	+	+	+	+	+	+
C203		OH, USA	1	+	+	+	+	+	+	+	+
C204		OH, USA	1	+	+	+	+	+	+	+	+
C205		OH, USA	1	+	+	+	+	+	+	+	+
C206		OH, USA	1	+	+	+	+	+	+	+	+
C207		OH, USA	1	+	+	+	+	+	+	+	+
C219		OH, USA	1	+	+	+	+	+	+	+	+
C220		OR, USA	1	+	+	+	+	+	+	+	+
C221		OR, USA	1	+	+	+	+	+	+	+	+
C224		OR, USA	1	+	+	+	+	+	+	+	+
C225		OR, USA	1	+	+	+	+	+	+	+	+
C226		OR, USA	1	+	+	+	+	+	+	+	+
K073*	H-160	ID, USA	1	+	+	+	+	+	+	+	+
K074*	N212	China	1	+	+	+	+	+	+	+	+
K075*	N7388A	Morocco	1	+	+	+	+	+	+	+	+
K081*	C222	OR, USA	1	+	+	+	+	+	+	+	+
K094*	71169	China	1	+	+	+	+	+	+	+	+
K387*	CM95	OH, USA	1	+	+	+	+	+	+	+	+
K389*	CM98	OH, USA	1	+	+	+	+	+	+	+	+
K390*	CM99	OH, USA	1	+	+	+	+	+	+	+	+
K392*	CM5	OH, USA	1	+	+	+	+	+	+	+	+
K398*	S53	CA, USA	1	+	+	+	+	+	+	+	+
K399*	CM36	NC, USA	1	+	+	+	+	+	+	+	+
K400*	CM33	NC, USA	1	+	+	+	+	+	+	+	+
K406*	1(A)	OH, USA	1	+	+	+	+	+	+	+	+
K407*	8(A-B)	OH, USA	1	+	+	+	+	+	+	+	+
K408*	17	OH, USA	1	+	+	+	+	+	+	+	+
K409*	75	OH, USA	1	+	+	+	+	+	+	+	+
K411*	57	OH, USA	1	+	+	+	+	+	+	+	+
K412*	73	OH, USA	1	+	+	+	+	+	+	+	+
K414*	34C	OH, USA	1	+	+	+	+	+	+	+	+
K415*	7	OH, USA	1	+	+	+	+	+	+	+	+
K416*	24	OH, USA	1	+	+	+	+	+	+	+	+
K417*	9	OH, USA	1	+	+	+	+	+	+	+	+
K418*	29	OH, USA	1	+	+	+	+	+	+	+	+
K419*	41	OH, USA	1	+	+	+	+	+	+	+	+
K420*	4	OH, USA	1	+	+	+	+	+	+	+	+
K421*	2(C)	OH, USA	1	+	+	+	+	+	+	+	+
K423*	5	OH, USA	1	+	+	+	+	+	+	+	+
K424*	19	OH, USA	1	+	+	+	+	+	+	+	+
K425*	10	OH, USA	1	+	+	+	+	+	+	+	+
K426*	27	OH, USA	1	+	+	+	+	+	+	+	+
K427*	20	OH, USA	1	+	+	+	+	+	+	+	+
K428*	6(A)	OH, USA	1	+	+	+	+	+	+	+	+
K429*	38	OH, USA	1	+	+	+	+	+	+	+	+
K430*	22	OH, USA	1	+	+	+	+	+	+	+	+
K431*	12	OH, USA	1	+	+	+	+	+	+	+	+
K433*	33	OH, USA	1	+	+	+	+	+	+	+	+

Strain	Other ID	Origin	Pattern #	dnaA	Cmm5/6	$CM_{3/4}$	chpC	tomA	ppaA	ppaC	LAMP
K434*	28	OH, USA	1	+	+	+	+	+	+	+	+
K435*	74	OH, USA	1	+	+	+	+	+	+	+	+
K436*	16	OH, USA	1	+	+	+	+	+	+	+	+
K438*	44	OH, USA	1	+	+	+	+	+	+	+	+
K439*	DR59	OH, USA	1	+	+	+	+	+	+	+	+
K441*	11	OH, USA	1	+	+	+	+	+	+	+	+
K442*	35	OH, USA	1	+	+	+	+	+	+	+	+
K443*	45	OH, USA	1	+	+	+	+	+	+	+	+
K444*	25	OH, USA	1	+	+	+	+	+	+	+	+
K445*	69	OH, USA	1	+	+	+	+	+	+	+	+
K446*	6(C)	OH, USA	1	+	+	+	+	+	+	+	+
K447*	1(H)	OH, USA	1	+	+	+	+	+	+	+	+
K450*	34(E)	OH, USA	1	+	+	+	+	+	+	+	+
K451*	36	OH, USA	1	+	+	+	+	+	+	+	+
K452*	8(C)	OH, USA	1	+	+	+	+	+	+	+	+
K456*	2(A)	OH, USA	1	+	+	+	+	+	+	+	+
K457*		OH, USA	1	+	+	+	+	+	+	+	+
K459*		OH, USA	1	+	+	+	+	+	+	+	+
K464*	cmm055	WA, USA	1	+	+	+	+	+	+	+	+
K470*	IPO542	Italy	1	+	+	+	+	+	+	+	+
K473*	IPO1799	Chile	1	+	+	+	+	+	+	+	+
K474*	N197	CA, USA	1	+	+	+	+	+	+	+	+
K476*	N202A	Chile	1	+	+	+	+	+	+	+	+
K477*	N202B	Chile	1	+	+	+	+	+	+	+	+
K478*	N211	Chile	1	+	+	+	+	+	+	+	+
K480*	N713P	Chile	1	+	+	+	+	+	+	+	+
T017	09-135A#1	FL, USA	1	+	+	+	+	+	+	+	+
T018	09-135A#2	FL, USA	1	+	+	+	+	+	+	+	+
T020	09-135D	FL, USA	1	+	+	+	+	+	+	+	+
T021	09-157B	TX, USA	1	+	+	+	+	+	+	+	+
T022	09-158A	TX, USA	1	+	+	+	+	+	+	+	+
T024	09-159A	TX, USA	1	+	+	+	+	+	+	+	+
T025	09-159B	TX, USA	1	+	+	+	+	+	+	+	+
T026	09-159C	TX, USA	1	+	+	+	+	+	+	+	+
T027	09-165A	TX, USA	1	+	+	+	+	+	+	+	+
T028	09-135C	FL, USA	1	+	+	+	+	+	+	+	+
T029	09-157A	TX, USA	1	+	+	+	+	+	+	+	+
T030	09-157C	TX, USA	1	+	+	+	+	+	+	+	+
T031	09-158C	TX, USA	1	+	+	+	+	+	+	+	+
T032	09-166A	TX, USA	1	+	+	+	+	+	+	+	+
T033	09-177A	TX, USA	1	+	+	+	+	+	+	+	+
T034	09-177B	TX, USA	1	+	+	+	+	+	+	+	+
T035	09-177C	TX, USA	1	+	+	+	+	+	+	+	+
T036	09-190A	TX, USA	1	+	+	+	+	+	+	+	+
T037	09-190B	TX, USA	1	+	+	+	+	+	+	+	+
T038	09-190C	TX, USA	1	+	+	+	+	+	+	+	+
T039	09-195A	TX, USA	1	+	+	+	+	+	+	+	+
T040	09-170A	VA, USA	1	+	+	+	+	+	+	+	+
T041	09-170B	VA, USA	1	+	+	+	+	+	+	+	+
T042	09-170C	VA, USA	1	+	+	+	+	+	+	+	+
T043	09-176A	TX, USA	1	+	+	+	+	+	+	+	+
T044	09-176B	TX, USA	1	+	+	+	+	+	+	+	+
T045	09-176C	TX, USA	1	+	+	+	+	+	+	+	+
T046	09-192A	BC	1	-	+	+	+	+	+	+	+

Strain	Other ID	Origin	Pattern #	dnaA	Cmm5/6	$CM_{3/4}$	chpC	tomA	ppaA	ppaC	LAMP
T047	09-192B	BC	1	+	+	+	+	+	+	+	+
T048	09-192C	BC	1	+	+	+	+	+	+	+	+
T049	09-197A	TX, USA	1	+	+	+	+	+	+	+	+
T050	09-197B	TX, USA	1	+	+	+	+	+	+	+	+
T051	09-197C	TX, USA	1	+	+	+	+	+	+	+	+
T052	09-239A	FL, USA	1	+	+	+	+	+	+	+	+
T053	09-241A	FL, USA	1	+	+	+	+	+	+	+	+
T054	09-199A	TX, USA	1	+	+	+	+	+	+	+	+
T055	09-198A	TX, USA	1	+	+	+	+	+	+	+	+
T063	06-VA1	VA, USA	1	+	+	+	+	+	+	+	+
T064	06-CDN1	Canada	1	+	+	+	+	+	+	+	+
T065	06-MX4	Mexico	1	+	+	+	+	+	+	+	+
T066	06-NE1	NE, USA	1	+	+	+	+	+	+	+	+
T067	06-TX1	TX, USA	1	+	+	+	+	+	+	+	+
T068	06-TX2	TX, USA	1	+	+	+	+	+	+	+	+
T069	06-TX3	TX, USA	1	+	+	+	+	+	+	+	+
T070	06-AZ2	AZ, USA	1	+	+	+	+	+	+	+	+
T071	06-MX2	Mexico	1	+	+	+	+	+	+	+	+
T072	07-MX2	Mexico	1	+	+	+	+	+	+	+	+
T073	07-MX1	Mexico	1	+	+	+	+	+	+	+	+
T074	07-MX3	Mexico	1	+	+	+	+	+	+	+	+
T075	07-TX1	TX, USA	1	+	+	+	+	+	+	+	+
T076	07-TX2	TX, USA	1	+	+	+	+	+	+	+	+
T077	07-TX3	TX, USA	1	+	+	+	+	+	+	+	+
T078	07-TX4	TX, USA	1	+	+	+	+	+	+	+	+
T079	07-MX4	Mexico	1	+	+	+	+	+	+	+	+
T080	07-MX5	Mexico	1	+	+	+	+	+	+	+	+
T081	07-MX6	Mexico	1	+	+	+	+	+	+	+	+
T082	07-MX7	Mexico	1	+	+	+	+	+	+	+	+
T083	07-MX8	Mexico	1	+	+	+	+	+	+	+	+
T084	07-MX9	Mexico	1	+	+	+	+	+	+	+	+
T087	07-TX6	TX, USA	1	+	+	+	+	+	+	+	+
T088	07TX-7	TX, USA	1	+	+	+	+	+	+	+	+
T089	07TX-8	TX, USA	1	+	+	+	+	+	+	+	+
T090	07TX-9	TX, USA	1	+	+	+	+	+	+	+	+
T091	07-MX11	Mexico	1	+	+	+	+	+	+	+	+
T093	07-MX13	Mexico	1	+	+	+	+	+	+	+	+
T094	07-MX14	Mexico	1	+	+	+	+	+	+	+	+
T097	07TX-10	TX, USA	1	+	+	+	+	+	+	+	+
T098	07TX-11	TX, USA	1	+	+	+	+	+	+	+	+
T099	07TX-12	TX, USA	1	+	+	+	+	+	+	+	+
T100	07TX-13	TX, USA	1	+	+	+	+	+	+	+	+
T101	07TX-14	TX, USA	1	+	+	+	+	+	+	+	+
T102	07TX-15	TX, USA	1	+	+	+	+	+	+	+	+
T103	07TX-16	TX, USA	1	+	+	+	+	+	+	+	+
T104	07TX-17	TX, USA	1	+	+	+	+	+	+	+	+
T105	07-CDN1	Canada	1	+	+	+	+	+	+	+	+
T106	07TX-18	TX, USA	1	+	+	+	+	+	+	+	+
T107	07TX-19	TX, USA	1	+	+	+	+	+	+	+	+
T108	07TX-20	TX, USA	1	+	+	+	+	+	+	+	+
T109	07TX-21	TX, USA	1	+	+	+	+	+	+	+	+
T110	07TX-22	TX, USA	1	+	+	+	+	+	+	+	+
T111	07-MI1	MI, USA	1	+	+	+	+	+	+	+	+
T112	07-OH1	OH, USA	1	+	+	+	+	+	+	+	+
T113	07-PA1	PA, USA	1	+	+	+	+	+	+	+	+
T114	07-OH2	OH, USA	1	+	+	+	+	+	+	+	+
T115	07-MI2	MI, USA	1	+	+	+	+	+	+	+	+
T116	07-MI3	MI, USA	1	+	+	+	+	+	+	+	+
T117	07-MI4	MI, USA	1	+	+	+	+	+	+	+	+
T121	07-MI6	MI, USA	1	+	+	+	+	+	+	+	+
T122	07-MI7	MI, USA	1	+	+	+	+	+	+	+	+

Strain	Other ID	Origin	Pattern #	dnaA	Cmm5/6	$CM_{3/4}$	chpC	tomA	ppaA	ppaC	LAMP
T124	07-MN1	MN, USA	1	+	+	+	+	+	+	+	+
T125	07-MN2	MN, USA	1	+	+	+	+	+	+	+	+
T127	07-MN4	MN, USA	1	+	+	+	+	+	+	+	+
T130	07TX-23	TX, USA	1	+	+	+	+	+	+	+	+
T131	07TX-24	TX, USA	1	+	+	+	+	+	+	+	+
T133	08-MX2	Mexico	1	+	+	+	+	+	+	+	+
T134	08-AZ1	AZ, USA	1	+	+	+	+	+	+	+	+
T135	08-MX3	Mexico	1	+	+	+	+	+	+	+	+
T136	08TX-1	TX, USA	1	+	+	+	+	+	+	+	+
T137	08TX-2	TX, USA	1	+	+	+	+	+	+	+	+
T138	08TX-3	TX, USA	1	+	+	+	+	+	+	+	+
T139	08TX-4	TX, USA	1	+	+	+	+	+	+	+	+
T141	08TX-6	TX, USA	1	+	+	+	+	+	+	+	+
T145	08TX-10	TX, USA	1	+	+	+	+	+	+	+	+
T146	08-CDN1	Canada	1	+	+	+	+	+	+	+	+
T147	08-CDN2	Canada	1	+	+	+	+	+	+	+	+
T148	08-MN1	MN, USA	1	+	+	+	+	+	+	+	+
T149	08-MN2	MN, USA	1	+	+	+	+	+	+	+	+
T150	08-MN3	MN, USA	1	+	+	+	+	+	+	+	+
T151	08-MN4	MN, USA	1	+	+	+	+	+	+	+	+
T152	08-MN5	MN, USA	1	+	+	+	+	+	+	+	+
T153	08-53A	MI, USA	1	+	+	+	+	+	+	+	+
T157	08-49A#1	MI, USA	1	+	+	+	+	+	+	+	+
T158	08-55A	TX, USA	1	+	+	+	+	+	+	+	+
T159	08-49A#2	MI, USA	1	+	+	+	+	+	+	+	+
T160	08-221A	PA, USA	1	+	+	+	+	+	+	+	+
T161	08-245A	VT, USA	1	+	+	+	+	+	+	+	+
T162	08-264A	PA, USA	1	+	+	+	+	+	+	+	+
T163	08-567	PA, USA	1	+	+	+	+	+	+	+	+
T165	08-632	OH, USA	1	+	+	+	+	+	+	+	+
T166	08-640A	NL	1	+	+	+	+	+	+	+	+
T168	08-645A	NL	1	+	+	+	+	+	+	+	+
T169	08-642A	NL	1	+	+	+	+	+	+	+	+
T170	08-639A	NL	1	+	+	+	+	+	+	+	+
T171	08-636A	NL	1	+	+	+	+	+	+	+	+
T172	08-641A	NL	1	+	+	+	+	+	+	+	+
T173	08-637A	NL	1	+	+	+	+	+	+	+	+
T174	08-567A	PA, USA	1	+	+	+	+	+	+	+	+
T175	08-568A	PA, USA	1	+	+	+	+	+	+	+	+
T176	08-632A	OH, USA	1	+	+	+	+	+	+	+	+
T177	08-791A	Canada	1	+	+	+	+	+	+	+	+
T184	06-AZ6	AZ, USA	1	+	+	+	+	+	+	+	+
T186	06-TX5	TX, USA	1	+	+	+	+	+	+	+	+
T187	06-TX6	TX, USA	1	+	+	+	+	+	+	+	+
T188	07-MX17	Mexico	1	+	+	+	+	+	+	+	+
T189	07-TX25	TX, USA	1	+	+	+	+	+	+	+	+
T191	06-AZ8	AZ, USA	1	+	+	+	+	+	+	+	+
T193	06-CDN8	Canada	1	+	+	+	+	+	+	+	+
T194	07-TX26	TX, USA	1	+	+	+	+	+	+	+	+
T195	07-AZ1	AZ, USA	1	+	+	+	+	+	+	+	+
T196	07-AZ2	AZ, USA	1	+	+	+	+	+	+	+	+
T197	07-MI9	MI, USA	1	+	+	+	+	+	+	+	+
T199	07-TX27	TX, USA	1	+	+	+	+	+	+	+	+
T200	07-TX28	TX, USA	1	+	+	+	+	+	+	+	+
T201	07-TX29	TX, USA	1	+	+	+	+	+	+	+	+
T203	08-TX11	TX, USA	1	+	+	+	+	+	+	+	+
T204	08-CDN3	Canada	1	+	+	+	+	+	+	+	+
T206	08-TX13	TX, USA	1	+	+	+	+	+	+	+	+
T207	08-TX14	TX, USA	1	+	+	+	+	+	+	+	+
T209	08-TX16	TX, USA	1	+	+	+	+	+	+	+	+
T210	08-TX17	TX, USA	1	+	+	+	+	+	+	+	+

Strain	Other ID	Origin	Pattern #	dnaA	Cmm5/6	$CM_{3/4}$	chpC	tomA	ppaA	ppaC	LAMP
T211	09-571E	OH, USA	1	+	+	+	+	+	+	+	+
T215	09-615A	OH, USA	1	+	+	+	+	+	+	+	+
T288	10-1052C	Canada	1	+	+	+	+	+	+	+	+
T289	10-1293A	AZ, USA	1	+	+	+	+	+	+	+	+
T290	10-1294A	AZ, USA	1	+	+	+	+	+	+	+	+
T292	10-1946A	Mexico	1	+	+	+	+	+	+	+	+
T293	10-1947A	Mexico	1	+	+	+	+	+	+	+	+
T294	10-2286A	Mexico	1	+	+	+	+	+	+	+	+
T295	10-2286B	Mexico	1	+	+	+	+	+	+	+	+
T297	10-2287A	Mexico	1	+	+	+	+	+	+	+	+
T298	10-2287B	Mexico	1	+	+	+	+	+	+	+	+
T299	10-2632A	Canada	1	+	+	+	+	+	+	+	+
T300	10-2633A	Canada	1	+	+	+	+	+	+	+	+
T301	10-2640A	PA, USA	1	+	+	+	+	+	+	+	+
T302	10-2646A	Canada	1	+	+	+	+	+	+	+	+
T303	10-2649E	Mexico	1	+	+	+	+	+	+	+	+
T305	10-2662A	Mexico	1	+	+	+	+	+	+	+	+
T306	10-2662B	Mexico	1	+	+	+	+	+	+	+	+
T308	10-2856B	Mexico	1	+	+	+	+	+	+	+	+
T309	10-2856C	Mexico	1	+	+	+	+	+	+	+	+
T310	10-2857A	Mexico	1	+	+	+	+	+	+	+	+
T312	10-2857C	Mexico	1	+	+	+	+	+	+	+	+
T313	08-922A	UK	1	+	+	+	+	+	+	+	+
C126-B		NL	2	+	-	+	+	+	+	+	+
C215		OR, USA	2	+	-	+	+	+	+	+	+
K078*	A518-5	HI, USA	2	+	-	+	+	+	+	+	+
K080*	E3	OH, USA	2	+	-	+	+	+	+	+	+
K082*	71169	China	2	+	-	+	+	+	+	+	+
K083*	A438-1	HI, USA	2	+	-	+	+	+	+	+	+
K093*	IPO545	S. Africa	2	+	-	+	+	+	+	+	+
K385*	A518-1	HI, USA	2	+	-	+	+	+	+	+	+
K386*	B-125	CA, USA	2	+	-	+	+	+	+	+	+
K388*	CM97	OH, USA	2	+	-	+	+	+	+	+	+
K393*	C12	CA, USA	2	+	-	+	+	+	+	+	+
K394*	C19	CA, USA	2	+	-	+	+	+	+	+	+
K402*	CM Kuykendall	NC, USA	2	+	-	+	+	+	+	+	+
K413*	18(E)	OH, USA	2	+	-	+	+	+	+	+	+
K448*	18(D)	OH, USA	2	+	-	+	+	+	+	+	+
K449*	26	OH, USA	2	+	-	+	+	+	+	+	+
K454*	14	OH, USA	2	+	-	+	+	+	+	+	+
K455*	43	OH, USA	2	+	-	+	+	+	+	+	+
K465*	cmm461	Portugal	2	+	-	+	+	+	+	+	+
K471*	IPO544	Hungary	2	+	-	+	+	+	+	+	+
K479*	N213	Chile	2	+	-	+	+	+	+	+	+
T019	09-135B	FL, USA	2	+	-	+	+	+	+	+	+
T058	06-CDN4	Canada	2	+	-	+	+	+	+	+	+
T128	07-MN5	MN, USA	2	+	-	+	+	+	+	+	+
T129	07-MN6	MN, USA	2	+	-	+	+	+	+	+	+
T311	10-2857B	Mexico	2	+	-	+	+	+	+	+	+
K410*	72	OH, USA	3	+	+	-	+	+	+	+	+
K422*	70	OH, USA	3	+	+	-	+	+	+	+	+
K467*	IPO500	UK	3	+	+	-	+	+	+	+	+
T085	07-MX10	Mexico	3	+	+	-	+	+	+	+	+
T086	07-TX5	TX, USA	3	+	+	-	+	+	+	+	+
T092	07-MX12	Mexico	3	+	+	-	+	+	+	+	+
T095	07-MX15	Mexico	3	+	+	-	+	+	+	+	+
T096	07-MX16	Mexico	3	+	+	-	+	+	+	+	+
T119	07-OH4	OH, USA	3	+	+	-	+	+	+	+	+
T120	07-MI5	MI, USA	3	+	+	-	+	+	+	+	+

Strain	Other ID	Origin	Pattern #	dnaA	Cmm5/6	$CM_{3/4}$	chpC	tomA	ppaA	ppaC	LAMP
T181	HIRC	Unknown	3	+	+	-	+	+	+	+	+
T182	06-CDN5	Canada	3	+	+	-	+	+	+	+	+
T183	06-CDN7	Canada	3	+	+	-	+	+	+	+	+
T185	06-TX4	TX, USA	3	+	+	-	+	+	+	+	+
T205	08-TX12	TX, USA	3	+	+	-	+	+	+	+	+
T212	09-572E	OH, USA	3	+	+	-	+	+	+	+	+
T213	09-592A	OH, USA	3	+	+	-	+	+	+	+	+
T214	09-586A	OH, USA	3	+	+	-	+	+	+	+	+
T216	09-617A	PA, USA	3	+	+	-	+	+	+	+	+
K077*	cmm462	Portugal	4	+	-	-	+	+	+	+	+
K395*	S44	CA, USA	4	+	-	-	+	+	+	+	+
K404*	DR73	IA, USA	4	+	-	-	+	+	+	+	+
K440*	BR4	IA, USA	4	+	-	-	+	+	+	+	+
A4868		CA, USA	5	+	+	+	-	+	+	+	+
K437*	2(C)	OH, USA	5	+	+	+	-	+	+	+	+
T118	07-OH3	OH, USA	5	+	+	+	-	+	+	+	+
T123	07-MI8	MI, USA	5	+	+	+	-	+	+	+	+
T126	07-MN3	MN, USA	5	+	+	+	-	+	+	+	+
T132	08-MX1	Mexico	5	+	+	+	-	+	+	+	+
T140	08TX-5	TX, USA	5	+	+	+	-	+	+	+	+
T142	08TX-7	TX, USA	5	+	+	+	-	+	+	+	+
T143	08TX-8	TX, USA	5	+	+	+	-	+	+	+	+
T144	08TX-9	TX, USA	5	+	+	+	-	+	+	+	+
T154	08-75A	MI, USA	5	+	+	+	-	+	+	+	+
T155	08-42A	MI, USA	5	+	+	+	-	+	+	+	+
T156	08-52A	MI, USA	5	+	+	+	-	+	+	+	+
T164	08-568	PA, USA	5	-	+	+	-	+	+	+	+
T167	08-643A	NL	5	+	+	+	-	+	+	+	+
T178	08-884A	TX, USA	5	+	+	+	-	+	+	+	+
T179	06-AZ5	AZ, USA	5	+	+	+	-	+	+	+	+
T190	06-AZ7	AZ, USA	5	+	+	+	-	+	+	+	+
A4775	F293	MI, USA	6	+	+	+	-	-	+	+	+
C216		OR, USA	6	+	+	+	-	-	+	+	+
K089*	C217	OR, USA	6	+	+	+	-	-	+	+	+
K432*	37	OH, USA	6	+	+	+	-	-	+	+	+
T192	06-AZ9	AZ, USA	6	+	+	+	-	-	+	+	+
T180	06-CDN6	Canada	7	+	+	+	+	+	-	+	+
T208	08-TX15	TX, USA	7	+	+	+	+	+	-	+	+
T217	09-614E	PA, USA	7	+	+	+	+	+	-	+	+
T291	10-1944A	IA, USA	7	-	+	+	+	+	-	+	+
T307	10-2856A	Mexico	7	+	+	+	+	+	-	+	+
A4004		OH, USA	8	+	+	+	-	-	-	-	+
A4818	29	OH, USA	8	+	+	+	-	-	-	-	+
A4833		MI, USA	8	+	+	+	-	-	-	-	+
C208		OH, USA	8	+	+	+	-	-	-	-	+
C209		OH, USA	8	+	+	+	-	-	-	-	+
C210		OH, USA	8	+	+	+	-	-	-	-	+
C211		OH, USA	8	+	+	+	-	-	-	-	+
C218		OR, USA	8	+	+	+	-	-	-	-	+
C223		OR, USA	8	+	+	+	-	-	-	-	+
K085*	S47	CA, USA	8	+	+	+	-	-	-	-	+
K086*	cmm016	WA, USA	8	+	+	+	-	-	-	-	+
K088*	ZUM3036	NL	8	+	+	+	-	-	-	-	+
K396*	S51	CA, USA	8	+	+	+	-	-	-	-	+
K397*	S52	CA, USA	8	+	+	+	-	-	-	-	+
K460*	cmm018	WA, USA	8	+	+	+	-	-	-	-	+
K461*	cmm024	WA, USA	8	+	+	+	-	-	-	-	+
K462*	cmm035	WA, USA	8	+	+	+	-	-	-	-	+
K463*	cmm037	WA, USA	8	+	+	+	-	-	-	-	+

Strain	Other ID	Origin	Pattern #	dnaA	Cmm5/6	$CM_{3/4}$	chpC	tomA	ppaA	ppaC	LAMP
T023	09-158B	TX, USA	8	+	+	+	-	-	-	-	+
A4598		WA, USA	9	+	-	+	-	-	-	-	+
A4820		OH, USA	9	+	-	+	-	-	-	-	+
A4830		MI, USA	10	+	-	+	-	-	+	+	+

[*] *dnaA* data was provided by Schneider et al. (2011).

Abbreviations: AZ – Arizona; BC – British Columbia, Canada; CA – California; FL – Florida; HI – Hawai‘i; IA – Iowa; ID – Idaho; MI – Michigan; MN – Minnesota; NC – North Carolina; NE – Nebraska; NL –Netherlands; OH – Ohio; OR – Oregon; PA – Pennsylvania; TX – Texas; UK – United Kingdom; USA – United States of America; VA – Virginia; VT – Vermont; WA – Washington.

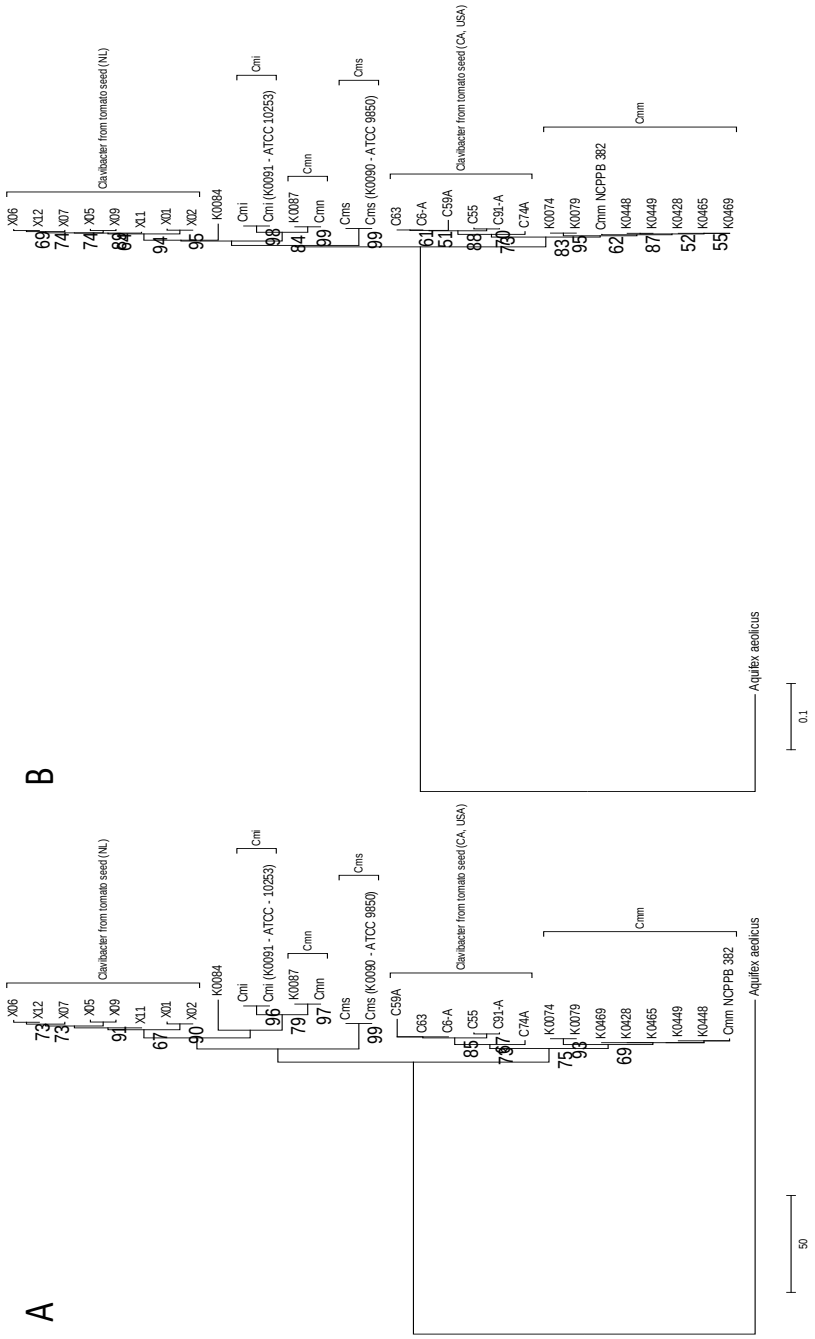


Figure B.1. Phylogenetic analysis of *Clavibacter* strains based on *dnaA* sequences. *dnaA* sequences were trimmed to 661 nt and multiple alignments were made using ClustalW, taking into account the corresponding amino acid alignments for protein-coding genes. Phylogenetic trees were constructed using A) MP and B) NJ analyses. MP analysis was used to construct trees with the closest neighbor interchange at search level 3. NJ trees were constructed using the Jukes-Cantor method to compute evolutionary distances. Confidence intervals were assessed using the bootstrap method with 1000 replications. Branch lengths were removed to view only the topology. Bootstrap values of 50% or more are shown to the left of corresponding nodes. Bars indicate 10% sequence divergence for B.

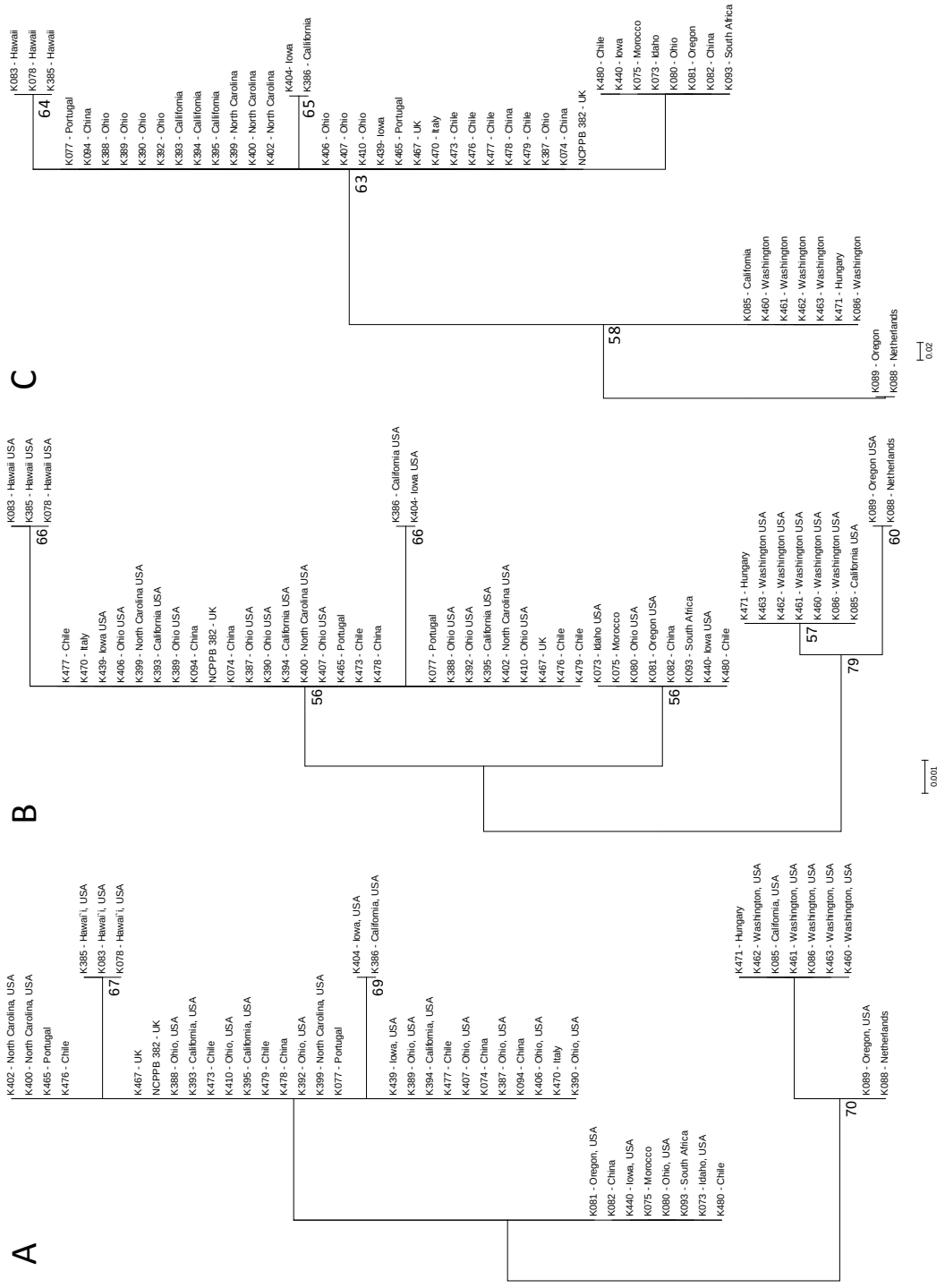


Figure B.2. Phylogenetic analysis of *Clavibacter michiganensis* subsp. *michiganensis* based on *clvA* sequences. *clvA* sequences were trimmed to 207 nt and multiple alignments were made using ClustalW, taking into account the corresponding amino acid alignments for protein-coding genes. Phylogenetic trees were constructed using A) MP, B) NJ and C) ML analyses with mid-point rooting. MP analysis was used to construct trees with the closest neighbor interchange at search level 1. Confidence intervals were assessed using the bootstrap method with 1000 replications. Bootstrap values of 50% or more are shown to the left of corresponding nodes. Bars indicate 0.1% and 2% sequence divergence for B and C, respectively.

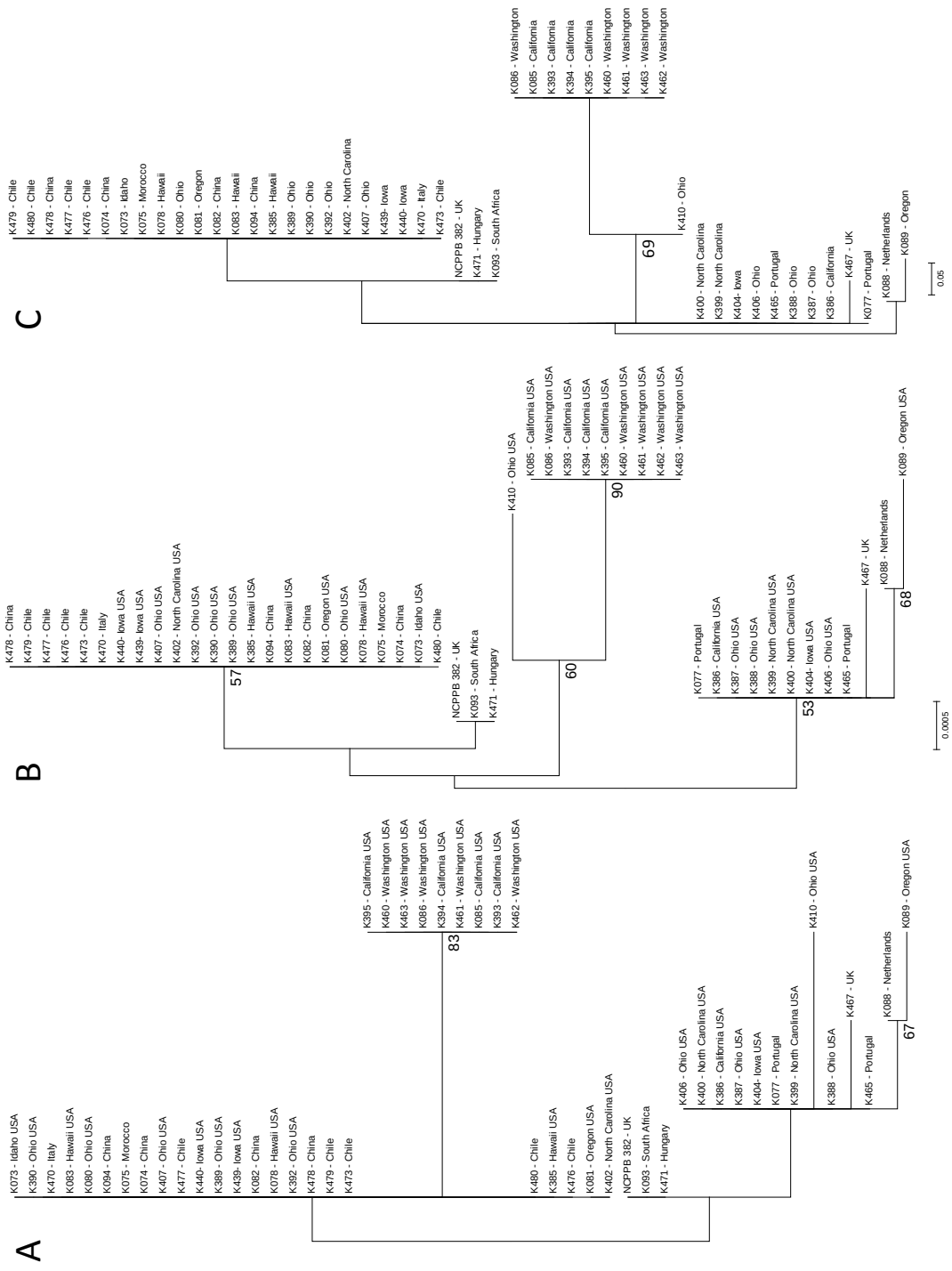


Figure B.3. Phylogenetic analysis of *Clavibacter michiganensis* subsp. *michiganensis* based on *clvF* sequences. *clvF* sequences were trimmed to 884 nt and multiple alignments were made using ClustalW, taking into account the corresponding amino acid alignments for protein-coding genes. Phylogenetic trees were constructed using A) MP, B) NJ and C) ML analyses with mid-point rooting. MP analysis was used to construct trees with the closest neighbor interchange at search level 1. Confidence intervals were assessed using the bootstrap method with 1000 replications. Bootstrap values of 50% or more are shown to the left of corresponding nodes. Bars indicate 0.05% and 5% sequence divergence for B and C, respectively.

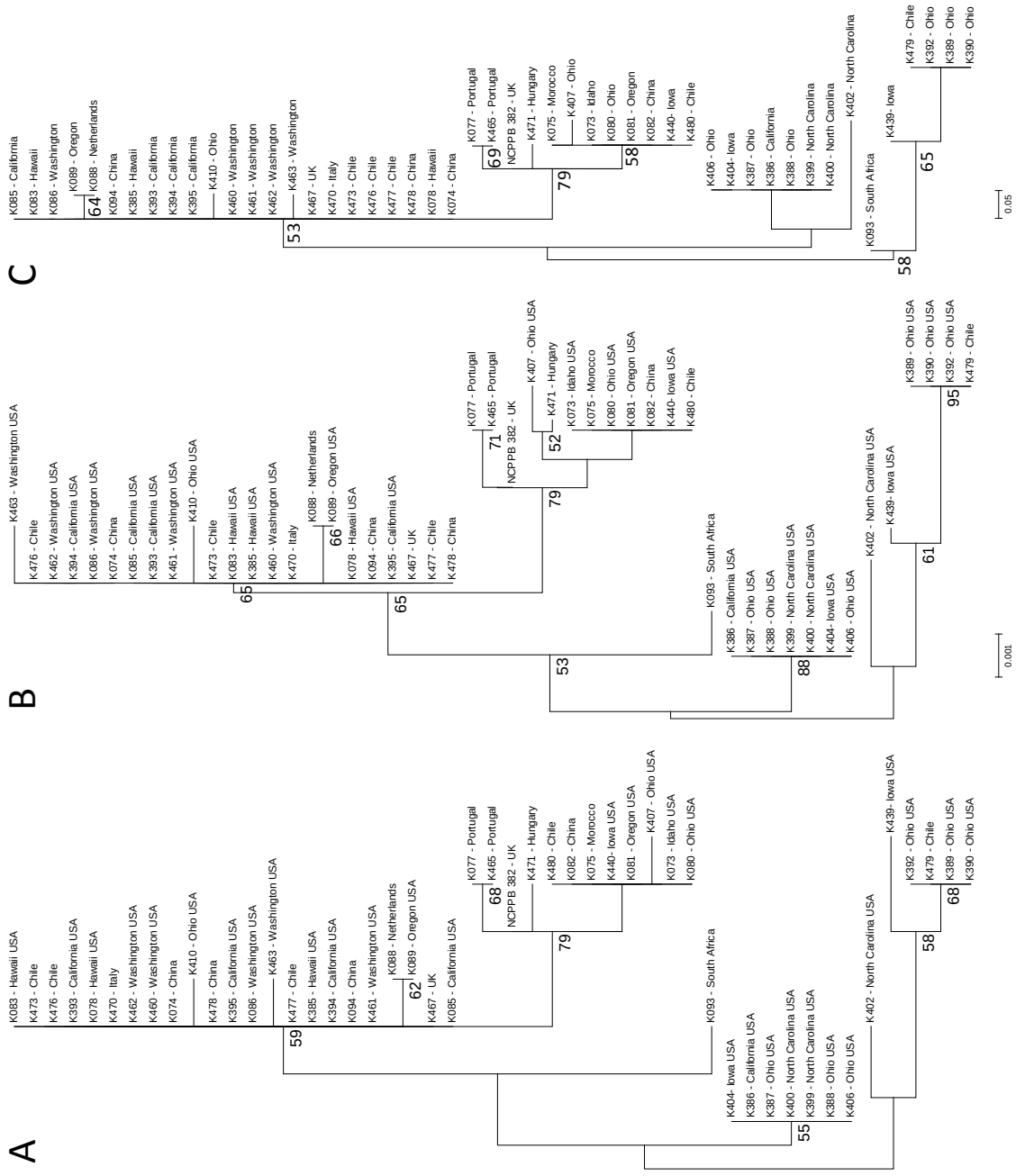


Figure B.4. Phylogenetic analysis of *Clavibacter michiganensis* subsp. *michiganensis* based on *clvG* sequences. *clvG* sequences were trimmed to 753 nt and multiple alignments were made using ClustalW, taking into account the corresponding amino acid alignments for protein-coding genes. Phylogenetic trees were constructed using A) MP, B) NJ and C) ML analyses with mid-point rooting. MP analysis was used to construct trees with the closest neighbor interchange at search level 1. Confidence intervals were assessed using the bootstrap method with 1000 replications. Bootstrap values of 50% or more are shown to the left of corresponding nodes. Bars indicate 0.1% and 5% sequence divergence for B and C, respectively.

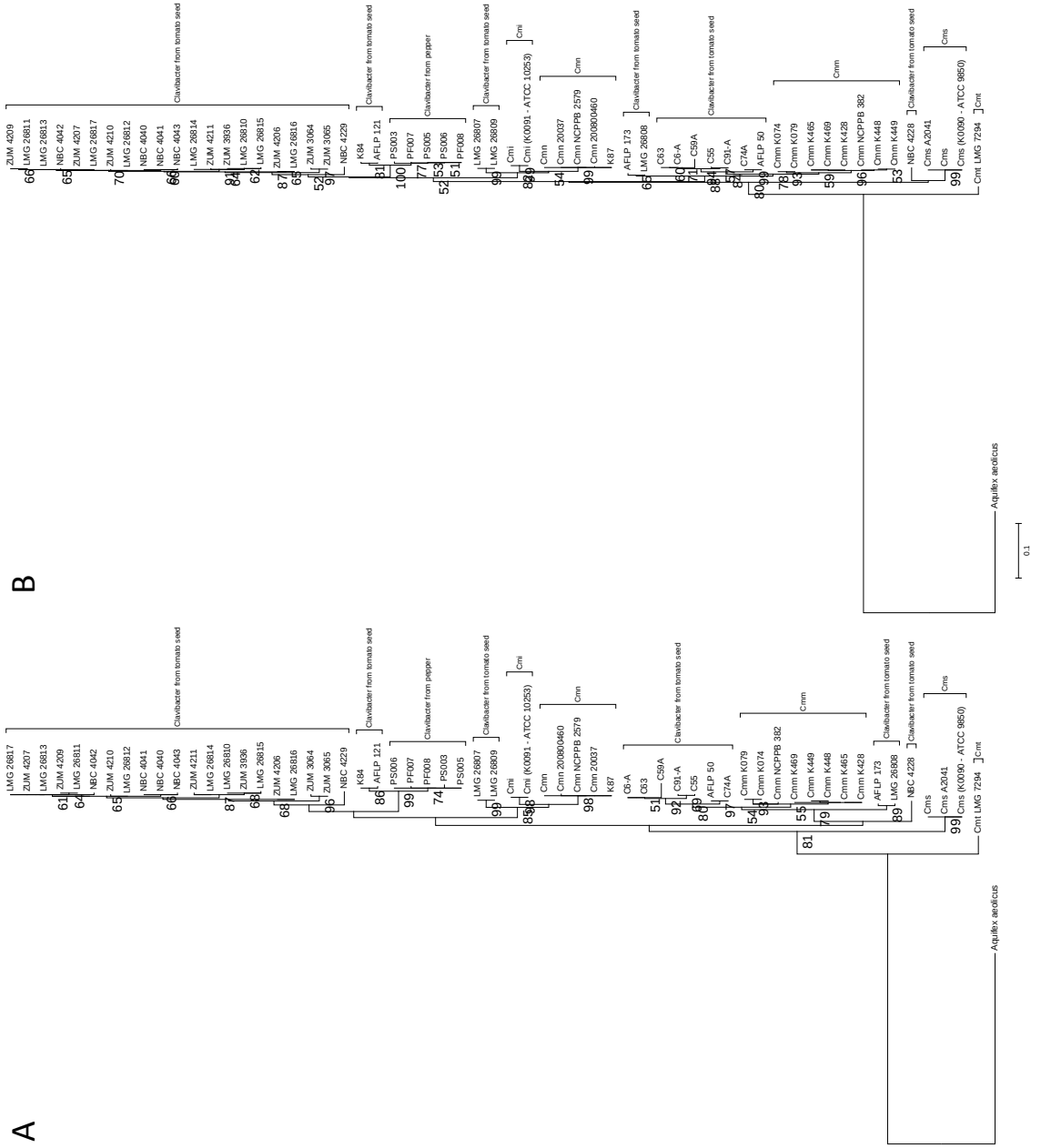


Figure B.5. Phylogenetic analysis of *Clavibacter* strains based on *dnaA* sequences. *dnaA* sequences were trimmed to 661 nt and multiple alignments were made using ClustalW, taking into account the corresponding amino acid alignments for protein-coding genes. Phylogenetic trees were constructed using A) MP and B) NJ analyses. MP analysis was used to construct trees with the closest neighbor interchange at search level 3. NJ trees were constructed using the Jukes-Cantor method to compute evolutionary distances. Confidence intervals were assessed using the bootstrap method with 1000 replications. Branch lengths were removed to view only the topology. Bootstrap values of 50% or more are shown to the left of corresponding nodes. Bars indicate 10% sequence divergence for B.

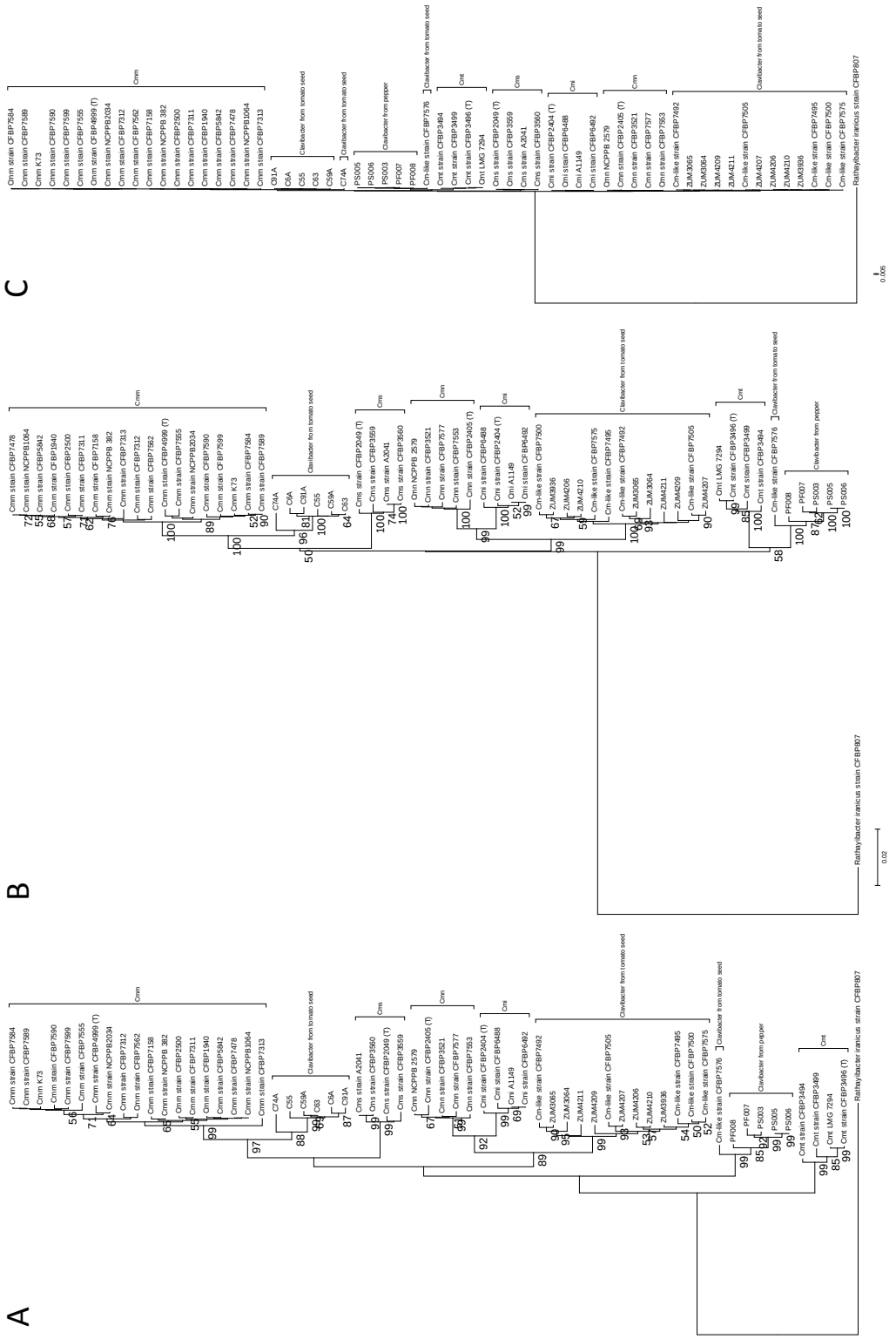


Figure B.6. Housekeeping gene MLSA. MSLA of concatenated *atpD*, *dnaK*, *gyrB*, *ppK*, *recA* and *rpoB* gene sequences. Phylogenetic trees were constructed using A) MP B) NJ and C) ML analyses. Confidence intervals were assessed using the bootstrap method with 1000 replications. Bootstrap values of 50% or more are shown to the left of corresponding nodes. Bars indicate 2% and 0.5% sequence divergence for B and C, respectively.

APPENDIX C: Pathogenicity Data.

Table C.1. Strains with available pathogenicity data.

Strain	Other Designation	Pattern #	dnaA	Cmm5/6	$CM_{3/4}$	chpC	tomA	ppaA	ppaC	LAMP	Pathogenicity
A4810[a]		1	+	+	+	+	+	+	+	+	+
A4825[a]		1	+	+	+	+	+	+	+	+	+
A4827[a]		1	+	+	+	+	+	+	+	+	+
C117[a]		1	+	+	+	+	+	+	+	+	+
C131-A[a]		1	+	+	+	+	+	+	+	+	+
C202[a]		1	+	+	+	+	+	+	+	+	+
C203[a]		1	+	+	+	+	+	+	+	+	+
C204[a]		1	+	+	+	+	+	+	+	+	+
C205[a]		1	+	+	+	+	+	+	+	+	+
C206[a]		1	+	+	+	+	+	+	+	+	+
C207[a]		1	+	+	+	+	+	+	+	+	+
C219[a]		1	+	+	+	+	+	+	+	+	+
C220[a]		1	+	+	+	+	+	+	+	+	+
C221[a]		1	+	+	+	+	+	+	+	+	+
C224[a]		1	+	+	+	+	+	+	+	+	+
C225[a]		1	+	+	+	+	+	+	+	+	+
C226[a]		1	+	+	+	+	+	+	+	+	+
K073[b]	H-160	1	+	+	+	+	+	+	+	+	+
K074[a,b]	N212	1	+	+	+	+	+	+	+	+	+
K075[b]	N7388A	1	+	+	+	+	+	+	+	+	+
K081[b]	C222	1	+	+	+	+	+	+	+	+	+
K094[b]	71169	1	+	+	+	+	+	+	+	+	+
K387[b]	CM95	1	+	+	+	+	+	+	+	+	+
C126-B[a]		2	+	-	+	+	+	+	+	+	+
C215		2	+	-	+	+	+	+	+	+	+
K078[b]	A518-5	2	+	-	+	+	+	+	+	+	+
K080[b]	E3	2	+	-	+	+	+	+	+	+	+
K082[b]	71169	2	+	-	+	+	+	+	+	+	+
K083[b]	A438-1	2	+	-	+	+	+	+	+	+	+
K093[b]	IPO545	2	+	-	+	+	+	+	+	+	+
K385[b]	A518-1	2	+	-	+	+	+	+	+	+	+
K386[b]	B-125	2	+	-	+	+	+	+	+	+	+
K388[b]	CM97	2	+	-	+	+	+	+	+	+	+
K393[b]	C12	2	+	-	+	+	+	+	+	+	+
K394[b]	C19	2	+	-	+	+	+	+	+	+	+
K402[b]	CM Kuykendall	2	+	-	+	+	+	+	+	+	+
K413[b]	18(E)	2	+	-	+	+	+	+	+	+	+
K448[b]	18(D)	2	+	-	+	+	+	+	+	+	+
K449[b]	26	2	+	-	+	+	+	+	+	+	+
K454[b]	14	2	+	-	+	+	+	+	+	+	+
K455[b]	43	2	+	-	+	+	+	+	+	+	+
K465[b]	cmm461	2	+	-	+	+	+	+	+	+	+
K471[b]	IPO544	2	+	-	+	+	+	+	+	+	+
K479[b]	N213	2	+	-	+	+	+	+	+	+	+
T019	09-135B	2	+	-	+	+	+	+	+	+	+
T058	06-CDN4	2	+	-	+	+	+	+	+	+	+
T128	07-MN5	2	+	-	+	+	+	+	+	+	+
T129	07-MN6	2	+	-	+	+	+	+	+	+	+
T311	10-2857B	2	+	-	+	+	+	+	+	+	+
K410[b]	72	3	+	+	-	+	+	+	+	+	+
K422[b]	70	3	+	+	-	+	+	+	+	+	+
K467[b]	IPO500	3	+	+	-	+	+	+	+	+	+
T085	07-MX10	3	+	+	-	+	+	+	+	+	+
T092	07-MX12	3	+	+	-	+	+	+	+	+	+
T095	07-MX15	3	+	+	-	+	+	+	+	+	+
T096	07-MX16	3	+	+	-	+	+	+	+	+	+
T119	07-OH4	3	+	+	-	+	+	+	+	+	+
T120	07-MI5	3	+	+	-	+	+	+	+	+	+

Strain	Other Designation	Pattern #	dnaA	Cmm5/6	$CM_{3/4}$	chpC	tomA	ppaA	ppaC	LAMP	Pathogenicity
T181	HIRC	3	+	+	-	+	+	+	+	+	+
T182	06-CDN5	3	+	+	-	+	+	+	+	+	+
T183	06-CDN7	3	+	+	-	+	+	+	+	+	+
T185	06-TX4	3	+	+	-	+	+	+	+	+	+
T205	08-TX12	3	+	+	-	+	+	+	+	+	+
T212	09-572E	3	+	+	-	+	+	+	+	+	+
T213	09-592A	3	+	+	-	+	+	+	+	+	+
T214	09-586A	3	+	+	-	+	+	+	+	+	+
T216	09-617A	3	+	+	-	+	+	+	+	+	+
K077[b]	cmm462	4	+	-	-	+	+	+	+	+	+
K395[b]	S44	4	+	-	-	+	+	+	+	+	+
K404[b]	DR73	4	+	-	-	+	+	+	+	+	+
K440[b]	BR4	4	+	-	-	+	+	+	+	+	+
A4868		5	+	+	+	-	+	+	+	+	+
K437[b]	2(C)	5	+	+	+	-	+	+	+	+	+
T118	07-OH3	5	+	+	+	-	+	+	+	+	+
T123	07-MI8	5	+	+	+	-	+	+	+	+	+
T126	07-MN3	5	+	+	+	-	+	+	+	+	+
T132	08-MX1	5	+	+	+	-	+	+	+	+	+
T140	08TX-5	5	+	+	+	-	+	+	+	+	+
T142	08TX-7	5	+	+	+	-	+	+	+	+	+
T143	08TX-8	5	+	+	+	-	+	+	+	+	+
T144	08TX-9	5	+	+	+	-	+	+	+	+	+
T154	08-75A	5	+	+	+	-	+	+	+	+	+
T155	08-42A	5	+	+	+	-	+	+	+	+	+
T156	08-52A	5	+	+	+	-	+	+	+	+	+
T164	08-568	5	+	+	+	-	+	+	+	+	+
T167	08-643A	5	+	+	+	-	+	+	+	+	+
T178	08-884A	5	+	+	+	-	+	+	+	+	+
T179	06-AZ5	5	+	+	+	-	+	+	+	+	+
T190	06-AZ7	5	+	+	+	-	+	+	+	+	+
A4775	F293	6	+	+	+	-	-	+	+	+	-
C216[a]		6	+	+	+	-	-	+	+	+	-
K089[b]	C217	6	+	+	+	-	-	+	+	+	-
T180	06-CDN6	7	+	+	+	+	+	-	+	+	+
T208	08-TX15	7	+	+	+	+	+	-	+	+	+
T217	09-614E	7	+	+	+	+	+	-	+	+	+
T291	10-1944A	7	-	+	+	+	+	-	+	+	+
T307	10-2856A	7	-	+	+	+	+	-	+	+	+
A4004		8	+	+	+	-	-	-	-	+	-
A4818	29	8	+	+	+	-	-	-	-	+	-
A4833		8	+	+	+	-	-	-	-	+	-
C208[a]		8	+	+	+	-	-	-	-	+	-
C209[a]		8	+	+	+	-	-	-	-	+	-
C210[a]		8	+	+	+	-	-	-	-	+	-
C211		8	+	+	+	-	-	-	-	+	-
C218[a]		8	+	+	+	-	-	-	-	+	-
C223[a]		8	+	+	+	-	-	-	-	+	-
K085[b]	S47	8	+	+	+	-	-	-	-	+	-
K086[b]	cmm016	8	+	+	+	-	-	-	-	+	-
K088[b]	ZUM3036	8	+	+	+	-	-	-	-	+	-
K396[b]	S51	8	+	+	+	-	-	-	-	+	-
K397[b]	S52	8	+	+	+	-	-	-	-	+	-
K460[b]	cmm018	8	+	+	+	-	-	-	-	+	-
K461[b]	cmm024	8	+	+	+	-	-	-	-	+	-
K462[b]	cmm035	8	+	+	+	-	-	-	-	+	-
K463[b]	cmm037	8	+	+	+	-	-	-	-	+	-
T023	09-195A	8	+	+	+	-	-	-	-	+	-
A4598		9	+	-	+	-	-	-	-	+	-
A4820		9	+	-	+	-	-	-	-	+	-
A4830		10	+	-	+	-	-	+	+	+	-

[a] Pathogenicity data was provided by Kaneshiro et al. (2006).
[b] *dnaA* data was provided by Schneider et al. (2011).

APPENDIX D: Gene Sequence Alignments.

```
NCPPB382 ATGAACGACATCCTCGAGACGGAGACCCCCGTCATGGTCAGCCCCCGGTGGGACATGCTGCTCGACGCGGGCGAGGAC
K073     ..........C..........................................................CG.....
K074     ..........C..........................................................CG.....
K075     ..........C..........................................................CG.....
K077     ..........C..........................................................CG.....
K078     ..........C..........................................................CG.....
K080     ..........C..........................................................CG.....
K081     ..........C..........................................................CG.....
K082     ..........C..........................................................CG.....
K083     ..........C..........................................................CG.....
K085     ..........C..........................................................CG.....
K086     ..........C..........................................................CG.....
K088     ..........C..........................................................CG.....
K089     ..........C..........................................................CG.....
K093     ..........C..........................................................CG.....
K094     ..........C..........................................................CG.....
K385     ..........C..........................................................CG.....
K386     ..........C..........................................................CG.....
K387     ..........C..........................................................CG.....
K388     ..........C..........................................................CG.....
K389     ..........C..........................................................CG.....
K390     ..........C..........................................................CG.....
K392     ..........C..........................................................CG.....
K393     ..........C..........................................................CG.....
K394     ..........C..........................................................CG.....
K395     ..........C..........................................................CG.....
K399     ..........C..........................................................CG.....
K400     ..........C..........................................................CG.....
K402     ..........C..........................................................CG.....
K404     ..........C..........................................................CG.....
K406     ..........C..........................................................CG.....
K407     ..........C..........................................................CG.....
K410     ..........C..........................................................CG.....
K439     ..........C..........................................................CG.....
K440     ..........C..........................................................CG.....
K460     ..........C..........................................................CG.....
K461     ..........C..........................................................CG.....
K462     ..........C..........................................................CG.....
K463     ..........C..........................................................CG.....
K465     ..........C..........................................................CG.....
K467     ..........C..........................................................CG.....
K470     ..........C..........................................................CG.....
K471     ..........C..........................................................CG.....
K473     ..........C..........................................................CG.....
K476     ..........C..........................................................CG.....
K477     ..........C..........................................................CG.....
K478     ..........C..........................................................CG.....
K479     ..........C..........................................................CG.....
K480     ..........C..........................................................CG.....
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```
NCPPB382 ACCAGCCCGTCCGTCCAGACCCAGATCGACGCGGAGTTCCGTCGCGTCGTGAGCCCGTACATGTCCAGCAGCGGCTGG
K073     ................................................................................
K074     ................................................................................
K075     ................................................................................
K077     ................................................................................
K078     ................................................................................
K080     ................................................................................
K081     ................................................................................
K082     ................................................................................
K083     ................................................................................
K085     ................................................................................
K086     ................................................................................
K088     ................................................................................
K089     ................................................................................
K093     ................................................................................
K094     ................................................................................
K385     ................................................................................
K386     ....................A...........................................................
K387     ................................................................................
K388     ................................................................................
K389     ................................................................................
K390     ................................................................................
K392     ................................................................................
K393     ................................................................................
K394     ................................................................................
K395     ................................................................................
K399     ................................................................................
K400     ................................................................................
K402     ................................................................................
K404     ....................A...........................................................
K406     ................................................................................
K407     ................................................................................
K410     ................................................................................
K439     ................................................................................
K440     ................................................................................
K460     ................................................................................
K461     ................................................................................
K462     ................................................................................
K463     ................................................................................
K465     ................................................................................
K467     ................................................................................
K470     ................................................................................
K471     ................................................................................
K473     ................................................................................
K476     ................................................................................
K477     ................................................................................
K478     ................................................................................
K479     ................................................................................
K480     ................................................................................
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```
NCPPB382 CTCTGCACGCTCACCATCGAATGCGGGACGATCATCTGCGCGTGTCGCTGA
K073     ..................................................
K074     ..................................................
K075     ..................................................
K077     ..................................................
K078     ................G.................................
K080     ..................................................
K081     ..................................................
K082     ..................................................
K083     ................G.................................
K085     ..................................................
K086     ..................................................
K088     ..................................................
K089     ..................................................
K093     ..................................................
K094     ..................................................
K385     ................G.................................
K386     ..................................................
K387     ..................................................
K388     ..................................................
K389     ..................................................
K390     ..................................................
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K440     ..................................................
K460     ..................................................
K461     ..................................................
K462     ..................................................
K463     ..................................................
K465     ..................................................
K467     ..................................................
K470     ..................................................
K471     ..................................................
K473     ..................................................
K476     ..................................................
K477     ..................................................
K478     ..................................................
K479     ..................................................
K480     ..................................................
```

Figure D.1. *clvA* nucleotide sequence alignment. The positions of the nucleotide differences relative to that published for strain NCPPB 382 are highlighted in yellow.

```
NCPPB382 MNDILETETPVMVSPRWDMLLDAGEDTSPSVQTQIDAEFRRVVSPYMSSSGWLCTLTIECGTIICACR*
K073     ...T.....................................................................
K074     .........................................................................
K075     ...T.....................................................................
K077     .........................................................................
K078     .........................................................................
K080     ...T.....................................................................
K081     ...T.....................................................................
K082     ...T.....................................................................
K083     .........................................................................
K085     ..................V......................................................
K086     ..................V......................................................
K088     ...T.............V......................................................
K089     ...T.............V......................................................
K093     ...T.....................................................................
K094     .........................................................................
K385     .........................................................................
K386     ..............................K..........................................
K387     .........................................................................
K388     .........................................................................
K389     .........................................................................
K390     .........................................................................
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K394     .........................................................................
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K399     .........................................................................
K400     .........................................................................
K402     .........................................................................
K404     ..............................K..........................................
K406     .........................................................................
K407     .........................................................................
K410     .........................................................................
K439     .........................................................................
K440     ...T.....................................................................
K460     ..................V......................................................
K461     ..................V......................................................
K462     ..................V......................................................
K463     ..................V......................................................
K465     .........................................................................
K467     .........................................................................
K470     .........................................................................
K471     ..................V......................................................
K473     .........................................................................
K476     .........................................................................
K477     .........................................................................
K478     .........................................................................
K479     .........................................................................
K480     ...T.....................................................................
```

Figure D.2. *clvA* amino acid sequence alignment. The positions of the amino acid differences relative to that published for strain NCPPB 382 are highlighted in yellow.

```
NCPPB382 ATGAGCGCCGTCATCGCCACCCGCGGGCTCACCAAGCGGTTCCGGGATCACGTCGCCGTCGACGCGCTCGACATCGAG
K073     ............................................................T.............
K074     ............................................................T.............
K075     ............................................................T.............
K077     ............................................................T.............
K078     ............................................................T.............
K080     ............................................................T.............
K081     ............................................................T.............
K082     ............................................................T.............
K083     ............................................................T.............
K085     ............................................................T.............
K086     ............................................................T.............
K088     ............................................................T.............
K089     ............................................................T.............
K093     ............................................................T.............
K094     ............................................................T.............
K385     ............................................................T.............
K386     ............................................................T.............
K387     ............................................................T.............
K388     ............................................................T.............
K389     ............................................................T.............
K390     ............................................................T.............
K392     ............................................................T.............
K393     ............................................................T.............
K394     ............................................................T.............
K395     ............................................................T.............
K399     ............................................................T.............
K400     ............................................................T.............
K402     ............................................................T.............
K404     ............................................................T.............
K406     ............................................................T.............
K407     ............................................................T.............
K410     ............................................................T.............
K439     ............................................................T.............
K440     ............................................................T.............
K460     ............................................................T.............
K461     ............................................................T.............
K462     ............................................................T.............
K463     ............................................................T.............
K465     ............................................................T.............
K467     ............................................................T.............
K470     ............................................................T.............
K471     ............................................................T.............
K473     ............................................................T.............
K476     ............................................................T.............
K477     ............................................................T.............
K478     ............................................................T.............
K479     ............................................................T.............
K480     ............................................................T.............
```

```
NCPPB382 GTGCCGCAGGGGTCCGTGTACGGGTTCCTCGGGCCCAACGGCTCCGGCAAGTCCACGACCATGAAGATGCTGCTCGGG
K073     ...............................................................................
K074     ...............................................................................
K075     ...............................................................................
K077     ...........C...................................................................
K078     ...............................................................................
K080     ...............................................................................
K081     ...............................................................................
K082     ...............................................................................
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K088     ...........C...................................................................
K089     ...........C...................................................................
K093     ...............................................................................
K094     ...............................................................................
K385     ...............................................................................
K386     ...........C...................................................................
K387     ...........C...................................................................
K388     ...........C...................................................................
K389     ...............................................................................
K390     ...............................................................................
K392     ...............................................................................
K393     ...............................................................................
K394     ...............................................................................
K395     ...............................................................................
K399     ...........C...................................................................
K400     ...........C...................................................................
K402     ...............................................................................
K404     ...........C...................................................................
K406     ...........C...................................................................
K407     ...............................................................................
K410     ...........C...................................................................
K439     ...............................................................................
K440     ...............................................................................
K460     ...............................................................................
K461     ...............................................................................
K462     ...............................................................................
K463     ...............................................................................
K465     ...........C...................................................................
K467     ...........C...................................................................
K470     ...............................................................................
K471     ...............................................................................
K473     ...............................................................................
K476     ...............................................................................
K477     ...............................................................................
K478     ...............................................................................
K479     ...............................................................................
K480     ...............................................................................
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```
NCPPB382 CTCACGCAGCCCACGTCGGGCGAGATCCACCTGTTCGGCCAGCGGCTCACGCCGGCGACGCGCGGCGGGCTCCTGCCG
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K074     ...............................................................................
K075     ...............................................................................
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K081     ...............................................................................
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K465     ...............................................................................
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K473     ...............................................................................
K476     ...............................................................................
K477     ...............................................................................
K478     ...............................................................................
K479     ...............................................................................
K480     ...............................................................................
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```
NCPPB382 TCCATCGGCTCCATGATCGAGGCGCCCGCCGGCTACGGCCACCTCACGGGGTGGGAGAACATGCGGATCGTGCGCGAC
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K074     ........................................................................
K075     ........................................................................
K077     ........................................................................
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K470     ........................................................................
K471     ........................................................................
K473     ........................................................................
K476     ........................................................................
K477     ........................................................................
K478     ........................................................................
K479     ........................................................................
K480     ........................................................................
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```
NCPPB382 ATGCTCGGGCTCGCGGAGGCCCAGGTGGAGCGGGCGTTCGCGACCGTGCGGCTCACGCAGCACCGCGACAAGCTGGTG
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K074     ........................................................................................
K075     ........................................................................................
K077     ........................................................................................
K078     ........................................................................................
K080     ........................................................................................
K081     ........................................................................................
K082     ........................................................................................
K083     ........................................................................................
K085     ..........................................C.............................................
K086     ..........................................C.............................................
K088     ........................................................................................
K089     ..............................A.........................................................
K093     ........................................................................................
K094     ........................................................................................
K385     ........................................................................................
K386     ........................................................................................
K387     ........................................................................................
K388     ........................................................................................
K389     ........................................................................................
K390     ........................................................................................
K392     ........................................................................................
K393     ..........................................C.............................................
K394     ..........................................C.............................................
K395     ..........................................C.............................................
K399     ........................................................................................
K400     ........................................................................................
K402     ........................................................................................
K404     ........................................................................................
K406     ........................................................................................
K407     ........................................................................................
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K440     ........................................................................................
K460     ..........................................C.............................................
K461     ..........................................C.............................................
K462     ..........................................C.............................................
K463     ..........................................C.............................................
K465     ........................................................................................
K467     ........................................................................................
K470     ........................................................................................
K471     ........................................................................................
K473     ........................................................................................
K476     ........................................................................................
K477     ........................................................................................
K478     ........................................................................................
K479     ........................................................................................
K480     ........................................................................................
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```
NCPPB382 CGCCGGTACTCGCTCGGAATGAAGCAGCGGCTGGGGATCGCCATGGCCCTCGCCCGCGACCCCTCGCTGCTCGTCCTC
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K074     ...............................................................................
K075     ...............................................................................
K077     ...............................................................................
K078     ...............................................................................
K080     ...............................................................................
K081     ...............................................................................
K082     ...............................................................................
K083     ...............................................................................
K085     ..............A................................................T..................
K086     ..............A................................................T..................
K088     ...............................................................................
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K093     ...............................................................................
K094     ...............................................................................
K385     ...............................................................................
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K392     ...............................................................................
K393     ..............A................................................T..................
K394     ..............A................................................T..................
K395     ..............A................................................T..................
K399     ...............................................................................
K400     ...............................................................................
K402     ...............................................................................
K404     ...............................................................................
K406     ...............................................................................
K407     ...............................................................................
K410     ..............A................................................................
K439     ...............................................................................
K440     ...............................................................................
K460     ..............A................................................T..................
K461     ..............A................................................T..................
K462     ..............A................................................T..................
K463     ..............A................................................T..................
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K467     ...............................................................................
K470     ...............................................................................
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K473     ...............................................................................
K476     ...............................................................................
K477     ...............................................................................
K478     ...............................................................................
K479     ...............................................................................
K480     ...............................................................................
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```
NCPPB382 GACGAGCCCACCAACGGGCTGGATCCGGCGGGCATCGAGGAGGTCCGCTCCCTCCTCATGGACCTGGCCGGCCAGGGC
K073     ............................................................................
K074     ............................................................................
K075     ............................................................................
K077     ............................................................................
K078     ............................................................................
K080     ............................................................................
K081     ............................................................................
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K473     ............................................................................
K476     ............................................................................
K477     ............................................................................
K478     ............................................................................
K479     ............................................................................
K480     ............................................................................
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```
NCPPB382 ATCACCGTGATGGTCTCCAGCCACCTCCTCGACGAGATCGAGAAGATGGCGGGCGTGCTCGGCATCCTGGCCGACGGC
K073     ...............................................................................
K074     ...............................................................................
K075     ...............................................................................
K077     ...............................................................................
K078     ...............................................................................
K080     ...............................................................................
K081     ...............................................................................
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K093     ...............................................................................
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K387     ...............................................................................
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K473     ...............................................................................
K476     ...............................................................................
K477     ...............................................................................
K478     ...............................................................................
K479     ...............................................................................
K480     ...............................................................................
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```
NCPPB382 CGGATGATCTTCCAGGGCACGCGCGCCGAGCTGTTCGAGCACTCCATCCCCGACCTCGTCATCGAGACGTCCGCGCCG
K073     ................................................................................
K074     ................................................................................
K075     ................................................................................
K077     ................................................................................
K078     ................................................................................
K080     ................................................................................
K081     ................................................................................
K082     ................................................................................
K083     ................................................................................
K085     ................................................................................
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K088     ................................................................................
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K407     ................................................................................
K410     ................................................................................
K439     ................................................................................
K440     ................................................................................
K460     ................................................................................
K461     ................................................................................
K462     ................................................................................
K463     ................................................................................
K465     ................................................................................
K467     ............................................................................G...
K470     ................................................................................
K471     ................................................................................
K473     ................................................................................
K476     ................................................................................
K477     ................................................................................
K478     ................................................................................
K479     ................................................................................
K480     ................................................................................
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```
NCPPB382 GACCGGGCCATGGCGGAGGTCGCCGGGGCGGCCAGGAGCCCGGAGGGGATCCGGCTCAGCGCCCGCTCCAAGGACGAG
K073     ........................................................................
K074     ........................................................................
K075     ........................................................................
K077     ........................................................................
K078     ........................................................................
K080     ........................................................................
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K082     ........................................................................
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K470     ........................................................................
K471     ........................................................................
K473     ........................................................................
K476     ........................................................................
K477     ........................................................................
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K479     ........................................................................
K480     ........................................................................
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```
NCPPB382 ACCGCGGACATCGTGCGCCGGCTCGTGGCGGCCGACGTGCCCGTGCACGAGGTGCGGCGGCTGCCGCAGAGCCTGGAG
K073     ................................................................................
K074     ................................................................................
K075     ................................................................................
K077     ................................................................................
K078     ................................................................................
K080     ................................................................................
K081     ................................................................................
K082     ................................................................................
K083     ................................................................................
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K407     ................................................................................
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K460     ................................................................................
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K467     ................................................................................
K470     ................................................................................
K471     ................................................................................
K473     ................................................................................
K476     ................................................................................
K477     ................................................................................
K478     ................................................................................
K479     ................................................................................
K480     ................................................................................
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```
NCPPB382 GACGTGTTCATGGACATCACCGGGC
K073     .........................
K074     .........................
K075     .........................
K077     .........................
K078     .........................
K080     .........................
K081     .........................
K082     .........................
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K086     .........................
K088     .........................
K089     .........................
K093     .........................
K094     .........................
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K476     .........................
K477     .........................
K478     .........................
K479     .........................
K480     .........................
```

Figure D.3. *clvF* nucleotide sequence alignment. The positions of the nucleotide differences relative to that published for strain NCPPB 382 are highlighted in yellow.

```
NCPPB382 MSAVIATRGLTKRFRDHVAVDALDIEVPQGSVYGFLGPNGSGKSTTMKMLLGLTQPTSGEIHLFGQRLTPATRGGLLPSI
K073     ........................................................................................
K074     ........................................................................................
K075     ........................................................................................
K077     ........................................................................................
K078     ........................................................................................
K080     ........................................................................................
K081     ........................................................................................
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K473     ........................................................................................
K476     ........................................................................................
K477     ........................................................................................
K478     ........................................................................................
K479     ........................................................................................
K480     ........................................................................................
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```
NCPPB382 GSMIEAPAGYGHLTGWENMRIVRDMLGLAEAQVERAFATVRLTQHRDKLVRRYSLGMKQRLGIAMALARDPSLLVLDEPT
K073     ................................................................................
K074     ................................................................................
K075     ................................................................................
K077     ................................................................................
K078     ................................................................................
K080     ................................................................................
K081     ................................................................................
K082     ................................................................................
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K473     ................................................................................
K476     ................................................................................
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K478     ................................................................................
K479     ................................................................................
K480     ................................................................................
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```
NCPPB382 NGLDPAGIEEVRSLLMDLAGQGITVMVSSHLLDEIEKMAGVLGILADGRMIFQGTRAELFEHSIPDLVIETSAPDRAMAE
K073     ................................................................................
K074     ................................................................................
K075     ................................................................................
K077     ................................................................................
K078     ................................................................................
K080     ................................................................................
K081     ................................................................................
K082     ................................................................................
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K463     ................................................................................
K465     ................................................................................
K467     ...............................................................................G.......
K470     ................................................................................
K471     ................................................................................
K473     ................................................................................
K476     ................................................................................
K477     ................................................................................
K478     ................................................................................
K479     ................................................................................
K480     ................................................................................
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```
NCPPB382 VAGAARSPEGIRLSARSKDETADIVRRLVAADVPVHEVRRLPQSLEDVFMDITG
K073     ..................................................
K074     ..................................................
K075     ..................................................
K077     ..................................................
K078     ..................................................
K080     ..................................................
K081     ..................................................
K082     ..................................................
K083     ..................................................
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K470     ..................................................
K471     ..................................................
K473     ..................................................
K476     ..................................................
K477     ..................................................
K478     ..................................................
K479     ..................................................
K480     ..................................................
```

Figure D.4. *clvF* amino acid sequence alignment. The positions of the amino acid differences relative to that published for strain NCPPB 382 are highlighted in yellow.

```
NCPPB382 GTGAGGCGCGCCGTGGCGATCGAGTTCCGCAAGATGCACCGGCTCCGCTCGCTCCCGCTGCTCATCGGCATGGTGGTG
K073     ........................................................................
K074     ........................................................................
K075     ........................................................................
K077     ........................................................................
K078     ........................................................................
K080     ........................................................................
K081     ........................................................................
K082     ........................................................................
K083     ........................................................................
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K394     ........................................................................
K395     ........................................................................
K399     ........................................................................
K400     ........................................................................
K402     ........................................................................
K404     ........................................................................
K406     ........................................................................
K407     ........................................................................
K410     ..........................................................A.............
K439     ........................................................................
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K465     ........................................................................
K467     ........................................................................
K470     ........................................................................
K471     ........................................................................
K473     ........................................................................
K476     ........................................................................
K477     ........................................................................
K478     ........................................................................
K479     ........................................................................
K480     ........................................................................
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```
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K074     ................................................................................
K075     ................................................................................
K077     ................................................................................
K078     ................................................................................
K080     ................................................................................
K081     ................................................................................
K082     ................................................................................
K083     ................................................................................
K085     ................................................................................
K086     ................................................................................
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K093     ................................................................................
K094     ................................................................................
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K386     ................................................................................
K387     ................................................................................
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K389     ...............................................................................G....
K390     ...............................................................................G....
K392     ...............................................................................G....
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K394     ................................................................................
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K407     ...............................................................................G....
K410     ................................................................................
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K463     ................G...............................................................
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K467     ................................................................................
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K471     ...............................................................................G....
K473     ................................................................................
K476     ................................................................................
K477     ................................................................................
K478     ................................................................................
K479     ...............................................................................G....
K480     ................................................................................
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```
NCPPB382 TGGGCCGCGCTCCTGCTCGGCTACACGCTCATGGCGGCGATGACCTCGCCGATCCTCACCGCGGTGATCGCGAGCCGC
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K077     ................................................................................
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K480     ................................................................................
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```
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K473     ........................................................................
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K477     ........................................................................
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K479     ........................................................................
K480     ........................................................................
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```
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K094     ....................................................................................
K385     ....................................................................................
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K388     ...................................................................G................
K389     ...................................................................G................
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K392     ...................................................................G................
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K400     ...................................................................G................
K402     ...................................................................G................
K404     ...................................................................G................
K406     ...................................................................G................
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K479     ...................................................................G................
K480     ....................................................................................
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K074     ................................................................................
K075     ................................................................................
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K088     ................................................................................
K089     ................................................................................
K093     ........C.......................................................................
K094     ................................................................................
K385     ................................................................................
K386     ........C.......................................................................
K387     ........C.......................................................................
K388     ........C.......................................................................
K389     ........C.....................................................C..G..............
K390     ........C.....................................................C..G..............
K392     ........C.....................................................C..G..............
K393     ................................................................................
K394     ................................................................................
K395     ................................................................................
K399     ........C.......................................................................
K400     ........C.......................................................................
K402     ........C.....................................................C..G..............
K404     ........C.......................................................................
K406     ........C.......................................................................
K407     ................................................................................
K410     ................................................................................
K439     ........C.....................................................C..................
K440     ................................................................................
K460     ................................................................................
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K465     ................................................................................
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K471     ................................................................................
K473     ................................................................................
K476     ................................................................................
K477     ................................................................................
K478     ................................................................................
K479     ........C.....................................................C..G..............
K480     ................................................................................
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```
NCPPB382 TTCTGCGCGCTGCACGTCTGGCTCGCGGCGCGCGTCGAGAACCAGCTCATCAGCGTGGGCGTGGGGATGCTGGGCGCC
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K094     ........................................................................
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K386     ..........................................................C.............
K387     ..........................................................C.............
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K476     ........................................................................
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K479     ..........................................................C.............
K480     ........................................................................
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```
NCPPB382 TTCCTGGCCGTCTTCTCCCTCCTGCTCCCGTCCGTCGCGAGCCGGGCCATCCCGTGGGGGTACTACGCCGTCATCTCG
K073     ........................................................................................
K074     ..........................................C.....................................
K075     ........................................................................................
K077     ........................................................................................
K078     ..........................................C.....................................
K080     ........................................................................................
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K086     ..........................................C.....................................
K088     ..........................................C.....................................
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K093     ..........................................C.....................................
K094     ..........................................C.....................................
K385     ..........................................C.....................................
K386     ..........................................C.....................................
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K395     ..........................................C.....................................
K399     ..........................................C.....................................
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K406     ..........................................C.....................................
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K439     ........................................................................................
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K462     ..........................................C.....................................
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K467     ..........................................C.....................................
K470     ..........................................C.....................................
K471     ........................................................................................
K473     ..........................................C.....................................
K476     ..........................................C.....................................
K477     ..........................................C.....................................
K478     ..........................................C.....................................
K479     ........................................................................................
K480     ........................................................................................
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```
NCPPB382 CAGGCCGGCCAGTCGGACGCGGGCGTGGGCTACGTCGCCTCGCCGCTCGGGTGGATCGCCGGCTTCCTCGTCGTGGTC
K073     ...........................................................................T.........
K074     ..........................................C................................................
K075     ...........................................................................T.........
K077     ...........................................................................T.........
K078     ..........................................C................................................
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K082     ...........................................................................T.........
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K407     ...........................................................................T.........
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K440     ...........................................................................T.........
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K462     ..........................................C................................................
K463     ..........................................C................................................
K465     ..........................................................................................
K467     ..........................................C................................................
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K471     ..........................................................................................
K473     ..........................................C................................................
K476     ..........................................C................................................
K477     ..........................................C................................................
K478     ..........................................C................................................
K479     ...............................A..........C................................................
K480     ...........................................................................T.........
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```
NCPPB382 GCGGCGGCCTTCGCGCTCGCGACGATCCGGCTCGACCGCGTGGAGAGGTGA
K073     ..................................................
K074     ..................................................
K075     ..................................................
K077     ..................................................
K078     ..................................................
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K385     ..................................................
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K388     ..................................................
K389     ..........................A.......................
K390     ..........................A.......................
K392     ..........................A.......................
K393     ..................................................
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K400     ..................................................
K402     ..................................................
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K406     ..................................................
K407     ..................................................
K410     ..................................................
K439     ..........................A.......................
K440     ..................................................
K460     ..................................................
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K477     ..................................................
K478     ..................................................
K479     ..........................A.......................
K480     ..................................................
```

Figure D.5. *clvG* nucleotide sequence alignment. The positions of the nucleotide differences relative to that published for strain NCPPB 382 are highlighted in yellow.

```
NCPPB382 MRRAVAIEFRKMHRLRSLPLLIGMVVAVAALSSASQFAGSTRAGFDDPGAHPWAALLLGYTLMAAMTSPILTAVIASRQT
K073     ................................................................................
K074     ................................................................................
K075     ................................................................................
K077     ................................................................................
K078     ................................................................................
K080     ................................................................................
K081     ................................................................................
K082     ................................................................................
K083     ................................................................................
K085     ................................................................................
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K088     ................................................................................
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K093     ................................................................................
K094     ................................................................................
K385     ................................................................................
K386     ................................................................................
K387     ................................................................................
K388     ................................................................................
K389     ...............................................R................................
K390     ...............................................R................................
K392     ...............................................R................................
K393     ................................................................................
K394     ................................................................................
K395     ................................................................................
K399     ................................................................................
K400     ................................................................................
K402     ...............................................R................................
K404     ................................................................................
K406     ................................................................................
K407     ...............................................R................................
K410     ..................M.............................................................
K439     ................................................................................
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K465     ................................................................................
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K471     ...............................................R................................
K473     ................................................................................
K476     ................................................................................
K477     ................................................................................
K478     ................................................................................
K479     ...............................................R................................
K480     ................................................................................
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```
NCPPB382 DIEHQAGGWILAAGAGRTAGELCRAKLVALAVLLVPAVLIQSLLVIAAGYAAGIRVPVEVGPWALYTLLLYLVDVAFCAL
K073     ................................................................................
K074     ................................................................................
K075     ................................................................................
K077     ............T...................................................................
K078     ................................................................................
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K081     ................................................................................
K082     ................................................................................
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K085     ................................................................................
K086     ................................................................................
K088     ................................................................................
K089     ................................................................................
K093     ................................................................................
K094     ................................................................................
K385     ................................................................................
K386     ..............................................V.................................
K387     ..............................................V.................................
K388     ..............................................V.................................
K389     ..............................................V.................................
K390     ..............................................V.................................
K392     ..............................................V.................................
K393     ................................................................................
K394     ................................................................................
K395     ................................................................................
K399     ..............................................V.................................
K400     ..............................................V.................................
K402     ..............................................V.................................
K404     ..............................................V.................................
K406     ..............................................V.................................
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K463     ................................................................................
K465     ............T...................................................................
K467     ................................................................................
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K471     ................................................................................
K473     ................................................................................
K476     ................................................................................
K477     ................................................................................
K478     ................................................................................
K479     ..............................................V.................................
K480     ................................................................................
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```
NCPPB382 HVWLAARVENQLISVGVGMLGAFLAVFSLLLPSVASRAIPWGYYAVISQAGQSDAGVGYVASPLGWIAGFLVVVAAAFAL
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K075     ................................................................................
K077     ................................................................................
K078     ................................................................................
K080     ................................................................................
K081     ................................................................................
K082     ................................................................................
K083     ................................................................................
K085     ................................................................................
K086     ................................................................................
K088     ................................................................................
K089     ................................................................................
K093     ..............................................................D.................
K094     ................................................................................
K385     ................................................................................
K386     ................................................................................
K387     ................................................................................
K388     ................................................................................
K389     ..............................................................D.................
K390     ..............................................................D.................
K392     ..............................................................D.................
K393     ................................................................................
K394     ................................................................................
K395     ................................................................................
K399     ................................................................................
K400     ................................................................................
K402     ................................................................................
K404     ................................................................................
K406     ................................................................................
K407     ................................................................................
K410     ................................................................................
K439     ..............................................................D.................
K440     ................................................................................
K460     ................................................................................
K461     ................................................................................
K462     ................................................................................
K463     ................................................................................
K465     ................................................................................
K467     ................................................................................
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K473     ................................................................................
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K477     ................................................................................
K478     ................................................................................
K479     ..............................................................D.................
K480     ................................................................................
```

```
NCPPB382 ATIRLDRVER*
K073     ..........
K074     ..........
K075     ..........
K077     ..........
K078     ..........
K080     ..........
K081     ..........
K082     ..........
K083     ..........
K085     ..........
K086     ..........
K088     ..........
K089     ..........
K093     ..........
K094     ..........
K385     ..........
K386     ..........
K387     ..........
K388     ..........
K389     ..........
K390     ..........
K392     ..........
K393     ..........
K394     ..........
K395     ..........
K399     ..........
K400     ..........
K402     ..........
K404     ..........
K406     ..........
K407     ..........
K410     ..........
K439     ..........
K440     ..........
K460     ..........
K461     ..........
K462     ..........
K463     ..........
K465     ..........
K467     ..........
K470     ..........
K471     ..........
K473     ..........
K476     ..........
K477     ..........
K478     ..........
K479     ..........
K480     ..........
```

Figure D.6. *clvG* amino acid sequence alignment. The positions of the amino acid differences relative to that published for strain NCPPB 382 are highlighted in yellow.

```
K73     TTGGCTGCTGCGCGCAACGCAACTCCCACTCCCCAGAACGGTCGCGACGCATCGCGGCTCTCGTTCGCGAAGATCACT
K73Rif  ..........................................................................

K73     GACACCCTCACCGTCCCCGACCTCCTCGCCCTGCAGACCGAGAGCTTCGACTGGCTCGTCGGCTCGGACGCGTGGAAG
K73Rif  ..........................................................................

K73     CGGCGCGTCGAGGAGGGCACCGCGCAGGGTCGCACCGACCTGGCGCTCAACTCGGGCCTCGAGGAGATCTTCGAGGAG
K73Rif  ..........................................................................

K73     ATCTCCCCCATCGAGGACCTGGGCGAGACCATGCAGCTCGGGTTCACCAACCCGTACCTCGAGGAGCAGAAGTACTCC
K73Rif  ..........................................................................

K73     ATCGACGAGTGCAAGGAGCGCGGCAAGACCTACTCCGCTCCCCTCTACGTCGAGGCCGAGTTCATGAACCACCTCACG
K73Rif  ..........................................................................

K73     GGTGAGATCAAGACCCAGACGGTCTTCATGGGCGACTTCCCCCTCATGACGGAGAAGGGCACGTTCATCATCAACGGC
K73Rif  ..........................................................................

K73     ACCGAGCGTGTCGTCGTGTCCCAGCTCGTCCGCTCGCCCGGCGTGTACTTCGAGCGCCAGCAGGAGAAGACCTCCGAC
K73Rif  ..........................................................................

K73     AAGGACATCTACTCCGCCCGCGTCATCCCGTCCCGCGGCGCCTGGCTCGAGTTCGAGATCGACAAGCGCGACCAGGTC
K73Rif  ..........................................................................

K73     GGCGTGCGCATCGACCGCAAGCGCAAGCAGTCGGTCACCGTGTTCCTGAAGGCCCTCGGCCTCACCAGCGAGCAGATC
K73Rif  ..........................................................................

K73     CTCGAGGAGTTCAAGGGCGTCGCGTCCATCGAGCTCACGCTCGAGAAGGACTCCATCCTCACCAAGGAGGAGGCCCTC
K73Rif  ..........................................................................

K73     AAGGACATCTACCGCAAGCTCCGTCCCGGCGAGCAGGTCGCCGCCGAGGCCGCCCGCGCGCTGCTGGACAACTTCTAC
K73Rif  ..........................................................................

K73     TTCAACCCGAAGCGCTACGACCTCGCGAAGGTGGGTCGCTACAAGATCAACCGCAAGCTCGGCATCGACAAGCAGCTC
K73Rif  ..........................................................................

K73     ACCGACTCGGTGCTGACGGTCGAGGACATCCTCGCGACCATCAAGTACCTCGTCTCGCTGCACGCGAACGAGACGAAG
K73Rif  ..........................................................................

K73     ATGAACGGCACGCGCGACGGCAAGCCCGTCGAGCTGCGCCTCGACGTGGACGACATCGACCACTTCGGCAACCGTCGC
K73Rif  ..........................................................................

K73     ATCCGCGCGGTCGGCGAGCTCATCCAGAACCAGGTGCGCACCGGCCTGTCCCGCATGGAGCGCGTCGTCCGCGAGCGC
K73Rif  ..........................................................................

K73     ATGACCACGCAGGACATCGAGGCCATCACGCCGCAGACCCTGATCAACGTGCGCCCCGTCGTCGCCGCGATCAAGGAG
K73Rif  ..........................................................................

K73     TTCTTCGGCACGAGCCAGCTGTCGCAGTTCATGGACCAGAACAATCCGCTCGCGGGCCTCACCCACAAGCGCCGCCTC
K73Rif  ..........................................................TG..............

K73     TCGGCGCTCGGCCCGGGTGGTCTGTCCCGTGAGCGCGCCGGCGTCGAGGTCCGCGACGTCCACCCGTCGCACTACGGC
K73Rif  ..........................................................................

K73     CGCATGTGCCCCATCGAGACCCCGGAAGGCCCGAACATCGGCCTGATCGGCTCGCTGGCGTCGTTCGCCCGCATCAAC
K73Rif  ..........................................................................

K73     TCGTTCGGCTTCATCGAGACCCCGTACCGCCGCGTCGTCGACGGCGTGGTCACGGACACGATCGACTACCTCACGGCC
K73Rif  ..........................................................................

K73     AGCGAGGAGGACGAGTTCCTCGTCGCCCAGGCCAACGCGCCCCTCACGAAGGACTTCCGCTTCGCGGAGGACCGCGTC
K73Rif  ..........................................................................

K73     CTCGTCCGCCCCAAGGGCGGTGAGGTGGAGCTCGTCGCGAAGGAGAACGTCCACTACATGGACGTCTCCCCGCGCCAG
K73Rif  ..........................................................................

K73     ATGGTGTCGGTCGCGACCTCGCTCATCCCGTTCCTCGAGCACGACGACGCGAACCGGGCCCTCATGGGCGCGAACATG
K73Rif  ..........................................................................

K73     CAGCGTCAGGCCGTCCCGCTGCTGCGCAGCGAGAGCCCGCTCGTCGGCACCGGCATGGAGGGCTACGCGGCGATCGAC
K73Rif  ..........................................................................
```

```
K73    GCCGGCGACGTCCTCACCGCCGACGCCTCGGGCGTCGTGCAAGAGGTGTCGGCCGAGGTCGTCACCATCCAGCTCGAC
K73Rif ..........................................................................

K73    GAGGGCGGCACGCAGACGTACTACCTGCGCAAGTTCGACCGCTCCAACCAGGGCACGAGCTACAACCACCGCGTCCTG
K73Rif ..........................................................................

K73    GTCTCGGCCGGCGACCGCATCGAGGCCGGCGAGGTCATCGCCGACGGTCCCGCCACGGAGAACGGCGAGCTCGCGCTC
K73Rif ..........................................................................

K73    GGCAAGAACCTGCTCGTCGCGTTCATGCCGTGGGAGGGCCACAACTTCGAGGACGCGATCATCCTGAGCCAGAACCTG
K73Rif ..........................................................................

K73    GTTAAGGACGACACCCTCTCCTCCATCCACATCGAGGAGTACGAGGTCGACGCGCGCGACACCAAGCTCGGCAAGGAG
K73Rif ..........................................................................

K73    GAGATCACCCGCGACCTCCCCAACGTCAGCCCGGAGCTGCTCGCCGACCTCGACGAGCGCGGCATCATCCGCATCGGC
K73Rif ..........................................................................

K73    GCCGAGGTCCGCCCCGGCGACATCCTCGTGGGCAAGGTCACGCCGAAGGGCGAGACCGAGCTCAGCGCCGAGGAGCGC
K73Rif ..........................................................................

K73    CTGCTGCGCGCGATCTTCAACGAGAAGAGCCGCGAGGTCCGCGACACGTCCCTGAAGGTGCCCCACGGCGAGCAGGGC
K73Rif ..........................................................................

K73    ACGATCATCGGCGTCAAGGTCTTCGACTCGCAGGACGGCGACGACGAGCTCGGCTCCGGCGTCAACCAGCGCGTCGTG
K73Rif ..........................................................................

K73    GTGTTCATCGCGCAGAAGCGCAAGATCACCGAGGGCGACAAGCTCGCCGGCCGTCACGGCAACAAGGGCGTCATCTCC
K73Rif ..........................................................................

K73    AAGATCCTGCCGGTCGAGGACATGCCGTTCCTCGCCGACGGGACCCCGGTCGACGTCATCCTCAATCCGCTCGGCATC
K73Rif ..........................................................................

K73    CCCGGCCGCATGAACTTCGGCCAGGTCCTGGAGACCCACCTCGGGTGGATCGCCAAGCAGGGCTGGGAGGTCGAGGGC
K73Rif ..........................................................................

K73    AAGCCGAAGTGGGCCGAGCGCCTGCCGGACCACGCGCGCCAGGCCCCGGCCGGCACGAAGGTCGCCACCCCGGTGTTC
K73Rif ..........................................................................

K73    GACGGAGCGCTCGAGGAGGAGATCGCCGGCCTGCTCGACTCGACGACGGTCACCCGCGACGGCGACCGCCTCATCGGG
K73Rif ..........................................................................

K73    TCCAGCGGCAAGACGCGCCTGTTCGACGGCCGCTCCGGCGAGCCGTTCCCGGAGCCCGTCTCGGTCGGCTACATGTAC
K73Rif ..........................................................................

K73    ATCCTGAAGCTGCACCACCTGGTGGACGACAAGATCCACGCGCGCTCCACGGGTCCCTACTCGATGATCACGCAGCAG
K73Rif ..........................................................................

K73    CCCCTGGGCGGTAAGGCCCAGTTCGGCGGCCAGCGGTTCGGCGAGATGGAGGTCTGGGCGCTCGAGGCCTACGGCGCC
K73Rif ..........................................................................

K73    GCGTACGCGCTGCAGGAGCTCCTCACCATCAAGTCGGACGACATCCTCGGCCGCGTGAAGGTGTACGAGGCCATCGTC
K73Rif ..........................................................................

K73    AAGGGCGAGAACATCCAGGAACCGGGTATCCCCGAGTCCTTCAAGGTCCTGATCAAGGAGATGCAGTCCCTCTGCCTG
K73Rif ..........................................................................

K73    AACGTCGAGGTCCTCTCGGCCGACGGCCAGGCGGTCAGCCTGCGCGACACGGATGACGAGGTCTTCCGCGCGGCGGAG
K73Rif ..........................................................................

K73    GAGCTCGGCATCAACATCTCCACCCGCTTCGAGTCGTCCAGCATCGACGACATCTAA
K73Rif ..................................................
```

Figure D.7. *rpoB* nucleotide sequence alignment for K73 and K73Rif. K73Rif contains a C→T and A→G transition at positions 1312 and 1313 (highlighted yellow), respectively.

```
K73    MAAARNATPTPQNGRDASRLSFAKITDTLTVPDLLALQTESFDWLVGSDAWKRRVEEGTAQGRTDLALNSGLEEIFEEIS
K73Rif ................................................................................

K73    PIEDLGETMQLGFTNPYLEEQKYSIDECKERGKTYSAPLYVEAEFMNHLTGEIKTQTVFMGDFPLMTEKGTFIINGTERV
K73Rif ................................................................................

K73    VVSQLVRSPGVYFERQQEKTSDKDIYSARVIPSRGAWLEFEIDKRDQVGVRIDRKRKQSVTVFLKALGLTSEQILEEFKG
K73Rif ................................................................................

K73    VASIELTLEKDSILTKEEALKDIYRKLRPGEQVAAEAARALLDNFYFNPKRYDLAKVGRYKINRKLGIDKQLTDSVLTVE
K73Rif ................................................................................

K73    DILATIKYLVSLHANETKMNGTRDGKPVELRLDVDDIDHFGNRRIRAVGELIQNQVRTGLSRMERVVRERMTTQDIEAIT
K73Rif ................................................................................

K73    PQTLINVRPVVAAIKEFFGTSQLSQFMDQNNPLAGLTHKRRLSALGPGGLSRERAGVEVRDVHPSHYGRMCPIETPEGPN
K73Rif ....................................C...........................................

K73    IGLIGSLASFARINSFGFIETPYRRVVDGVVTDTIDYLTASEEDEFLVAQANAPLTKDFRFAEDRVLVRPKGGEVELVAK
K73Rif ................................................................................

K73    ENVHYMDVSPRQMVSVATSLIPFLEHDDANRALMGANMQRQAVPLLRSESPLVGTGMEGYAAIDAGDVLTADASGVVQEV
K73Rif ................................................................................

K73    SAEVVTIQLDEGGTQTYYLRKFDRSNQGTSYNHRVLVSAGDRIEAGEVIADGPATENGELALGKNLLVAFMPWEGHNFED
K73Rif ................................................................................

K73    AIILSQNLVKDDTLSSIHIEEYEVDARDTKLGKEEITRDLPNVSPELLADLDERGIIRIGAEVRPGDILVGKVTPKGETE
K73Rif ................................................................................

K73    LSAEERLLRAIFNEKSREVRDTSLKVPHGEQGTIIGVKVFDSQDGDDELGSGVNQRVVVFIAQKRKITEGDKLAGRHGNK
K73Rif ................................................................................

K73    GVISKILPVEDMPFLADGTPVDVILNPLGIPGRMNFGQVLETHLGWIAKQGWEVEGKPKWAERLPDHARQAPAGTKVATP
K73Rif ................................................................................

K73    VFDGALEEEIAGLLDSTTVTRDGDRLIGSSGKTRLFDGRSGEPFPEPVSVGYMYILKLHHLVDDKIHARSTGPYSMITQQ
K73Rif ................................................................................

K73    PLGGKAQFGGQRFGEMEVWALEAYGAAYALQELLTIKSDDILGRVKVYEAIVKGENIQEPGIPESFKVLIKEMQSLCLNV
K73Rif ................................................................................

K73    EVLSADGQAVSLRDTDDEVFRAAEELGINISTRFESSSIDDI*
K73Rif ........................................
```

Figure D.8. *rpoB* amino acid sequence alignment for K73 and K73Rif. K73Rif contains an H→C amino acid change at position 438 (highlighted yellow).

```
A4775     TTGGCTGCTGCGCGCAACGCAACTCCCACTCCCCAGAACGGTCGCGACGCATCGCGGCTCTCGTTCGCGAAGATCACT
A4775Rif  ..........................................................................

A4775     GACACCCTCACCGTCCCCGACCTCCTCGCCCTGCAGACCGAGAGCTTCGACTGGCTCGTCGGCTCGGACGCGTGGAAG
A4775Rif  ..........................................................................

A4775     CGGCGCGTCGAGGAGGGCACGAAGCAGGGTCGCACCGACCTGGCGCTCAACTCGGGCCTCGAGGAGATCTTCGAGGAG
A4775Rif  ..........................................................................

A4775     ATCTCCCCCATCGAGGACCTGGGCGAGACCATGCAGCTCGGGTTCACCAACCCGTACCTCGAGGAGCAGAAGTACTCC
A4775Rif  ..........................................................................

A4775     ATCGACGAGTGCAAGGAGCGCGGCAAGACCTACTCCGCTCCCCTCTACGTCGAGGCCGAGTTCATGAACCACCTCACG
A4775Rif  ..........................................................................

A4775     GGTGAGATCAAGACCCAGACGGTCTTCATGGGCGACTTCCCCCTCATGACGGAGAAGGGCACGTTCATCATCAACGGC
A4775Rif  ..........................................................................

A4775     ACCGAGCGTGTCGTCGTGTCCCAGCTCGTCCGCTCGCCCGGCGTGTACTTCGAGCGCCAGCAGGAGAAGACCTCCGAC
A4775Rif  ..........................................................................

A4775     AAGGACATCTACTCCGCCCGCGTCATCCCGTCCCGCGGCGCCTGGCTCGAGTTCGAGATCGACAAGCGCGACCAGGTC
A4775Rif  ..........................................................................

A4775     GGCGTGCGCATCGACCGCAAGCGCAAGCAGTCGGTCACCGTGTTCCTGAAGGCCCTCGGCCTCACCAGCGAGCAGATC
A4775Rif  ..........................................................................

A4775     CTCGAGGAGTTCAAGGGCGTCGCGTCCATCGAGCTCACGCTCGAGAAGGACTCCATCCTCACCAAGGAGGAGGCCCTC
A4775Rif  ..........................................................................

A4775     AAGGACATCTACCGCAAGCTCCGTCCCGGCGAGCAGGTCGCCGCCGAGGCCGCCCGCGCGCTGCTGGACAACTTCTAC
A4775Rif  ..........................................................................

A4775     TTCAACCCGAAGCGCTACGACCTGGCGAAGGTGGGTCGCTACAAGATCAACCGCAAGCTCGGCATCGACAAGCAGCTC
A4775Rif  ..........................................................................

A4775     ACCGACTCGGTGCTGACGGTCGAGGACATCCTCGCGACCATCAAGTACCTCGTCTCGCTGCACGCGAACGAGACGAAG
A4775Rif  ..........................................................................

A4775     ATGAACGGCACGCGCGACGGCAAGCCCGTCGAGCTGCGCCTCGACGTGGACGACATCGACCACTTCGGCAACCGTCGC
A4775Rif  ..........................................................................

A4775     ATCCGCGCGGTCGGCGAGCTCATCCAGAACCAGGTGCGCACCGGCCTGTCCCGCATGGAGCGCGTCGTCCGCGAGCGC
A4775Rif  ..........................................................................

A4775     ATGACCACGCAGGACATCGAGGCCATCACGCCGCAGACCCTGATCAACGTGCGCCCCGTCGTCGCCGCGATCAAGGAG
A4775Rif  ..........................................................................

A4775     TTCTTCGGCACGAGCCAGCTGTCGCAGTTCATGGACCAGAACAACCCGCTCGCGGGCCTCACCCACAAGCGCCGCCTC
A4775Rif  ..........................................................G.............

A4775     TCGGCGCTCGGCCCGGGTGGTCTGTCCCGTGAGCGCGCCGGCGTCGAGGTCCGCGACGTCCACCCGTCGCACTACGGC
A4775Rif  ..........................................................................

A4775     CGCATGTGCCCCATCGAGACCCCGGAAGGCCCGAACATCGGCCTGATCGGCTCGCTGGCGTCGTTCGCCCGCATCAAC
A4775Rif  ..........................................................................

A4775     TCGTTCGGCTTCATCGAGACCCCGTACCGTCGCGTCGTCGACGGCGTGGTCACGGACACGATCGACTACCTCACGGCC
A4775Rif  ..........................................................................

A4775     AGCGAGGAGGACGAGTTCCTCGTCGCCCAGGCCAACGCGCCCCTCACGAAGGACTTCCGCTTCGCGGAGGACCGCGTC
A4775Rif  ..........................................................................

A4775     CTCGTCCGCCCCAAGGGCGGTGAGGTGGAGCTCGTCGCGAAGGAGAACGTCCACTACATGGACGTCTCCCCGCGCCAG
A4775Rif  ..........................................................................

A4775     ATGGTGTCGGTCGCGACCTCGCTCATCCCGTTCCTCGAGCACGACGACGCGAACCGGGCCCTCATGGGCGCGAACATG
A4775Rif  ..........................................................................

A4775     CAGCGTCAGGCCGTCCCGCTGCTGCGCAGCGAGAGCCCGCTCGTCGGCACCGGCATGGAGGGCTACGCGGCGATCGAC
A4775Rif  ..........................................................................
```

```
A4775     GCCGGCGACGTCCTCACCGCCGACGCCTCGGGCGTCGTGCAGGAGGTGTCGGCCGAGGTCGTCACCATCCAGCTCGAC
A4775Rif  ................................................................................

A4775     GAGGGCGGCACGCAGACGTACTACCTGCGCAAGTTCGACCGCTCCAACCAGGGCACGAGCTACAACCACCGCGTCCTG
A4775Rif  ................................................................................

A4775     GTCTCGGCCGGCGACCGCATCGAGGCCGGCGAGGTCATCGCCGACGGCCCCGCCACGGAGAACGGCGAGCTCGCGCTC
A4775Rif  ................................................................................

A4775     GGCAAGAACCTGCTCGTCGCGTTCATGCCGTGGGAGGGCCACAACTTCGAGGACGCGATCATCCTGAGCCAGAACCTG
A4775Rif  ................................................................................

A4775     GTCAAGGACGACACCCTCTCCTCCATCCACATCGAGGAGTACGAGGTCGACGCGCGCGACACCAAGCTCGGCAAGGAG
A4775Rif  ................................................................................

A4775     GAGATCACCCGCGACCTCCCCAACGTCAGCCCGGAGCTGCTCGCCGACCTCGACGAGCGCGGCATCATCCGCATCGGC
A4775Rif  ................................................................................

A4775     GCCGAGGTCCGCCCCGGCGACATCCTCGTGGGCAAGGTCACGCCGAAGGGCGAGACCGAGCTCAGCGCCGAGGAGCGC
A4775Rif  ................................................................................

A4775     CTGCTGCGCGCGATCTTCAACGAGAAGAGCCGCGAGGTCCGCGACACGTCCCTGAAGGTGCCCCACGGCGAGCAGGGC
A4775Rif  ................................................................................

A4775     ACGATCATCGGCGTCAAGGTCTTCGACTCGCAGGACGGCGACGACGAGCTCGGCTCCGGCGTCAACCAGCGCGTCGTG
A4775Rif  ................................................................................

A4775     GTGTTCATCGCGCAGAAGCGCAAGATCACCGAGGGCGACAAGCTCGCCGGCCGTCACGGCAACAAGGGCGTCATCTCC
A4775Rif  ................................................................................

A4775     AAGATCCTGCCGGTCGAGGACATGCCGTTCCTCGCCGACGGGACCCCGGTCGACGTCATCCTCAATCCGCTCGGCATC
A4775Rif  ..........................................T.....................................

A4775     CCCGGCCGCATGAACTTCGGCCAGGTCCTGGAGACCCACCTCGGGTGGATCGCCAAGCAGGGCTGGGAGGTCGAGGGC
A4775Rif  ................................................................................

A4775     AAGCCGAAGTGGGCCGAGCGCCTGCCGGACCACGCGCGCCAGGCTCCGGCCGGCACGAAGGTCGCCACCCCGGTGTTC
A4775Rif  ................................................................................

A4775     GACGGAGCGCTCGAGGAGGAGATCGCCGGCCTGCTCGACTCGACGACGGTCACCCGCGACGGCGACCGCCTCATCGGG
A4775Rif  ................................................................................

A4775     TCCAGCGGCAAGACGCGCCTGTTCGACGGCCGCTCCGGCGAGCCGTTCCCGGAGCCCGTCTCGGTCGGCTACATGTAC
A4775Rif  ................................................................................

A4775     ATCCTGAAGCTGCACCACCTGGTGGACGACAAGATCCACGCGCGCTCCACGGGTCCCTACTCGATGATCACGCAGCAG
A4775Rif  ................................................................................

A4775     CCCCTGGGCGGTAAGGCCCAGTTCGGCGGCCAGCGGTTCGGCGAGATGGAGGTCTGGGCGCTCGAGGCCTACGGCGCC
A4775Rif  ................................................................................

A4775     GCGTACGCGCTGCAGGAGCTCCTCACCATCAAGTCGGACGACATCCTCGGCCGCGTGAAGGTGTACGAGGCCATCGTC
A4775Rif  ................................................................................

A4775     AAGGGCGAGAACATCCAGGAACCGGGTATCCCCGAGTCCTTCAAGGTCCTGATCAAGGAGATGCAGTCCCTCTGCCTG
A4775Rif  ................................................................................

A4775     AACGTCGAGGTCCTCTCGGCCGACGGCCAGGCGGTCAGCCTGCGCGACACGGATGACGAGGTCTTCCGCGCGGCGGAG
A4775Rif  ................................................................................

A4775     GAGCTCGGCATCAACATCTCCACCCGCTTCGAGTCGTCCAGCATCGACGACATCTAA
A4775Rif  ............................................................
```

Figure D.9. *rpoB* nucleotide sequence alignment for A4775 and A4775Rif. A4775Rif contains an A→G and C→T transition at positions 1313 and 2697 (highlighted yellow), respectively.

```
A4775    MAAARNATPTPQNGRDASRLSFAKITDTLTVPDLLALQTESFDWLVGSDAWKRRVEEGTKQGRTDLALNSGLEEIFEEIS
A4775Rif ................................................................................

A4775    PIEDLGETMQLGFTNPYLEEQKYSIDECKERGKTYSAPLYVEAEFMNHLTGEIKTQTVFMGDFPLMTEKGTFIINGTERV
A4775Rif ................................................................................

A4775    VVSQLVRSPGVYFERQQEKTSDKDIYSARVIPSRGAWLEFEIDKRDQVGVRIDRKRKQSVTVFLKALGLTSEQILEEFKG
A4775Rif ................................................................................

A4775    VASIELTLEKDSILTKEEALKDIYRKLRPGEQVAAEAARALLDNFYFNPKRYDLAKVGRYKINRKLGIDKQLTDSVLTVE
A4775Rif ................................................................................

A4775    DILATIKYLVSLHANETKMNGTRDGKPVELRLDVDDIDHFGNRRIRAVGELIQNQVRTGLSRMERVVRERMTTQDIEAIT
A4775Rif ................................................................................

A4775    PQTLINVRPVVAAIKEFFGTSQLSQFMDQNNPLAGLTHKRRLSALGPGGLSRERAGVEVRDVHPSHYGRMCPIETPEGPN
A4775Rif ................................R...............................................

A4775    IGLIGSLASFARINSFGFIETPYRRVVDGVVTDTIDYLTASEEDEFLVAQANAPLTKDFRFAEDRVLVRPKGGEVELVAK
A4775Rif ................................................................................

A4775    ENVHYMDVSPRQMVSVATSLIPFLEHDDANRALMGANMQRQAVPLLRSESPLVGTGMEGYAAIDAGDVLTADASGVVQEV
A4775Rif ................................................................................

A4775    SAEVVTIQLDEGGTQTYYLRKFDRSNQGTSYNHRVLVSAGDRIEAGEVIADGPATENGELALGKNLLVAFMPWEGHNFED
A4775Rif ................................................................................

A4775    AIILSQNLVKDDTLSSIHIEEYEVDARDTKLGKEEITRDLPNVSPELLADLDERGIIRIGAEVRPGDILVGKVTPKGETE
A4775Rif ................................................................................

A4775    LSAEERLLRAIFNEKSREVRDTSLKVPHGEQGTIIGVKVFDSQDGDDELGSGVNQRVVVFIAQKRKITEGDKLAGRHGNK
A4775Rif ................................................................................

A4775    GVISKILPVEDMPFLADGTPVDVILNPLGIPGRMNFGQVLETHLGWIAKQGWEVEGKPKWAERLPDHARQAPAGTKVATP
A4775Rif ................................................................................

A4775    VFDGALEEEIAGLLDSTTVTRDGDRLIGSSGKTRLFDGRSGEPFPEPVSVGYMYILKLHHLVDDKIHARSTGPYSMITQQ
A4775Rif ................................................................................

A4775    PLGGKAQFGGQRFGEMEVWALEAYGAAYALQELLTIKSDDILGRVKVYEAIVKGENIQEPGIPESFKVLIKEMQSLCLNV
A4775Rif ................................................................................

A4775    EVLSADGQAVSLRDTDDEVFRAAEELGINISTRFESSSIDDI*
A4775Rif ....................................................
```

Figure D.10. *rpoB* amino acid sequence alignment for A4775 and A4775Rif. A4775Rif contains an H→R amino acid change at position 438 (highlighted yellow).

```
A4818     TTGGCTGCTGCGCGCAACGCAACTCCCACTCCCCAGAACGGTCGCGACGCATCGCGGCTCTCGTTCGCGAAGATCACT
A4818Rif  ..........................................................................

A4818     GACACCCTCACCGTCCCCGACCTCCTCGCCCTGCAGACCGAGAGCTTCGACTGGCTCGTCGGCTCGGACGCGTGGAAG
A4818Rif  ..........................................................................

A4818     CGGCGCGTCGAGGAGGGCACGAAGCAGGGTCGCACCGACCTGGCGCTCAACTCGGGCCTCGAGGAGATCTTCGAGGAG
A4818Rif  ..........................................................................

A4818     ATCTCCCCCATCGAGGACCTGGGCGAGACCATGCAGCTCGGGTTCACCAACCCGTACCTCGAGGAGCAGAAGTACTCC
A4818Rif  ..........................................................................

A4818     ATCGACGAGTGCAAGGAGCGCGGCAAGACCTACTCCGCTCCCCTCTACGTCGAGGCCGAGTTCATGAACCACCTCACG
A4818Rif  ..........................................................................

A4818     GGTGAGATCAAGACCCAGACGGTCTTCATGGGCGACTTCCCCCTCATGACGGAGAAGGGCACGTTCATCATCAACGGC
A4818Rif  ..........................................................................

A4818     ACCGAGCGTGTCGTCGTGTCCCAGCTCGTCCGCTCGCCCGGCGTGTACTTCGAGCGCCAGCAGGAGAAGACCTCCGAC
A4818Rif  ..........................................................................

A4818     AAGGACATCTACTCCGCCCGCGTCATCCCGTCCCGCGGCGCCTGGCTCGAGTTCGAGATCGACAAGCGCGACCAGGTC
A4818Rif  ..........................................................................

A4818     GGCGTGCGCATCGACCGCAAGCGCAAGCAGTCGGTCACCGTGTTCCTGAAGGCCCTCGGCCTCACCAGCGAGCAGATC
A4818Rif  ..........................................................................

A4818     CTCGAGGAGTTCAAGGGCGTCGCGTCCATCGAGCTCACGCTCGAGAAGGACTCCATCCTCACCAAGGAGGAGGCCCTC
A4818Rif  ..........................................................................

A4818     AAGGACATCTACCGCAAGCTCCGTCCCGGCGAGCAGGTCGCCGCCGAGGCCGCCCGCGCGCTGCTGGACAACTTCTAC
A4818Rif  ..........................................................................

A4818     TTCAACCCGAAGCGCTACGACCTGGCGAAGGTGGGTCGCTACAAGATCAACCGCAAGCTCGGCATCGACAAGCAGCTC
A4818Rif  ..........................................................................

A4818     ACCGACTCGGTGCTGACGGTCGAGGACATCCTCGCGACCATCAAGTACCTCGTCTCGCTGCACGCGAACGAGACGAAG
A4818Rif  ..........................................................................

A4818     ATGAACGGCACGCGCGACGGCAAGCCCGTCGAGCTGCGCCTCGACGTGGACGACATCGACCACTTCGGCAACCGTCGC
A4818Rif  ..........................................................................

A4818     ATCCGCGCGGTCGGCGAGCTCATCCAGAACCAGGTGCGCACCGGCCTGTCCCGCATGGAGCGCGTCGTCCGCGAGCGC
A4818Rif  ..........................................................................

A4818     ATGACCACGCAGGACATCGAGGCCATCACGCCGCAGACCCTGATCAACGTGCGCCCCGTCGTCGCCGCGATCAAGGAG
A4818Rif  ..........................................................................

A4818     TTCTTCGGCACGAGCCAGCTGTCGCAGTTCATGGACCAGAACAACCCGCTCGCGGGCCTCACCCACAAGCGCCGCCTC
A4818Rif  ......................T...................................................

A4818     TCGGCGCTCGGCCCGGGTGGTCTGTCCCGTGAGCGCGCCGGCGTCGAGGTCCGCGACGTCCACCCGTCGCACTACGGC
A4818Rif  ..........................................................................

A4818     CGCATGTGCCCCATCGAGACCCCGGAAGGCCCGAACATCGGCCTGATCGGCTCGCTGGCGTCGTTCGCCCGCATCAAC
A4818Rif  ..........................................................................

A4818     TCGTTCGGCTTCATCGAGACCCCGTACCGTCGCGTCGTCGACGGCGTGGTCACGGACACGATCGACTACCTCACGGCC
A4818Rif  ..........................................................................

A4818     AGCGAGGAGGACGAGTTCCTCGTCGCCCAGGCCAACGCGCCCCTCACGAAGGACTTCCGCTTCGCGGAGGACCGCGTC
A4818Rif  ..........................................................................

A4818     CTCGTCCGCCCCAAGGGCGGTGAGGTGGAGCTCGTCGCGAAGGAGAACGTCCACTACATGGACGTCTCCCCGCGCCAG
A4818Rif  ..........................................................................

A4818     ATGGTGTCGGTCGCGACCTCGCTCATCCCGTTCCTCGAGCACGACGACGCGAACCGGGCCCTCATGGGCGCGAACATG
A4818Rif  ..........................................................................

A4818     CAGCGTCAGGCCGTCCCGCTGCTGCGCAGCGAGAGCCCGCTCGTCGGCACCGGCATGGAGGGCTACGCGGCGATCGAC
A4818Rif  ..........................................................................
```

```
A4818     GCCGGCGACGTCCTCACCGCCGACGCCTCGGGCGTCGTGCAGGAGGTGTCGGCCGAGGTCGTCACCATCCAGCTCGAC
A4818Rif  ..........................................................................

A4818     GAGGGCGGCACGCAGACGTACTACCTGCGCAAGTTCGACCGCTCCAACCAGGGCACGAGCTACAACCACCGCGTCCTG
A4818Rif  ..........................................................................

A4818     GTCTCGGCCGGCGACCGCATCGAGGCCGGCGAGGTCATCGCCGACGGCCCCGCCACGGAGAACGGCGAGCTCGCGCTC
A4818Rif  ..........................................................................

A4818     GGCAAGAACCTGCTCGTCGCGTTCATGCCGTGGGAGGGCCACAACTTCGAGGACGCGATCATCCTGAGCCAGAACCTG
A4818Rif  ..........................................................................

A4818     GTCAAGGACGACACCCTCTCCTCCATCCACATCGAGGAGTACGAGGTCGACGCGCGCGACACCAAGCTCGGCAAGGAG
A4818Rif  ..........................................................................

A4818     GAGATCACCCGCGACCTCCCCAACGTCAGCCCGGAGCTGCTCGCCGACCTCGACGAGCGCGGCATCATCCGCATCGGC
A4818Rif  ..........................................................................

A4818     GCCGAGGTCCGCCCCGGCGACATCCTCGTGGGCAAGGTCACGCCGAAGGGCGAGACCGAGCTCAGCGCCGAGGAGCGC
A4818Rif  ..........................................................................

A4818     CTGCTGCGCGCGATCTTCAACGAGAAGAGCCGCGAGGTCCGCGACACGTCCCTGAAGGTGCCCCACGGCGAGCAGGGC
A4818Rif  ..........................................................................

A4818     ACGATCATCGGCGTCAAGGTCTTCGACTCGCAGGACGGCGACGACGAGCTCGGCTCCGGCGTCAACCAGCGCGTCGTG
A4818Rif  ..........................................................................

A4818     GTGTTCATCGCGCAGAAGCGCAAGATCACCGAGGGCGACAAGCTCGCCGGCCGTCACGGCAACAAGGGCGTCATCTCC
A4818Rif  ..........................................................................

A4818     AAGATCCTGCCGGTCGAGGACATGCCGTTCCTCGCCGACGGGACCCCGGTCGACGTCATCCTCAATCCGCTCGGCATC
A4818Rif  ..........................................................................

A4818     CCCGGCCGCATGAACTTCGGCCAGGTCCTGGAGACCCACCTCGGGTGGATCGCCAAGCAGGGCTGGGAGGTCGAGGGC
A4818Rif  ..........................................................................

A4818     AAGCCGAAGTGGGCCGAGCGCCTGCCGGACCACGCGCGCCAGGCTCCGGCCGGCACGAAGGTCGCCACCCCGGTGTTC
A4818Rif  ..........................................................................

A4818     GACGGAGCGCTCGAGGAGGAGATCGCCGGCCTGCTCGACTCGACGACGGTCACCCGCGACGGCGACCGCCTCATCGGG
A4818Rif  ..........................................................................

A4818     TCCAGCGGCAAGACGCGCCTGTTCGACGGCCGCTCCGGCGAGCCGTTCCCGGAGCCCGTCTCGGTCGGCTACATGTAC
A4818Rif  ..........................................................................

A4818     ATCCTGAAGCTGCACCACCTGGTGGACGACAAGATCCACGCGCGCTCCACGGGTCCCTACTCGATGATCACGCAGCAG
A4818Rif  ..........................................................................

A4818     CCCCTGGGCGGTAAGGCCCAGTTCGGCGGCCAGCGGTTCGGCGAGATGGAGGTCTGGGCGCTCGAGGCCTACGGCGCC
A4818Rif  ..........................................................................

A4818     GCGTACGCGCTGCAGGAGCTCCTCACCATCAAGTCGGACGACATCCTCGGCCGCGTGAAGGTGTACGAGGCCATCGTC
A4818Rif  ..........................................................................

A4818     AAGGGCGAGAACATCCAGGAACCGGGTATCCCCGAGTCCTTCAAGGTCCTGATCAAGGAGATGCAGTCCCTCTGCCTG
A4818Rif  ..........................................................................

A4818     AACGTCGAGGTCCTCTCGGCCGACGGCCAGGCGGTCAGCCTGCGCGACACGGATGACGAGGTCTTCCGCGCGGCGGAG
A4818Rif  ..........................................................................

A4818     GAGCTCGGCATCAACATCTCCACCCGCTTCGAGTCGTCCAGCATCGACGACATCTAA
A4818Rif  ..................................................
```

Figure D.11. *rpoB* nucleotide sequence alignment for A4818 and A4818Rif. A4818Rif contains an A→T transversion at position 1274 (highlighted yellow).

```
A4818    MAAARNATPTPQNGRDASRLSFAKITDTLTVPDLLALQTESFDWLVGSDAWKRRVEEGTKQGRTDLALNSGLEEIFEEIS
A4818Rif ................................................................................

A4818    PIEDLGETMQLGFTNPYLEEQKYSIDECKERGKTYSAPLYVEAEFMNHLTGEIKTQTVFMGDFPLMTEKGTFIINGTERV
A4818Rif ................................................................................

A4818    VVSQLVRSPGVYFERQQEKTSDKDIYSARVIPSRGAWLEFEIDKRDQVGVRIDRKRKQSVTVFLKALGLTSEQILEEFKG
A4818Rif ................................................................................

A4818    VASIELTLEKDSILTKEEALKDIYRKLRPGEQVAAEAARALLDNFYFNPKRYDLAKVGRYKINRKLGIDKQLTDSVLTVE
A4818Rif ................................................................................

A4818    DILATIKYLVSLHANETKMNGTRDGKPVELRLDVDDIDHFGNRRIRAVGELIQNQVRTGLSRMERVVRERMTTQDIEAIT
A4818Rif ................................................................................

A4818    PQTLINVRPVVAAIKEFFGTSQLSQFMDQNNPLAGLTHKRRLSALGPGGLSRERAGVEVRDVHPSHYGRMCPIETPEGPN
A4818Rif ......................L.........................................................

A4818    IGLIGSLASFARINSFGFIETPYRRVVDGVVTDTIDYLTASEEDEFLVAQANAPLTKDFRFAEDRVLVRPKGGEVELVAK
A4818Rif ................................................................................

A4818    ENVHYMDVSPRQMVSVATSLIPFLEHDDANRALMGANMQRQAVPLLRSESPLVGTGMEGYAAIDAGDVLTADASGVVQEV
A4818Rif ................................................................................

A4818    SAEVVTIQLDEGGTQTYYLRKFDRSNQGTSYNHRVLVSAGDRIEAGEVIADGPATENGELALGKNLLVAFMPWEGHNFED
A4818Rif ................................................................................

A4818    AIILSQNLVKDDTLSSIHIEEYEVDARDTKLGKEEITRDLPNVSPELLADLDERGIIRIGAEVRPGDILVGKVTPKGETE
A4818Rif ................................................................................

A4818    LSAEERLLRAIFNEKSREVRDTSLKVPHGEQGTIIGVKVFDSQDGDDELGSGVNQRVVVFIAQKRKITEGDKLAGRHGNK
A4818Rif ................................................................................

A4818    GVISKILPVEDMPFLADGTPVDVILNPLGIPGRMNFGQVLETHLGWIAKQGWEVEGKPKWAERLPDHARQAPAGTKVATP
A4818Rif ................................................................................

A4818    VFDGALEEEIAGLLDSTTVTRDGDRLIGSSGKTRLFDGRSGEPFPEPVSVGYMYILKLHHLVDDKIHARSTGPYSMITQQ
A4818Rif ................................................................................

A4818    PLGGKAQFGGQRFGEMEVWALEAYGAAYALQELLTIKSDDILGRVKVYEAIVKGENIQEPGIPESFKVLIKEMQSLCLNV
A4818Rif ................................................................................

A4818    EVLSADGQAVSLRDTDDEVFRAAEELGINISTRFESSSIDDI*
A4818Rif ....................................................
```

Figure D.12. *rpoB* amino acid sequence alignment for A4818 and A4818Rif. A4818Rif contains a Q→L amino acid change at position 425 (highlighted yellow).

```
C55    TTGGCTGCTGCGCGCAACGCAACTCCCACTCCCCAGAACGGTCGCGACGCATCGCGGCTCTCGTTCGCGAAGATCACT
C55Rif ..........................................................................

C55    GACACCCTCACCGTCCCCGACCTCCTCGCCCTGCAGACCGAGAGCTTCGACTGGCTCGTCGGCTCGGACGCGTGGAAG
C55Rif ..........................................................................

C55    CGGCGCGTCGAGGAGGGCACCGCGCAGGGTCGCACCGACCTGGCGCTCAACTCGGGCCTCGAGGAGATCTTCGAGGAG
C55Rif ..........................................................................

C55    ATCTCCCCCATCGAGGACCTGGGCGAGACCATGCAGCTCGGGTTCACGAACCCGTACCTCGAGGAGCAGAAGTACTCC
C55Rif ..........................................................................

C55    ATCGACGAGTGCAAGGAGCGCGGCAAGACCTACTCCGCTCCCCTCTACGTCGAGGCCGAGTTCATGAACCACCTCACG
C55Rif ..........................................................................

C55    GGTGAGATCAAGACCCAGACGGTCTTCATGGGCGACTTCCCCCTCATGACGGAGAAGGGCACGTTCATCATCAACGGC
C55Rif ..........................................................................

C55    ACCGAGCGTGTCGTCGTGTCCCAGCTCGTCCGCTCGCCCGGCGTGTACTTCGAGCGCCAGCAGGAGAAGACCTCCGAC
C55Rif ..........................................................................

C55    AAGGACATCTACTCCGCCCGCGTCATCCCCTCCCGTGGCGCCTGGCTCGAGTTCGAGATCGACAAGCGCGACCAGGTC
C55Rif ..........................................................................

C55    GGCGTGCGCATCGACCGCAAGCGCAAGCAGTCGGTCACCGTGTTCCTCAAGGCCCTCGGCCTCACCAGCGAGCAGATC
C55Rif ..........................................................................

C55    CTCGAGGAGTTCAAGGGCGTCGCGTCCATCGAGCTCACGCTCGAGAAGGACTCCATCCTCACCAAGGAGGAGGCCCTC
C55Rif ..........................................................................

C55    AAGGACATCTACCGCAAGCTCCGTCCGGGCGAGCAGGTCGCCGCCGAGGCCGCCCGCGCGCTGCTGGACAACTTCTAC
C55Rif ..........................................................................

C55    TTCAACCCCAAGCGCTACGACCTGGCGAAGGTGGGTCGCTACAAGATCAACCGCAAGCTCGGCATCGACAAGCAGCTC
C55Rif ..........................................................................

C55    ACCGACTCGGTGCTCACGGTCGAGGACATCCTCGCGACCATCAAGTACCTCGTCTCGCTGCACGCGAACGAGACGAAG
C55Rif ..........................................................................

C55    ATGAACGGCACGCGCGACGGCAAGGCCGTCGAGCTGCGCCTCGACGTGGACGACATCGACCACTTCGGCAACCGCCGC
C55Rif ..........................................................................

C55    ATCCGCGCGGTCGGCGAGCTCATCCAGAACCAGGTGCGCACCGGCCTGTCCCGCATGGAGCGCGTCGTCCGCGAGCGC
C55Rif ..........................................................................

C55    ATGACCACGCAGGACATCGAGGCCATCACGCCCCAGACCCTGATCAACGTGCGCCCCGTCGTCGCCGCGATCAAGGAG
C55Rif ..........................................................................

C55    TTCTTCGGCACGAGCCAGCTGTCGCAGTTCATGGACCAGAACAACCCGCTCGCGGGCCTCACCCACAAGCGCCGCCTC
C55Rif ..........................................................T.............

C55    TCGGCGCTCGGCCCGGGTGGTCTGTCCCGTGAGCGCGCCGGCGTCGAGGTCCGCGACGTCCACCCGTCGCACTACGGC
C55Rif ..........................................................................

C55    CGCATGTGCCCCATCGAGACCCCGGAAGGCCCGAACATCGGCCTGATCGGCTCGCTGGCGTCGTTCGCCCGCATCAAC
C55Rif ..........................................................................

C55    TCGTTCGGCTTCATCGAGACCCCGTACCGTCGCGTCGTCGACGGCGTGGTCACGGACACGATCGACTACCTCACGGCC
C55Rif ..........................................................................

C55    AGCGAGGAGGACGAGTTCCTCGTCGCCCAGGCCAACGCGCCCCTCACGAAGGACTTCCGCTTCGCGGAGGACCGCGTC
C55Rif ..........................................................................

C55    CTCGTCCGCCCGAAGGGCGGTGAGGTCGAGCTCGTCGCGAAGGAGAACGTCCACTACATGGACGTCTCCCCGCGCCAG
C55Rif ..........................................................................

C55    ATGGTGTCGGTCGCGACCTCGCTCATCCCCTTCCTCGAGCACGACGACGCGAACCGGGCCCTCATGGGCGCGAACATG
C55Rif ..........................................................................

C55    CAGCGTCAGGCCGTCCCGCTGCTGCGCAGCGAGAGCCCGCTCGTCGGCACCGGCATGGAGGGCTACGCGGCGGTCGAC
C55Rif ..........................................................................
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```
C55    GCCGGCGACGTCCTCACGGCCGACGCCTCGGGCGTCGTGCAGGAGGTGTCGGCCGAGGTCGTCACCATCCAGCTCGAC
C55Rif ..........................................................................

C55    GAGGGCGGCACGCAGACCTACTACCTGCGCAAGTTCGACCGCTCGAACCAGGGCACGAGCTACAACCACCGCGTCCTG
C55Rif ..........................................................................

C55    GTCTCGGCCGGCGACCGCATCGAGGCCGGCGAGGTCATCGCCGACGGCCCCGCCACGGAGAACGGCGAGCTCGCGCTC
C55Rif ..........................................................................

C55    GGCAAGAACCTGCTCGTCGCGTTCATGCCGTGGGAGGGCCACAACTTCGAGGACGCGATCATCCTGAGCCAGAACCTG
C55Rif ..........................................................................

C55    GTCAAGGACGACACCCTCTCCTCCATCCACATCGAGGAGTACGAGGTCGACGCGCGCGACACCAAGCTCGGCAAGGAG
C55Rif ..........................................................................

C55    GAGATCACCCGCGACCTCCCCAACGTCAGCCCGGAGCTGCTCGCCGACCTCGACGAGCGCGGCATCATCCGCATCGGC
C55Rif ..........................................................................

C55    GCCGAGGTCCGCCCCGGCGACATCCTCGTGGGCAAGGTCACGCCGAAGGGCGAGACCGAGCTCAGCGCCGAGGAGCGC
C55Rif ..........................................................................

C55    CTGCTGCGCGCGATCTTCAACGAGAAGAGCCGCGAGGTCCGCGACACGTCCCTGAAGGTGCCCCACGGCGAGCAGGGC
C55Rif ..........................................................................

C55    ACGATCATCGGCGTCAAGGTCTTCGACTCGCAGGACGGCGACGACGAGCTCGGCTCCGGCGTCAACCAGCGGGTCGTG
C55Rif ..........................................................................

C55    GTGTTCATCGCCCAGAAGCGCAAGATCACCGAGGGCGACAAGCTCGCCGGCCGTCACGGCAACAAGGGCGTCATCTCC
C55Rif ..........................................................................

C55    AAGATCCTGCCGGTCGAGGACATGCCGTTCCTCGCCGACGGGACCCCGGTCGACGTCATCCTCAACCCGCTCGGCATC
C55Rif ..........................................................................

C55    CCCGGCCGCATGAACTTCGGCCAGGTCCTGGAGACCCACCTCGGGTGGATCGCCAAGCAGGGCTGGGAGGTCGAGGGC
C55Rif ..........................................................................

C55    AAGCCGAAGTGGGCCGAGCGCCTGCCGGACCACGCGCGCCAGGCCCCGGCCGGCACGAAGGTCGCCACCCCGGTGTTC
C55Rif ..........................................................................

C55    GACGGAGCGCTCGAGGAGGAGATCGCCGGCCTGCTCGACTCGACGACGGTCACCCGCGACGGCGACCGCCTCATCGGG
C55Rif ..........................................................................

C55    TCCAGCGGCAAGACGCGCCTGTTCGACGGCCGCTCCGGCGAGCCGTTCCCCGAGCCCGTCTCGGTCGGCTACATGTAC
C55Rif ..........................................................................

C55    ATCCTGAAGCTGCACCACCTGGTGGACGACAAGATCCACGCGCGCTCGACGGGTCCCTACTCGATGATCACGCAGCAG
C55Rif ..........................................................................

C55    CCCCTGGGCGGTAAGGCCCAGTTCGGCGGCCAGCGGTTCGGCGAGATGGAGGTCTGGGCGCTCGAGGCCTACGGCGCC
C55Rif ..........................................................................

C55    GCGTACGCGCTGCAGGAGCTCCTCACCATCAAGTCGGACGACATCCTCGGCCGCGTCAAGGTGTACGAGGCCATCGTC
C55Rif ..........................................................................

C55    AAGGGCGAGAACATCCAGGAACCGGGTATCCCCGAGTCCTTCAAGGTCCTGATCAAGGAGATGCAGTCCCTCTGCCTG
C55Rif ..........................................................................

C55    AACGTCGAGGTCCTCTCGGCCGACGGCCAGGCGGTCAGCCTGCGCGACACGGATGACGAGGTCTTCCGCGCGGCGGAG
C55Rif ..........................................................................

C55    GAGCTCGGCATCAACATCTCCACCCGCTTCGAGTCGTCCAGCATCGACGACATCTAA
C55Rif ..................................................
```

Figure D.13. *rpoB* nucleotide sequence alignment for C55 and C55Rif. C55Rif contains a C→T transition at position 1312 (highlighted yellow).

```
C55    MAAARNATPTPQNGRDASRLSFAKITDTLTVPDLLALQTESFDWLVGSDAWKRRVEEGTAQGRTDLALNSGLEEIFEEIS
C55Rif ................................................................................

C55    PIEDLGETMQLGFTNPYLEEQKYSIDECKERGKTYSAPLYVEAEFMNHLTGEIKTQTVFMGDFPLMTEKGTFIINGTERV
C55Rif ................................................................................

C55    VVSQLVRSPGVYFERQQEKTSDKDIYSARVIPSRGAWLEFEIDKRDQVGVRIDRKRKQSVTVFLKALGLTSEQILEEFKG
C55Rif ................................................................................
C55    VASIELTLEKDSILTKEEALKDIYRKLRPGEQVAAEAARALLDNFYFNPKRYDLAKVGRYKINRKLGIDKQLTDSVLTVE
C55Rif ................................................................................

C55    DILATIKYLVSLHANETKMNGTRDGKAVELRLDVDDIDHFGNRRIRAVGELIQNQVRTGLSRMERVVRERMTTQDIEAIT
C55Rif ................................................................................

C55    PQTLINVRPVVAAIKEFFGTSQLSQFMDQNNPLAGLTHKRRLSALGPGGLSRERAGVEVRDVHPSHYGRMCPIETPEGPN
C55Rif ....................................Y...........................................

C55    IGLIGSLASFARINSFGFIETPYRRVVDGVVTDTIDYLTASEEDEFLVAQANAPLTKDFRFAEDRVLVRPKGGEVELVAK
C55Rif ................................................................................

C55    ENVHYMDVSPRQMVSVATSLIPFLEHDDANRALMGANMQRQAVPLLRSESPLVGTGMEGYAAVDAGDVLTADASGVVQEV
C55Rif ................................................................................

C55    SAEVVTIQLDEGGTQTYYLRKFDRSNQGTSYNHRVLVSAGDRIEAGEVIADGPATENGELALGKNLLVAFMPWEGHNFED
C55Rif ................................................................................

C55    AIILSQNLVKDDTLSSIHIEEYEVDARDTKLGKEEITRDLPNVSPELLADLDERGIIRIGAEVRPGDILVGKVTPKGETE
C55Rif ................................................................................

C55    LSAEERLLRAIFNEKSREVRDTSLKVPHGEQGTIIGVKVFDSQDGDDELGSGVNQRVVVFIAQKRKITEGDKLAGRHGNK
C55Rif ................................................................................

C55    GVISKILPVEDMPFLADGTPVDVILNPLGIPGRMNFGQVLETHLGWIAKQGWEVEGKPKWAERLPDHARQAPAGTKVATP
C55Rif ................................................................................

C55    VFDGALEEEIAGLLDSTTVTRDGDRLIGSSGKTRLFDGRSGEPFPEPVSVGYMYILKLHHLVDDKIHARSTGPYSMITQQ
C55Rif ................................................................................

C55    PLGGKAQFGGQRFGEMEVWALEAYGAAYALQELLTIKSDDILGRVKVYEAIVKGENIQEPGIPESFKVLIKEMQSLCLNV
C55Rif ................................................................................

C55    EVLSADGQAVSLRDTDDEVFRAAEELGINISTRFESSSIDDI*
C55Rif ....................................
```

Figure D.14. *rpoB* amino acid sequence alignment for C55 and C55Rif. C55Rif contains an H→Y amino acid change at position 438 (highlighted yellow).

```
ZUM3936     TTGGCTGCTGCGCGCAACGCAACTCCCACTCCCCAGAACGGTCGCGACGCATCGCGGCTCTCGTTCGCGAAGATCACT
ZUM3936Rif ..........................................................................

ZUM3936     GACACCCTCACCGTCCCCGACCTGCTCGCCCTGCAGACCGAGAGCTTCGACTGGCTCGTCGGCTCGGACGTCTGGAAG
ZUM3936Rif ..........................................................................

ZUM3936     CGGCGCGTCGAGGAGGGCACGAAGCAGGGTCGCACCGACCTGGCGCTCAACTCCGGCCTCGAGGAGATCTTCGAGGAG
ZUM3936Rif ..........................................................................

ZUM3936     ATCTCCCCCATCGAGGACCTGGGCGAGACCATGCAGCTCGGGTTCACGAACCCGTACCTCGAGGAGCAGAAGTACTCC
ZUM3936Rif ..........................................................................

ZUM3936     ATCGACGAGTGCAAGGAGCGCGGCAAGACCTACTCCGCTCCCCTCTACGTCGAGGCCGAGTTCATGAACCACCTCACG
ZUM3936Rif ..........................................................................

ZUM3936     GGTGAGATCAAGACCCAGACGGTCTTCATGGGCGACTTCCCCCTCATGACGGAGAAGGGCACGTTCATCATCAACGGC
ZUM3936Rif ..........................................................................

ZUM3936     ACCGAGCGTGTCGTCGTGTCCCAGCTCGTCCGCTCGCCCGGCGTGTACTTCGAGCGCCAGCAGGAGAAGACCAGCGAC
ZUM3936Rif ..........................................................................

ZUM3936     AAGGACATCTACTCCGCCCGCGTCATCCCGTCCCGCGGCGCATGGCTCGAGTTCGAGATCGACAAGCGCGACCAGGTC
ZUM3936Rif ..........................................................................

ZUM3936     GGCGTGCGCATCGACCGCAAGCGCAAGCAGTCGGTCACGGTGTTCCTGAAGGCCCTCGGCCTCACCAGCGAGCAGATC
ZUM3936Rif ..........................................................................

ZUM3936     CTGGAGGAGTTCAAGGGCGTCGCGTCCATCGAGCTCACGCTCGAGAAGGACTCCATCCTCACCAAGGAGGAGGCCCTC
ZUM3936Rif ..........................................................................

ZUM3936     AAGGACATCTACCGCAAGCTCCGCCCGGGCGAGCAGGTCGCCGCCGAGGCCGCCCGCGCGCTGCTGGACAACTTCTAC
ZUM3936Rif ..........................................................................

ZUM3936     TTCAACCCGAAGCGCTACGACCTGGCGAAGGTGGGTCGCTACAAGATCAACCGCAAGCTCGGCATCGACAAGCAGCTC
ZUM3936Rif ..........................................................................

ZUM3936     ACCGACTCGGTGCTGACGGTCGAGGACATCCTCGCGACCATCAAGTACCTAGTCTCGCTGCACGCGAACGAGACGAAG
ZUM3936Rif ..........................................................................

ZUM3936     ATGAACGGCACGCGCGACGGCAAGCCCGTCGAGCTGCGCCTCGACGTGGACGACATCGACCACTTCGGCAACCGCCGC
ZUM3936Rif ..........................................................................

ZUM3936     ATCCGCGCGGTCGGCGAGCTCATCCAGAACCAGGTCCGCACCGGCCTGTCCCGCATGGAGCGCGTCGTCCGCGAGCGC
ZUM3936Rif ..........................................................................

ZUM3936     ATGACCACGCAGGACATCGAGGCCATCACGCCGCAGACCCTGATCAACGTGCGCCCCGTCGTCGCCGCGATCAAGGAG
ZUM3936Rif ..........................................................................

ZUM3936     TTCTTCGGCACGAGCCAGCTGTCGCAGTTCATGGACCAGAACAACCCGCTCGCGGGTCTCACCCACAAGCGCCGCCTC
ZUM3936Rif ..........................................................T.............

ZUM3936     TCGGCGCTCGGCCCGGGTGGTCTGTCCCGTGAGCGCGCCGGCGTCGAGGTCCGCGACGTCCACCCGTCGCACTACGGC
ZUM3936Rif ..........................................................................

ZUM3936     CGCATGTGCCCCATCGAGACCCCGGAAGGCCCGAACATCGGCCTGATCGGCTCGCTGGCGTCGTTCGCCCGCATCAAC
ZUM3936Rif ..........................................................................

ZUM3936     TCGTTCGGCTTCATCGAGACCCCGTACCGTCGCGTCGTCGACGGCGTGGTCACCGAGCAGATCGACTACCTCACGGCC
ZUM3936Rif ..........................................................................

ZUM3936     AGCGAGGAGGACGAGTTCCTCGTCGCCCAGGCCAACGCGCCCCTCACGAAGGACTTCCGCTTCGCGGAGGACCGCGTC
ZUM3936Rif ..........................................................................

ZUM3936     CTCGTCCGCCCCAAGGGCGGTGAGGTGGAGCTCGTCGCGAAGGAGAACGTCCACTACATGGACGTCTCCCCGCGCCAG
ZUM3936Rif ..........................................................................

ZUM3936     ATGGTGTCGGTCGCGACCTCGCTCATCCCGTTCCTCGAGCACGACGACGCGAACCGGGCCCTCATGGGCGCGAACATG
ZUM3936Rif ..........................................................................

ZUM3936     CAGCGTCAGGCCGTCCCGCTGCTGCGCAGCGAGAGCCCGCTCGTCGGCACCGGCATGGAGGGCTACGCGGCGATCGAC
ZUM3936Rif ..........................................................................
```

```
ZUM3936     GCCGGCGACGTCCTCACCGCCGACGCCTCGGGCGTCGTGCAGGAGGTGTCGGCCGAGGTCGTCACCATCCAGCTCGAC
ZUM3936Rif ..........................................................................

ZUM3936     GAGGGCGGCACGCAGACCTACTACCTGCGCAAGTTCGACCGCTCCAACCAGGGCACGAGCTACAACCACCGCGTCCTG
ZUM3936Rif ..........................................................................

ZUM3936     GTCTCGGCCGGCGACCGCATCGAGGCCGGCGAGGTCATCGCCGACGGCCCCGCCACGGAGAACGGCGAGCTCGCGCTC
ZUM3936Rif ..........................................................................

ZUM3936     GGCAAGAACCTGCTCGTCGCGTTCATGCCGTGGGAGGGCCACAACTTCGAGGACGCGATCATCCTGAGCCAGAACCTG
ZUM3936Rif ..........................................................................

ZUM3936     GTCAAGGACGACACCCTCTCCTCCATCCACATCGAGGAGTACGAGGTCGACGCGCGCGACACCAAGCTCGGCAAGGAG
ZUM3936Rif ..........................................................................

ZUM3936     GAGATCACCCGCGACCTCCCCAACGTCAGCCCGGAGCTGCTCGCCGACCTCGACGAGCGCGGCATCATCCGCATCGGC
ZUM3936Rif ..........................................................................

ZUM3936     GCCGAGGTCCGCCCCGGCGACATCCTCGTGGGCAAGGTCACGCCGAAGGGCGAGACCGAGCTCAGCGCCGAGGAGCGC
ZUM3936Rif ..........................................................................

ZUM3936     CTGCTGCGCGCGATCTTCAACGAGAAGAGCCGCGAGGTCCGCGACACGTCCCTGAAGGTGCCCCACGGCGAGCAGGGC
ZUM3936Rif ..........................................................................

ZUM3936     ACGATCATCGGCGTCAAGGTCTTCGACTCGCAGGACGGCGACGACGAGCTCGGCTCGGGCGTCAACCAGCGCGTCGTG
ZUM3936Rif ..........................................................................

ZUM3936     GTGTTCATCGCGCAGAAGCGCAAGATCACCGAGGGCGACAAGCTGGCCGGCCGTCACGGCAACAAGGGCGTCATCTCC
ZUM3936Rif ..........................................................................

ZUM3936     AAGATCCTGCCGGTCGAGGACATGCCGTTCCTCGCCGACGGGACCCCGGTCGACGTCATCCTCAACCCGCTCGGCATC
ZUM3936Rif ..........................................................................

ZUM3936     CCCGGCCGCATGAACTTCGGCCAGGTCCTGGAGACCCACCTCGGGTGGATCGCCAAGCAGGGCTGGGAGGTCGAGGGC
ZUM3936Rif ..........................................................................

ZUM3936     AAGCCGAAGTGGGCCGAGCGCCTGCCGGACCACGCGCGCCAGGCCCCGGCCGGCACCAAGGTCGCCACCCCGGTGTTC
ZUM3936Rif ..........................................................................

ZUM3936     GACGGAGCGCTCGAGGAGGAGATCGCCGGCCTGCTCGACTCGACGACGGTCACCCGCGACGGCGACCGCCTCATCGGG
ZUM3936Rif ..........................................................................

ZUM3936     TCCAGCGGCAAGACGCGCCTGTTCGACGGCCGCTCCGGCGAGCCGTTCCCCGAGCCCGTCTCGGTCGGCTACATGTAC
ZUM3936Rif ..........................................................................

ZUM3936     ATCCTGAAGCTGCACCACCTGGTGGATGACAAGATCCACGCGCGCTCGACGGGTCCCTACTCGATGATCACGCAGCAG
ZUM3936Rif ..........................................................................

ZUM3936     CCCCTGGGCGGTAAGGCCCAGTTCGGCGGCCAGCGGTTCGGCGAGATGGAGGTCTGGGCGCTCGAGGCCTACGGCGCC
ZUM3936Rif ..........................................................................

ZUM3936     GCGTACGCGCTGCAGGAGCTCCTCACCATCAAGTCGGACGACATCCTCGGCCGCGTGAAGGTGTACGAGGCCATCGTC
ZUM3936Rif ..........................................................................

ZUM3936     AAGGGCGAGAACATCCAGGAACCGGGTATCCCCGAGTCCTTCAAGGTCCTGATCAAGGAGATGCAGTCCCTCTGCCTG
ZUM3936Rif ..........................................................................

ZUM3936     AACGTCGAGGTCCTCTCGGCCGACGGCCAGGCGGTCAGCCTGCGCGACACGGATGACGAGGTCTTCCGCGCGGCGGAG
ZUM3936Rif ..........................................................................

ZUM3936     GAGCTCGGCATCAACATCTCCACCCGCTTCGAGTCGTCCAGCATCGACGACATCTAA
ZUM3936Rif ..................................................
```

Figure D.15. *rpoB* nucleotide sequence alignment for ZUM3936 and ZUM3936Rif. ZUM3936Rif contains a C→T transition at positions 1312 (highlighted yellow).

```
ZUM3936    MAAARNATPTPQNGRDASRLSFAKITDTLTVPDLLALQTESFDWLVGSDVWKRRVEEGTKQGRTDLALNSGLEEIFEEIS
ZUM3936Rif ................................................................................

ZUM3936    PIEDLGETMQLGFTNPYLEEQKYSIDECKERGKTYSAPLYVEAEFMNHLTGEIKTQTVFMGDFPLMTEKGTFIINGTERV
ZUM3936Rif ................................................................................

ZUM3936    VVSQLVRSPGVYFERQQEKTSDKDIYSARVIPSRGAWLEFEIDKRDQVGVRIDRKRKQSVTVFLKALGLTSEQILEEFKG
ZUM3936Rif ................................................................................

ZUM3936    VASIELTLEKDSILTKEEALKDIYRKLRPGEQVAAEAARALLDNFYFNPKRYDLAKVGRYKINRKLGIDKQLTDSVLTVE
ZUM3936Rif ................................................................................

ZUM3936    DILATIKYLVSLHANETKMNGTRDGKPVELRLDVDDIDHFGNRRIRAVGELIQNQVRTGLSRMERVVRERMTTQDIEAIT
ZUM3936Rif ................................................................................

ZUM3936    PQTLINVRPVVAAIKEFFGTSQLSQFMDQNNPLAGLTHKRRLSALGPGGLSRERAGVEVRDVHPSHYGRMCPIETPEGPN
ZUM3936Rif ..................................Y.............................................

ZUM3936    IGLIGSLASFARINSFGFIETPYRRVVDGVVTEQIDYLTASEEDEFLVAQANAPLTKDFRFAEDRVLVRPKGGEVELVAK
ZUM3936Rif ................................................................................

ZUM3936    ENVHYMDVSPRQMVSVATSLIPFLEHDDANRALMGANMQRQAVPLLRSESPLVGTGMEGYAAIDAGDVLTADASGVVQEV
ZUM3936Rif ................................................................................

ZUM3936    SAEVVTIQLDEGGTQTYYLRKFDRSNQGTSYNHRVLVSAGDRIEAGEVIADGPATENGELALGKNLLVAFMPWEGHNFED
ZUM3936Rif ................................................................................

ZUM3936    AIILSQNLVKDDTLSSIHIEEYEVDARDTKLGKEEITRDLPNVSPELLADLDERGIIRIGAEVRPGDILVGKVTPKGETE
ZUM3936Rif ................................................................................

ZUM3936    LSAEERLLRAIFNEKSREVRDTSLKVPHGEQGTIIGVKVFDSQDGDDELGSGVNQRVVVFIAQKRKITEGDKLAGRHGNK
ZUM3936Rif ................................................................................

ZUM3936    GVISKILPVEDMPFLADGTPVDVILNPLGIPGRMNFGQVLETHLGWIAKQGWEVEGKPKWAERLPDHARQAPAGTKVATP
ZUM3936Rif ................................................................................

ZUM3936    VFDGALEEEIAGLLDSTTVTRDGDRLIGSSGKTRLFDGRSGEPFPEPVSVGYMYILKLHHLVDDKIHARSTGPYSMITQQ
ZUM3936Rif ................................................................................

ZUM3936    PLGGKAQFGGQRFGEMEVWALEAYGAAYALQELLTIKSDDILGRVKVYEAIVKGENIQEPGIPESFKVLIKEMQSLCLNV
ZUM3936Rif ................................................................................

ZUM3936    EVLSADGQAVSLRDTDDEVFRAAEELGINISTRFESSSIDDI*
ZUM3936Rif ........................................
```

Figure D.16. *rpoB* amino acid sequence alignment for ZUM3936 and ZUM3936Rif. ZUM3936Rif contains an H→Y amino acid change at position 438 (highlighted yellow).

APPENDIX E: Seed LAMP Data.

Table E.1. Seed germination, Immunostrip and LAMP data Trial 1*.

Seed	Germ?	Colony		Immun		LAMP		Seed	Germ?	Colony		Immun		LAMP	
1	+	+	+	+	+	+	+	51	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	52	-	+	+	+	+	+	+
3	+	+	+	+	+	+	+	53	-	+	+	+	+	+	+
4	+	+	+	+	+	+	+	54	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	55	+	+	+	+	+	+	+
6	-	?	+	+	+	+	+	56	-	+	+	+	+	+	+
7	+	+	+	+	+	+	+	57	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	58	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	59	-	+	+	+	+	+	+
10	-	+	+	+	+	+	+	60	+	+	+	+	+	+	+
11	-	+	+	+	+	+	+	61	-	+	+	+	+	+	+
12	+	?	+	+	+	+	+	62	+	+	+	+	+	+	+
13	+	+	+	+	+	+	+	63	+	+	+	+	+	+	+
14	-	+	+	+	+	+	+	64	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	65	+	+	+	+	+	+	+
16	+	+	+	+	+	+	+	66	+	+	+	+	+	+	+
17	+	?	+	+	+	+	+	67	+	?	+	+	+	+	+
18	-	?	+	+	+	+	+	68	+	+	+	+	+	+	+
19	+	+	+	+	+	+	+	69	+	+	+	+	+	+	+
20	+	?	+	+	+	+	+	70	+	+	+	+	+	+	+
21	+	?	+	+	+	+	+	71	+	+	+	+	+	+	+
22	+	?	+	+	+	+	+	72	+	+	+	+	+	+	+
23	+	+	+	+	+	+	+	73	+	+	+	+	+	+	+
24	+	+	+	+	+	+	+	74	+	+	+	+	+	+	+
25	+	+	+	+	+	+	+	75	+	+	+	+	+	+	+
26	+	+	+	+	+	+	+	76	+	+	+	+	+	+	+
27	+	+	+	+	+	+	+	77	+	+	+	+	+	+	+
28	-	+	+	+	+	+	+	78	-	+	+	+	+	+	+
29	+	+	+	+	+	+	+	79	-	+	+	+	+	+	+
30	-	+	+	+	+	+	+	80	+	+	+	+	+	+	+
31	+	+	+	+	+	+	+	81	+	+	+	+	+	+	+
32	+	+	+	+	+	+	+	82	+	+	+	+	+	+	+
33	+	+	+	+	+	+	+	83	-	+	+	+	+	+	+
34	+	+	+	+	+	+	+	84	+	+	+	+	+	+	+
35	+	+	+	+	+	+	+	85	-	+	+	+	+	+	+
36	+	+	+	+	+	+	+	86	-	?	+	+	+	+	+
37	-	+	+	+	+	+	+	87	-	+	+	+	+	+	+
38	-	+	+	+	+	+	+	88	+	+	+	+	+	+	+
39	-	+	+	+	+	+	+	89	+	?	+	+	+	+	+
40	+	+	+	+	+	+	+	90	+	+	+	+	+	+	+
41	+	?	+	+	+	+	+	91	+	+	+	+	+	+	+
42	+	?	+	+	+	+	+	92	+	+	+	+	+	+	+
43	+	+	+	+	+	+	+	93	-	?	+	+	+	+	+
44	+	?	+	+	+	+	+	94	+	?	+	+	+	+	+
45	+	+	+	+	+	+	+	95	+	?	+	+	+	+	+
46	+	+	+	+	+	+	+	96	+	?	-	+	-	+	-
47	+	+	+	+	+	+	+	97	+	?	+	+	+	+	+
48	-	+	+	+	+	+	+	98	+	+	+	+	+	+	+
49	+	?	+	+	+	+	+	99	+	?	+	+	+	+	+
50	-	+	+	+	+	+	+	100	-	+	+	+	+	+	+
		4d	7d	sd	col	sd	col			4d	7d	sd	col	sd	col

* Germ – germination; Immun – Immunostrip result; ? - no discernible colonies observed; sd = seed; col - colony.

Table E.2. Seed germination, Immunostrip and LAMP data Trial 2*.

Seed	Germ?	Colony		Immun		LAMP		Seed	Germ?	Colony		Immun		LAMP	
1	+	+	+	+	+	+	+	51	-	+	+	+	+	+	+
2	+	?	+	+	+	+	+	52	-	+	+	+	+	+	+
3	+	+	+	+	+	+	+	53	-	+	+	+	+	+	+
4	-	+	+	+	+	+	+	54	+	?	+	+	+	+	+
5	-	?	+	+	+	+	+	55	-	?	+	+	+	+	+
6	+	+	+	+	+	+	+	56	+	+	+	+	+	+	+
7	-	+	+	+	+	+	+	57	-	+	+	+	+	+	+
8	+	?	+	+	+	+	+	58	+	+	+	+	+	+	+
9	+	?	+	+	+	+	+	59	-	+	+	+	+	+	+
10	+	?	+	+	+	+	+	60	+	?	+	+	+	+	+
11	-	?	+	+	+	+	+	61	+	?	+	+	+	+	+
12	-	?	+	+	+	+	+	62	-	+	+	+	+	+	+
13	+	?	+	+	+	+	+	63	+	?	+	+	+	+	+
14	-	+	+	+	+	+	+	64	-	?	+	+	+	+	+
15	-	?	+	+	+	+	+	65	+	?	+	+	+	+	+
16	-	?	+	+	+	+	+	66	+	?	+	+	+	+	+
17	-	+	+	+	+	+	+	67	-	?	+	+	+	+	+
18	+	+	+	+	+	+	+	68	-	+	+	+	+	+	+
19	+	+	+	+	+	+	+	69	+	?	+	+	+	+	+
20	-	+	+	+	+	+	+	70	+	?	+	+	+	+	+
21	+	?	+	+	+	+	+	71	-	+	+	+	+	+	+
22	+	?	+	+	+	+	+	72	-	?	+	+	+	+	+
23	-	?	+	+	+	+	+	73	-	?	+	+	+	+	+
24	+	?	+	+	+	+	+	74	+	+	+	+	+	+	+
25	+	+	+	+	+	+	+	75	+	?	+	+	+	+	+
26	+	?	+	+	+	+	+	76	+	+	+	+	+	+	+
27	+	?	+	+	+	+	+	77	-	+	+	+	+	+	+
28	+	?	+	+	+	+	+	78	+	?	+	+	+	+	+
29	+	+	+	+	+	+	+	79	-	?	+	+	+	+	+
30	+	?	+	+	+	+	+	80	+	+	+	+	+	+	+
31	+	?	+	+	+	+	+	81	+	?	+	+	+	+	+
32	+	+	+	+	+	+	+	82	-	?	+	+	+	+	+
33	-	+	+	+	+	+	+	83	+	+	+	+	+	+	+
34	-	+	+	+	+	+	+	84	-	?	+	+	+	+	+
35	-	+	+	+	+	+	+	85	+	?	+	+	+	+	+
36	+	+	+	+	+	+	+	86	-	?	+	+	+	+	+
37	-	?	+	+	+	+	+	87	+	?	+	+	+	+	+
38	+	?	+	+	+	+	+	88	-	?	+	+	+	+	+
39	-	+	+	+	+	+	+	89	-	?	+	+	+	+	+
40	+	?	+	+	+	+	+	90	-	?	+	+	+	+	+
41	+	+	+	+	+	+	+	91	-	?	+	+	+	+	+
42	-	?	+	+	+	+	+	92	+	+	+	+	+	+	+
43	-	?	+	+	+	+	+	93	-	+	+	+	+	+	+
44	-	+	+	+	+	+	+	94	+	+	+	+	+	+	+
45	-	+	+	+	+	+	+	95	+	?	+	+	+	+	+
46	+	?	+	+	+	+	+	96	-	?	+	+	+	+	+
47	-	?	+	+	+	+	+	97	+	?	+	+	+	+	+
48	-	+	+	+	+	+	+	98	+	?	+	+	+	+	+
49	+	?	+	+	+	+	+	99	-	?	+	+	+	+	+
50	-	+	+	+	+	+	+	100	-	?	+	+	+	+	+
		4d	7d	sd	col	sd	col			4d	7d	sd	col	sd	col

* Germ – germination; Immun – Immunostrip result; ? - no discernible colonies observed; sd = seed; col - colony.

Table E.3. Seed germination, Immunostrip and LAMP data Trial 3*.

Seed	Germ?	Colony		Immun		LAMP		Seed	Germ?	Colony		Immun		LAMP	
1	+	?	+	+	+	+	+	51	+	+	+	+	+	+	+
2	-	?	+	+	+	+	+	52	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	53	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	54	-	?	+	+	+	+	+
5	+	?	+	+	+	+	+	55	+	?	+	+	+	+	+
6	+	?	+	+	+	+	+	56	+	?	+	+	+	+	+
7	+	+	+	+	+	+	+	57	-	?	+	+	+	+	+
8	+	+	+	+	+	+	+	58	-	?	+	+	+	+	+
9	+	+	+	+	+	+	+	59	-	?	-	+	-	+	-
10	+	+	+	+	+	+	+	60	-	?	+	+	+	+	+
11	+	?	+	+	+	+	+	61	+	+	+	+	+	+	+
12	+	?	+	+	+	+	+	62	-	+	+	+	+	+	+
13	+	+	+	+	+	+	+	63	-	+	+	+	+	+	+
14	+	?	+	+	+	+	+	64	+	?	+	+	+	+	+
15	+	?	+	+	+	+	+	65	+	?	+	+	+	+	+
16	+	+	+	+	+	+	+	66	+	?	+	+	+	+	+
17	+	?	+	+	+	+	+	67	-	+	+	+	+	+	+
18	-	+	+	+	+	+	+	68	+	?	+	+	+	+	+
19	-	+	+	+	+	+	+	69	+	?	+	+	+	+	+
20	+	+	+	+	+	+	+	70	+	?	+	+	+	+	+
21	-	+	+	+	+	+	+	71	+	?	+	+	+	+	+
22	+	+	+	+	+	+	+	72	+	?	+	+	+	+	+
23	+	+	+	+	+	+	+	73	-	?	+	+	+	+	+
24	+	+	+	+	+	+	+	74	-	?	+	+	+	+	+
25	+	?	+	+	+	+	+	75	-	+	+	+	+	+	+
26	+	+	+	+	+	+	+	76	-	+	+	+	+	+	+
27	-	+	+	+	+	+	+	77	+	+	+	+	+	+	+
28	+	+	+	+	+	+	+	78	-	+	+	+	+	+	+
29	+	+	+	+	+	+	+	79	+	+	+	+	+	+	+
30	+	?	+	+	+	+	+	80	+	+	+	+	+	+	+
31	-	?	+	+	+	+	+	81	+	+	+	+	+	+	+
32	+	?	+	+	+	+	+	82	+	?	+	+	+	+	+
33	+	?	+	+	+	+	+	83	-	?	+	+	+	+	+
34	+	+	+	+	+	+	+	84	+	?	+	+	+	+	+
35	-	?	+	+	+	+	+	85	-	+	+	+	+	+	+
36	-	+	+	+	+	+	+	86	-	?	+	+	+	+	+
37	+	+	+	+	+	+	+	87	+	+	+	+	+	+	+
38	+	+	+	+	+	+	+	88	-	?	+	+	+	+	+
39	+	+	+	+	+	+	+	89	+	+	+	+	+	+	+
40	+	+	+	+	+	+	+	90	+	+	+	+	+	+	+
41	+	+	+	+	+	+	+	91	+	?	-	+	-	+	-
42	+	?	+	+	+	+	+	92	-	+	+	+	+	+	+
43	-	?	+	+	+	+	+	93	+	+	+	+	+	+	+
44	-	?	+	+	+	+	+	94	+	+	+	+	+	+	+
45	-	?	+	+	+	+	+	95	+	+	+	+	+	+	+
46	+	?	+	+	+	+	+	96	-	+	+	+	+	+	+
47	-	+	+	+	+	+	+	97	-	+	+	+	+	+	+
48	+	+	+	+	+	+	+	98	+	+	+	+	+	+	+
49	-	?	+	+	+	+	+	99	+	?	+	+	+	+	+
50	+	+	+	+	+	+	+	100	+	+	+	+	+	+	+
		4d	7d	sd	col	sd	col			4d	7d	sd	col	sd	col

* Germ – germination; Immun – Immunostrip result; ? - no discernible colonies observed; sd = seed; col - colony.

APPENDIX F: Growth on Media.

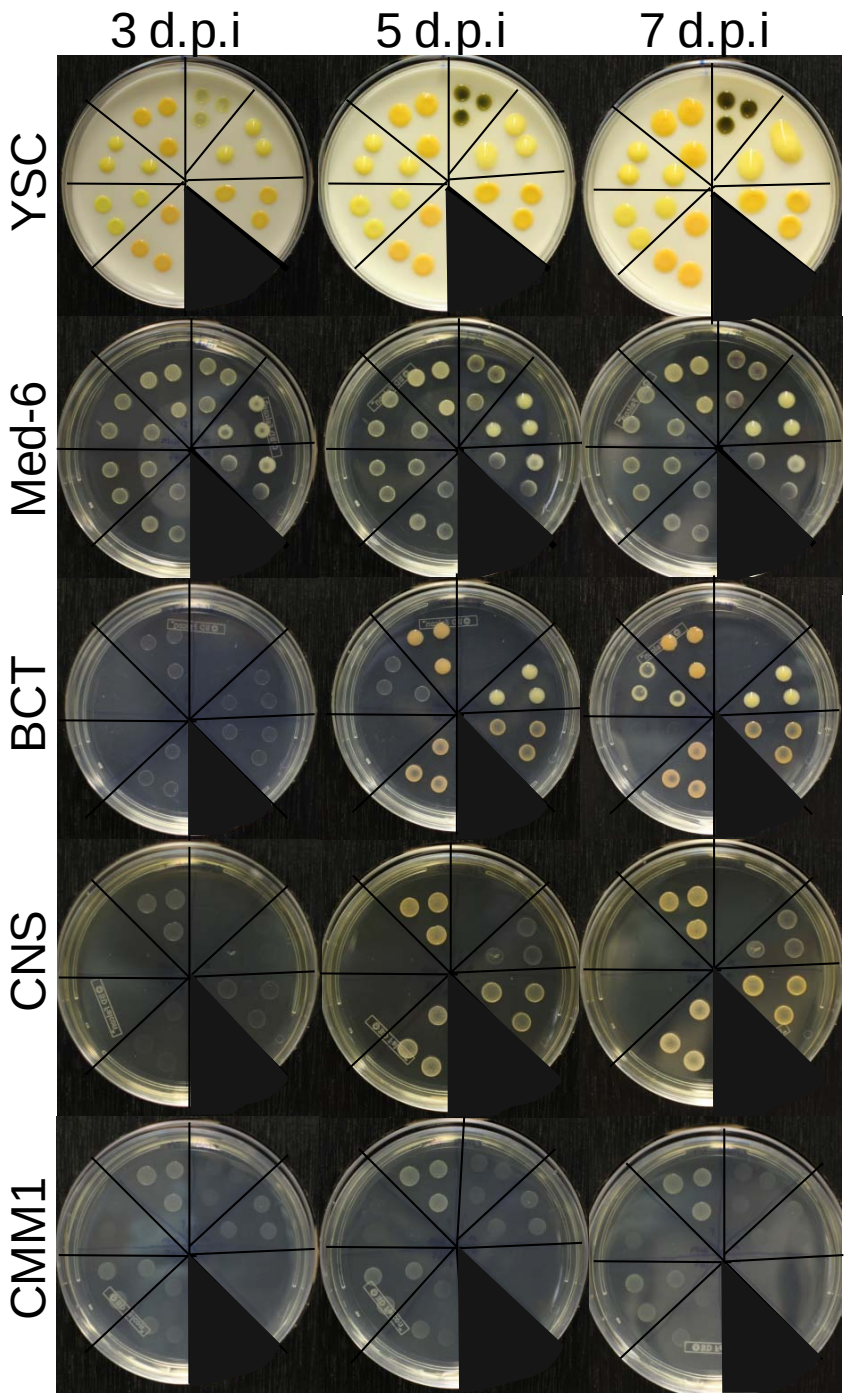

Figure F.1. Bacterial growth on various media, first trial. Different subspecies of *Clavibacter* were spot plated onto two growth media (YSC and Medium 6 [Med-6]) and three selective media (BCT, CNS and CMM1) to show differential growth, color and selectivity of media. Plates were divided into eight quadrants. Starting with the top right quadrant (12 o'clock to 1:30) and moving clockwise, *Cmi*, *Cmm*, *Cmn*, *Cms* (not included), *Cmt*, C55, ZUM3936, and PS005 were plated, respectively.

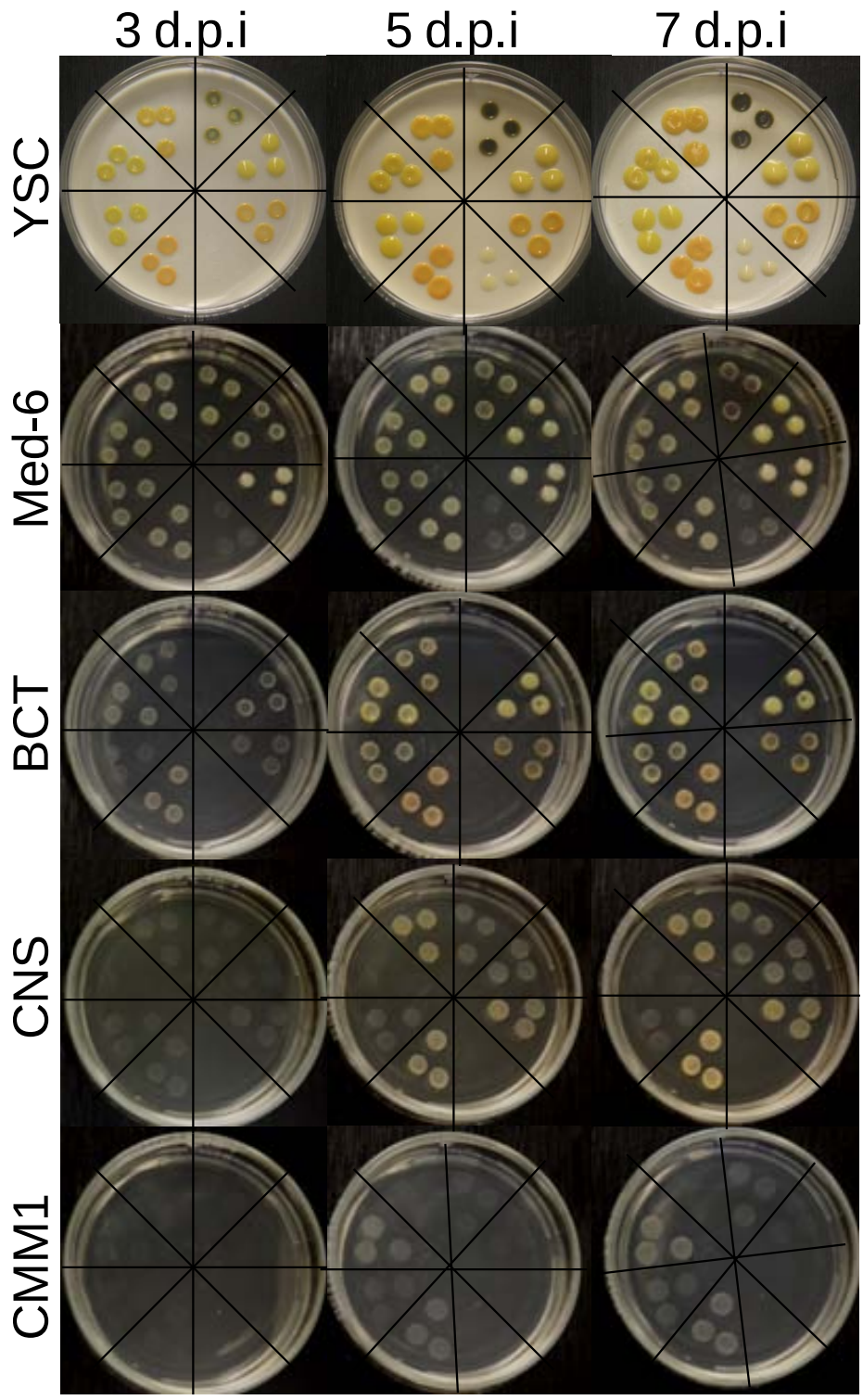

Figure F.2. Bacterial growth on various media, second trial. Different subspecies of *Clavibacter* were spot plated onto two growth media (YSC and Medium 6 [Med-6]) and three selective media (BCT, CNS and CMM1) to show differential growth, color and selectivity of media. Plates were divided into eight quadrants. Starting with the top right quadrant (12 o'clock to 1:30) and moving clockwise, *Cmi*, *Cmm*, *Cmn*, *Cms*, *Cmt*, C55, ZUM3936, and PS005 were plated, respectively.

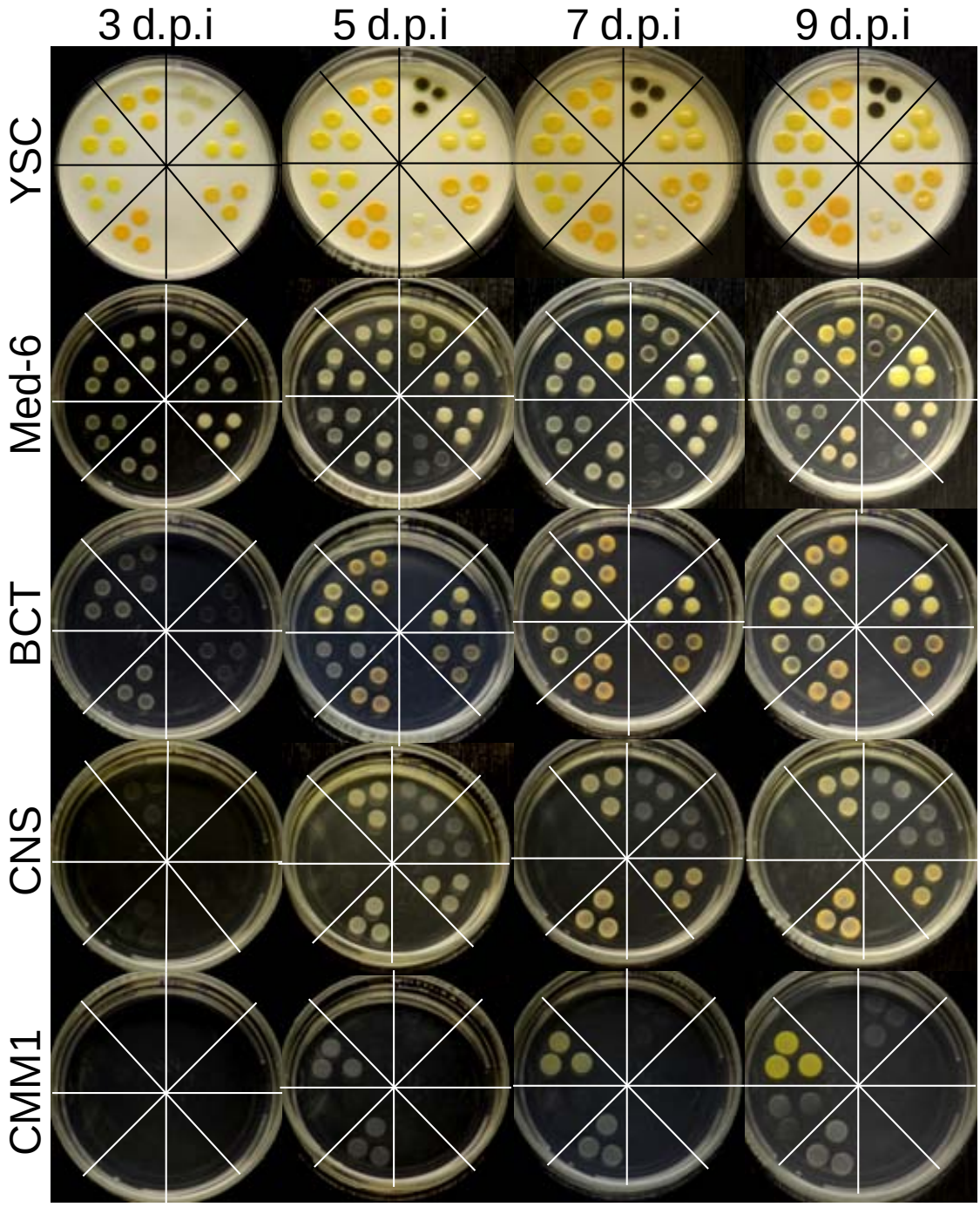

Figure F.3. Bacterial growth on various media, final trial. Different subspecies of *Clavibacter* were spot plated onto two growth media (YSC and Medium 6 [Med-6]) and three selective media (BCT, CNS and CMM1) to show differential growth, color and selectivity of media. Plates were divided into eight quadrants. Starting with the top right quadrant (12 o'clock to 1:30) and moving clockwise, *Cmi*, *Cmm*, *Cmn*, *Cms*, *Cmt*, C55, ZUM3936, and PS005 were plated, respectively.

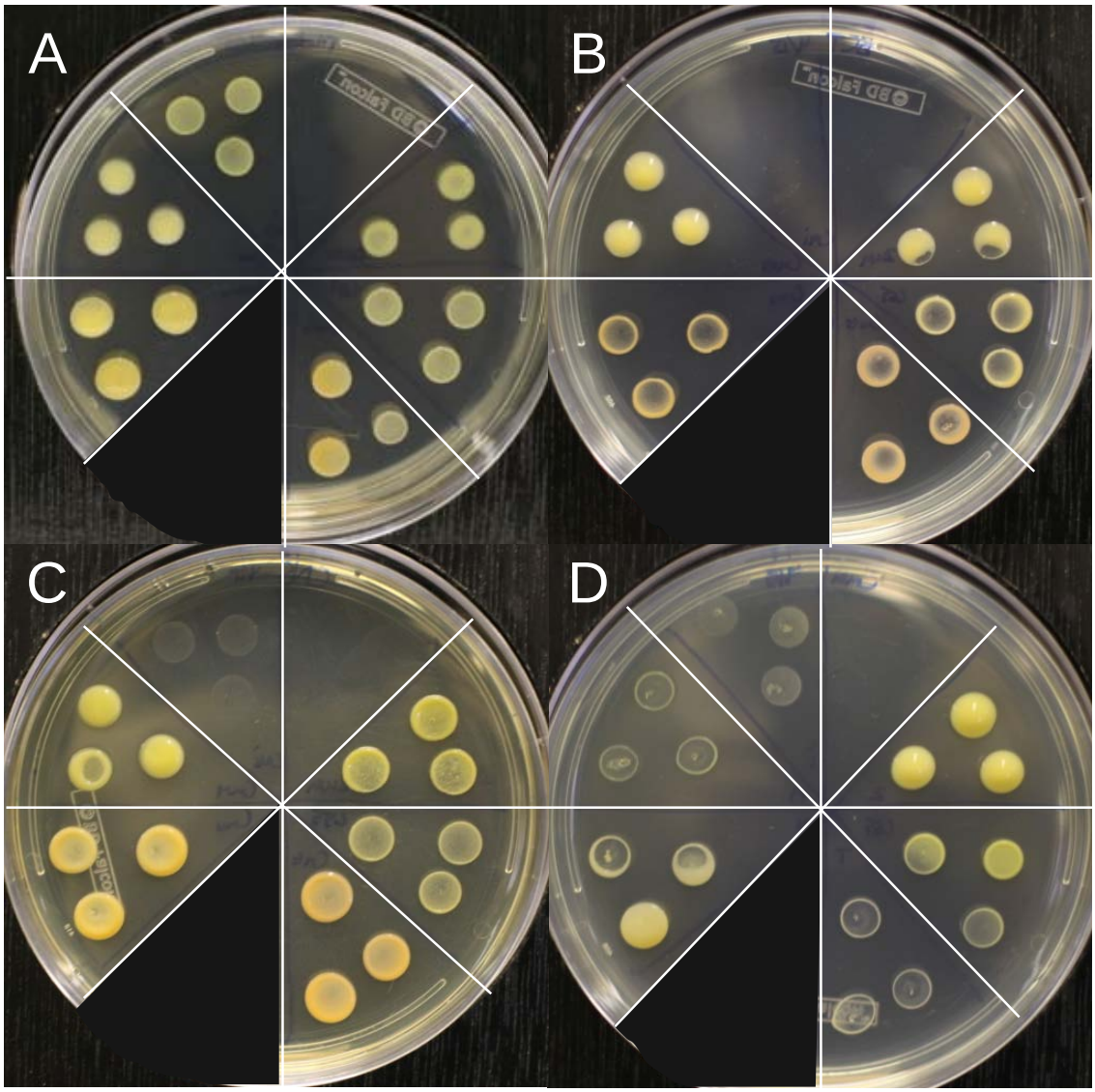

Figure F.4. Bacterial growth on various media at high inoculum. Different subspecies of *Clavibacter* were spot plated onto A) Medium 6 and selective media B) BCT, C) CNS and D) CMM1 at a high inoculum (~5 x 10^5 CFU/spot) to show differential growth, color and selectivity of media. Pictures were taken at 3 d.p.i. for A and at 7 d.p.i for B, C and D. Plates were divided into eight quadrants. Starting with the top left quadrant (11:30 to 12 o'clock) and moving counterclockwise, *Cmi*, *Cmm*, *Cmn*, *Cms* (not included), *Cmt*, C55, ZUM3936, and PS005 (not included) were plated, respectively.

APPENDIX G: BIOLOG Data.

Table G.1. Strain C55 BIOLOG data.

C55 Test	Plate 1	Plate 2	Plate 3	Plate 4	*COMBINED*
Carbon Source Utilization:					
Dextrin	+	+	+	+	+
D-Maltose	+	+	+	+	+
D-Trehalose	+	+	+	+	+
D-Cellobiose	+	+	+	+	+
Gentiobiose	+	+	+	+	+
Sucrose	+	+	+	+	+
D-Turanose	+	+	+	+	+
Stachyose	+	+	+	+	+
D-Raffinose	+	+	+	+	+
α-D-Lactose	+	+	+	+	+
D-Melebiose	+	+	+	+	+
β-Methyl-D-Glucoside	+	+	+	+	+
D-Salicin	+	+	+	+	+
N-Acetyl-D-Glucosamine	-	-	-	-	-
N-Acetyl-β-D-Mannosamine	-	-	-	-	-
N-Acetyl-D-Galactosamine	-	-	-	-	-
N-Acetyl-Neuraminic Acid	-	-	-	-	-
α-D-Glucose	+	+	+	+	+
D-Mannose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Galactose	+	+	+	+	+
3-Methyl Glucose	-	-	-	-	-
D-Fucose	-	w	w	w	w
L-Fucose	-	-	-	-	-
L-Rhamnose	-	-	-	w	-
Inosine	-	w	+	w	w/v
D-Sorbitol	+	+	w	w	+/w
D-Mannitol	+	+	+	+	+
D-Arabitol	-	-	-	-	-
myo-Inositol	+	+	+	+	+
Glycerol	+	+	+	+	+
D-Glucose-6-Phosphate	-	-	-	-	-
D-Fructose-6-Phosphate	-	-	-	-	-
D-Aspartic Acid	-	-	-	-	-
D-Serine	-	-	-	-	-
Gelatin	-	-	-	-	-
Glycyl-L-Proline	-	-	-	-	-
L-Alanine	+	-	-	w	v
L-Arginine	-	-	-	-	-
L-Aspartic Acid	+	+	+	+	+
L-Glutamic Acid	+	+	+	+	+
L-Histidine	-	-	-	-	-
L-Pyroglutamic Acid	-	-	-	-	-
L-Serine	-	-	-	-	-
Pectin	+	+	+	+	+
D-Galacturonic Acid	-	-	-	-	-
L-Galactonic Acid Lactone	-	-	-	-	-
D-Gluconic Acid	+	+	+	+	+

C55 Test	Plate 1	Plate 2	Plate 3	Plate 4	*COMBINED*
D-Glucuronic Acid	-	-	-	-	-
Glucuronamide	w	w	w	w	w
Mucic Acid	-	-	-	-	-
Quinic Acid	+	+	+	+	+
D-Saccharic Acid	-	-	-	-	-
p-Hydroxy-Phenylacetc Acid	-	-	-	-	-
Methyl Pyruvate	-	-	-	-	-
D-Lactic Acid Methyl Ester	-	-	-	-	-
L-Lactic Acid	-	-	-	-	-
Citric Acid	-	-	-	-	-
α-Ketoglutaric Acid	w	-	-	-	-
D-Malic Acid	-	-	-	-	-
L-Malic Acid	+	+	+	+	+
Bromo-Succinic Acid	-	-	-	-	-
Tween 40	-	-	-	-	-
γ-Amino-Butyric Acid	-	-	-	-	-
α-Hydroxy-Butyric Acid	-	-	-	-	-
β-Hydroxy-D,L-Butyric Acid	-	-	-	-	-
α-Keto-Butyric Acid	-	-	-	-	-
Acetoacetic Acid	+	+	+	+	+
Propionic Acid	-	-	-	-	-
Acetic Acid	+	w	w	w	w
Formic Acid	-	-	-	-	-
Chemical Sensitivity:					
pH6	+	+	+	+	+
pH5	w	+	+	w	+/w
1% NaCl	+	+	+	+	+
4% NaCl	+	w	w	+	+/w
8% NaCl	-	-	-	-	-
1% Sodium Lactate	+	+	+	+	+
Fusidic Acid	-	-	-	-	-
D-Serine	-	-	-	-	-
Troleandomycin	-	-	-	-	-
Rifamycin SV	-	-	-	-	-
Minocycline	-	-	-	-	-
Lincomycin	-	-	-	-	-
Guanidine HCl	-	-	-	-	-
Niaproof 4	-	-	-	-	-
Vancomycin	-	-	-	-	-
Tetrazolium Violet	-	-	-	-	-
Tetrazolium Blue	-	-	-	-	-
Nalidixic Acid	+	+	+	+	+
Lithium Chloride	+	+	+	+	+
Potassium Tellurite	w	+	+	+	+
Aztreonam	+	+	+	+	+
Sodium Butyrate	-	-	-	-	-
Sodium Bromate	w	w	w	w	w

Table G.2. Strain ZUM 3936 BIOLOG data.

ZUM 3936 Test	Plate 1	Plate 2	Plate 3	Plate 4	*COMBINED*
Carbon Source Utilization:					
Dextrin	+	+	+	+	+
D-Maltose	+	+	+	+	+
D-Trehalose	+	+	+	+	+
D-Cellobiose	+	+	+	+	+
Gentiobiose	+	+	+	+	+
Sucrose	+	+	+	+	+
D-Turanose	+	+	+	+	+
Stachyose	+	+	+	+	+
D-Raffinose	+	w	w	w	w
α-D-Lactose	+	+	+	+	+
D-Melebiose	+	+	+	+	+
β-Methyl-D-Glucoside	+	+	+	+	+
D-Salicin	+	+	+	+	+
N-Acetyl-D-Glucosamine	+	+	+	+	+
N-Acetyl-β-D-Mannosamine	w	-	-	w	w/-
N-Acetyl-D-Galactosamine	-	-	-	-	-
N-Acetyl-Neuraminic Acid	-	-	-	-	-
α-D-Glucose	+	+	+	+	+
D-Mannose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Galactose	+	+	+	+	+
3-Methyl Glucose	-	-	-	-	-
D-Fucose	w	-	-	-	-
L-Fucose	-	-	-	-	-
L-Rhamnose	+	w	+	+	+
Inosine	+	+	+	+	+
D-Sorbitol	+	+	+	+	+
D-Mannitol	+	+	+	+	+
D-Arabitol	-	-	-	-	-
myo-Inositol	+	+	+	+	+
Glycerol	+	+	+	+	+
D-Glucose-6-Phosphate	-	-	-	-	-
D-Fructose-6-Phosphate	-	-	-	-	-
D-Aspartic Acid	-	-	-	-	-
D-Serine	-	-	-	-	-
Gelatin	-	-	-	-	-
Glycyl-L-Proline	-	-	-	-	-
L-Alanine	+	+	+	+	+
L-Arginine	-	-	-	-	-
L-Aspartic Acid	w	w	w	w	w
L-Glutamic Acid	+	+	+	+	+
L-Histidine	-	-	-	-	-
L-Pyroglutamic Acid	-	-	-	-	-
L-Serine	+	+	+	+	+
Pectin	+	+	+	+	+
D-Galacturonic Acid	-	-	-	-	-
L-Galactonic Acid Lactone	-	-	-	-	-
D-Gluconic Acid	+	+	+	+	+

ZUM 3936 Test	Plate 1	Plate 2	Plate 3	Plate 4	*COMBINED*
D-Glucuronic Acid	-	-	-	-	-
Glucuronamide	-	-	-	-	-
Mucic Acid	w	-	-	-	-
Quinic Acid	-	-	-	-	-
D-Saccharic Acid	-	-	-	-	-
p-Hydroxy-Phenylacetc Acid	-	-	-	-	-
Methyl Pyruvate	w	w	w	w	w
D-Lactic Acid Methyl Ester	-	-	-	-	-
L-Lactic Acid	+	w	w	w	w
Citric Acid	+	+	+	+	+
α-Ketoglutaric Acid	w	w	w	w	w
D-Malic Acid	-	-	-	-	-
L-Malic Acid	+	+	+	+	+
Bromo-Succinic Acid	+	+	+	+	+
Tween 40	+	+	+	+	+
γ-Amino-Butyric Acid	-	-	-	-	-
α-Hydroxy-Butyric Acid	-	-	-	-	-
β-Hydroxy-D,L-Butyric Acid	-	-	-	-	-
α-Keto-Butyric Acid	w	w	w	w	w
Acetoacetic Acid	+	+	+	+	+
Propionic Acid	-	-	-	-	-
Acetic Acid	-	-	-	-	-
Formic Acid	-	-	-	-	-
Chemical Sensitivity:					
pH6	+	+	+	+	+
pH5	+	+	+	+	+
1% NaCl	+	+	+	+	+
4% NaCl	+	+	+	+	+
8% NaCl	w	+	w	+	+/w
1% Sodium Lactate	+	+	+	+	+
Fusidic Acid	-	-	-	-	-
D-Serine	-	-	-	-	-
Troleandomycin	-	-	-	-	-
Rifamycin SV	-	-	-	-	-
Minocycline	-	-	-	-	-
Lincomycin	-	-	-	-	-
Guanidine HCl	-	-	-	-	-
Niaproof 4	-	-	-	-	-
Vancomycin	-	-	-	-	-
Tetrazolium Violet	-	-	-	-	-
Tetrazolium Blue	-	-	-	-	-
Nalidixic Acid	+	+	+	+	+
Lithium Chloride	+	+	+	+	+
Potassium Tellurite	+	+	+	+	+
Aztreonam	+	+	+	+	+
Sodium Butyrate	w	+	+	+	+
Sodium Bromate	+	+	+	+	+

Table G.3. *Clavibacter michiganensis* subsp. *insidiosus* strain A1149 BIOLOG data.

Cmi Test	Plate 1	Plate 2	Plate 3	Plate 4	*COMBINED*
Carbon Source Utilization:					
Dextrin	w	+	+	+	+
D-Maltose	-	+	w	w	w/v
D-Trehalose	-	-	-	w	-
D-Cellobiose	-	+	+	+	+
Gentiobiose	-	-	-	-	-
Sucrose	+	+	+	+	+
D-Turanose	+	+	+	+	+
Stachyose	-	-	-	-	-
D-Raffinose	-	-	-	-	-
α-D-Lactose	+	+	+	+	+
D-Melebiose	-	-	-	-	-
β-Methyl-D-Glucoside	-	-	-	-	-
D-Salicin	-	-	-	-	-
N-Acetyl-D-Glucosamine	-	-	-	-	-
N-Acetyl-β-D-Mannosamine	-	-	-	-	-
N-Acetyl-D-Galactosamine	-	-	-	-	-
N-Acetyl-Neuraminic Acid	-	-	-	-	-
α-D-Glucose	+	+	+	+	+
D-Mannose	w	+	+	+	+
D-Fructose	+	+	+	+	+
D-Galactose	+	+	+	+	+
3-Methyl Glucose	-	w	+	+	+/v
D-Fucose	-	w	w	+	w/v
L-Fucose	-	w	+	+	+/v
L-Rhamnose	-	w	+	w	w/v
Inosine	-	-	-	-	-
D-Sorbitol	-	w	w	w	w
D-Mannitol	+	+	+	+	+
D-Arabitol	-	-	-	-	-
myo-Inositol	-	+	+	+	+
Glycerol	+	+	+	+	+
D-Glucose-6-Phosphate	-	-	-	-	-
D-Fructose-6-Phosphate	-	-	-	-	-
D-Aspartic Acid	-	-	-	-	-
D-Serine	-	-	-	-	-
Gelatin	-	-	-	-	-
Glycyl-L-Proline	-	-	-	-	-
L-Alanine	-	-	-	-	-
L-Arginine	-	-	-	-	-
L-Aspartic Acid	-	+	+	+	+
L-Glutamic Acid	-	-	-	w	-
L-Histidine	-	-	-	-	-
L-Pyroglutamic Acid	-	-	-	-	-
L-Serine	-	-	-	-	-
Pectin	+	+	+	+	+
D-Galacturonic Acid	-	-	-	-	-
L-Galactonic Acid Lactone	-	-	-	-	-
D-Gluconic Acid	+	-	-	-	-

Cmi Test	Plate 1	Plate 2	Plate 3	Plate 4	*COMBINED*
D-Glucuronic Acid	-	-	-	-	-
Glucuronamide	w	w	+	+	+/w
Mucic Acid	-	-	-	-	-
Quinic Acid	-	+	+	+	+
D-Saccharic Acid	-	-	-	-	-
p-Hydroxy-Phenylacetc Acid	-	-	-	-	-
Methyl Pyruvate	-	-	-	-	-
D-Lactic Acid Methyl Ester	-	-	-	-	-
L-Lactic Acid	-	-	-	-	-
Citric Acid	-	-	-	-	-
α-Ketoglutaric Acid	w	-	-	-	-
D-Malic Acid	-	-	-	-	-
L-Malic Acid	+	+	+	+	+
Bromo-Succinic Acid	-	-	-	-	-
Tween 40	w	+	+	+	+
γ-Amino-Butyric Acid	-	-	-	-	-
α-Hydroxy-Butyric Acid	-	-	-	-	-
β-Hydroxy-D,L-Butyric Acid	-	-	-	-	-
α-Keto-Butyric Acid	w	-	-	-	-
Acetoacetic Acid	+	+	+	+	+
Propionic Acid	+	-	-	-	-
Acetic Acid	+	+	+	+	+
Formic Acid	+	-	-	-	-
Chemical Sensitivity:					
pH6	w	+	+	+	+
pH5	-	-	-	-	-
1% NaCl	+	+	+	+	+
4% NaCl	-	-	-	-	-
8% NaCl	-	-	-	-	-
1% Sodium Lactate	-	-	-	-	-
Fusidic Acid	-	-	-	-	-
D-Serine	-	-	-	-	-
Troleandomycin	-	-	-	-	-
Rifamycin SV	-	-	-	-	-
Minocycline	-	-	-	-	-
Lincomycin	-	-	-	-	-
Guanidine HCl	-	-	-	-	-
Niaproof 4	-	-	-	-	-
Vancomycin	-	-	-	-	-
Tetrazolium Violet	-	-	-	-	-
Tetrazolium Blue	-	-	-	-	-
Nalidixic Acid	+	+	+	+	+
Lithium Chloride	+	w	w	w	w
Potassium Tellurite	+	+	+	+	+
Aztreonam	+	+	+	+	+
Sodium Butyrate	+	w	w	w	w
Sodium Bromate	-	-	-	-	-

Table G.4. *Clavibacter michiganensis* subsp. *michiganensis* strain K73 BIOLOG data.

Cmm Test	Plate 1	Plate 2	Plate 3	Plate 4	*COMBINED*
Carbon Source Utilization:					
Dextrin	+	+	+	+	+
D-Maltose	+	+	+	+	+
D-Trehalose	+	+	+	+	+
D-Cellobiose	+	+	+	+	+
Gentiobiose	+	+	+	+	+
Sucrose	+	+	+	+	+
D-Turanose	+	+	+	+	+
Stachyose	+	+	+	+	+
D-Raffinose	+	+	+	+	+
α-D-Lactose	+	+	+	+	+
D-Melebiose	+	+	+	+	+
β-Methyl-D-Glucoside	+	+	+	+	+
D-Salicin	+	+	+	+	+
N-Acetyl-D-Glucosamine	-	-	-	-	-
N-Acetyl-β-D-Mannosamine	-	-	-	-	-
N-Acetyl-D-Galactosamine	-	-	-	-	-
N-Acetyl-Neuraminic Acid	-	-	-	-	-
α-D-Glucose	+	+	+	+	+
D-Mannose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Galactose	+	+	+	+	+
3-Methyl Glucose	-	-	w	-	-
D-Fucose	-	w	+	w	w/v
L-Fucose	w	w	w	+	w
L-Rhamnose	-	-	-	-	-
Inosine	+	w	w	+	+/w
D-Sorbitol	+	w	+	+	+
D-Mannitol	+	+	+	+	+
D-Arabitol	-	-	-	-	-
myo-Inositol	+	+	+	+	+
Glycerol	+	+	+	+	+
D-Glucose-6-Phosphate	-	w	w	-	w/-
D-Fructose-6-Phosphate	-	w	w	w	w
D-Aspartic Acid	-	-	-	-	-
D-Serine	-	-	-	-	-
Gelatin	w	-	-	-	-
Glycyl-L-Proline	-	-	-	-	-
L-Alanine	+	-	w	w	w/v
L-Arginine	-	-	w	-	-
L-Aspartic Acid	+	+	+	+	+
L-Glutamic Acid	+	+	+	+	+
L-Histidine	-	-	-	-	-
L-Pyroglutamic Acid	-	-	-	-	-
L-Serine	+	+	+	+	+
Pectin	+	+	+	+	+
D-Galacturonic Acid	-	-	-	-	-
L-Galactonic Acid Lactone	-	-	-	-	-
D-Gluconic Acid	+	+	+	+	+

Cmm Test	Plate 1	Plate 2	Plate 3	Plate 4	*COMBINED*
D-Glucuronic Acid	-	-	-	-	-
Glucuronamide	w	+	w	+	+/w
Mucic Acid	-	w	w	w	w
Quinic Acid	+	+	+	+	+
D-Saccharic Acid	-	-	-	-	-
p-Hydroxy-Phenylacetc Acid	-	-	-	-	-
Methyl Pyruvate	-	-	-	-	-
D-Lactic Acid Methyl Ester	-	w	w	-	w/-
L-Lactic Acid	-	-	-	-	-
Citric Acid	+	+	+	+	+
α-Ketoglutaric Acid	w	w	w	w	w
D-Malic Acid	+	w	w	w	w
L-Malic Acid	+	+	+	+	+
Bromo-Succinic Acid	+	-	+	+	+
Tween 40	+	+	+	+	+
γ-Amino-Butyric Acid	-	-	-	-	-
α-Hydroxy-Butyric Acid	-	-	-	-	-
β-Hydroxy-D,L-Butyric Acid	-	-	-	-	-
α-Keto-Butyric Acid	-	-	-	-	-
Acetoacetic Acid	+	+	+	+	+
Propionic Acid	w	w	w	w	w
Acetic Acid	+	+	+	+	+
Formic Acid	-	-	-	-	-
Chemical Sensitivity:					
pH6	+	+	+	+	+
pH5	+	+	+	+	+
1% NaCl	+	+	+	+	+
4% NaCl	+	+	+	+	+
8% NaCl	-	-	-	-	-
1% Sodium Lactate	+	-	+	w	+/v
Fusidic Acid	-	-	-	-	-
D-Serine	-	-	-	-	-
Troleandomycin	-	-	-	-	-
Rifamycin SV	-	-	-	-	-
Minocycline	-	-	-	-	-
Lincomycin	-	-	-	-	-
Guanidine HCl	+	+	+	+	+
Niaproof 4	-	-	-	-	-
Vancomycin	-	-	-	-	-
Tetrazolium Violet	-	-	-	-	-
Tetrazolium Blue	-	-	-	-	-
Nalidixic Acid	+	+	+	+	+
Lithium Chloride	+	+	+	+	+
Potassium Tellurite	+	+	+	+	+
Aztreonam	+	+	+	+	+
Sodium Butyrate	-	-	-	-	-
Sodium Bromate	+	+	+	+	+

Table G.5. *Clavibacter michiganensis* subsp. *nebraskensis* strain NCPPB 2579 BIOLOG data.

Cmn Test	Plate 1	Plate 2	Plate 3	Plate 4	*COMBINED*
Carbon Source Utilization:					
Dextrin	+	+	+	+	+
D-Maltose	+	+	+	+	+
D-Trehalose	+	+	+	+	+
D-Cellobiose	+	+	+	+	+
Gentiobiose	+	+	+	+	+
Sucrose	+	+	+	+	+
D-Turanose	+	+	+	+	+
Stachyose	+	+	+	+	+
D-Raffinose	-	-	-	-	-
α-D-Lactose	+	+	+	+	+
D-Melebiose	w	+	+	+	+
β-Methyl-D-Glucoside	+	+	+	+	+
D-Salicin	+	+	+	+	+
N-Acetyl-D-Glucosamine	-	-	-	-	-
N-Acetyl-β-D-Mannosamine	-	-	-	-	-
N-Acetyl-D-Galactosamine	-	-	-	-	-
N-Acetyl-Neuraminic Acid	-	-	-	-	-
α-D-Glucose	+	+	+	+	+
D-Mannose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Galactose	+	+	+	+	+
3-Methyl Glucose	-	-	-	-	-
D-Fucose	-	-	-	-	-
L-Fucose	-	-	-	-	-
L-Rhamnose	-	-	-	-	-
Inosine	-	-	-	-	-
D-Sorbitol	+	+	+	+	+
D-Mannitol	+	+	+	+	+
D-Arabitol	-	-	-	-	-
myo-Inositol	+	+	+	+	+
Glycerol	+	+	+	+	+
D-Glucose-6-Phosphate	-	-	-	-	-
D-Fructose-6-Phosphate	-	-	-	-	-
D-Aspartic Acid	-	-	-	-	-
D-Serine	-	-	-	-	-
Gelatin	-	-	-	-	-
Glycyl-L-Proline	-	-	-	w	-
L-Alanine	+	+	+	+	+
L-Arginine	-	-	-	-	-
L-Aspartic Acid	w	w	w	w	w
L-Glutamic Acid	w	w	w	+	w
L-Histidine	-	-	-	-	-
L-Pyroglutamic Acid	-	-	-	-	-
L-Serine	+	+	+	+	+
Pectin	+	+	+	+	+
D-Galacturonic Acid	-	-	-	-	-
L-Galactonic Acid Lactone	-	-	-	-	-
D-Gluconic Acid	+	+	+	+	+

Cmn Test	Plate 1	Plate 2	Plate 3	Plate 4	*COMBINED*
D-Glucuronic Acid	-	-	-	-	-
Glucuronamide	-	-	-	-	-
Mucic Acid	-	-	-	-	-
Quinic Acid	-	-	-	-	-
D-Saccharic Acid	-	-	-	-	-
p-Hydroxy-Phenylacetc Acid	-	-	-	-	-
Methyl Pyruvate	-	-	-	-	-
D-Lactic Acid Methyl Ester	-	w	-	-	-
L-Lactic Acid	-	-	-	-	-
Citric Acid	w	w	w	w	w
α-Ketoglutaric Acid	-	-	-	-	-
D-Malic Acid	-	-	-	-	-
L-Malic Acid	-	-	-	-	-
Bromo-Succinic Acid	-	-	-	-	-
Tween 40	+	+	+	+	+
γ-Amino-Butyric Acid	-	-	-	-	-
α-Hydroxy-Butyric Acid	-	-	-	-	-
β-Hydroxy-D,L-Butyric Acid	-	-	-	-	-
α-Keto-Butyric Acid	-	-	-	-	-
Acetoacetic Acid	+	+	+	+	+
Propionic Acid	-	w	w	-	w/-
Acetic Acid	w	w	-	-	w/-
Formic Acid	-	-	-	-	-
Chemical Sensitivity:					
pH6	+	+	+	+	+
pH5	w	w	w	w	w
1% NaCl	+	+	+	+	+
4% NaCl	w	-	-	+	v
8% NaCl	-	-	-	-	-
1% Sodium Lactate	-	+	+	+	+
Fusidic Acid	-	-	-	-	-
D-Serine	-	-	-	-	-
Troleandomycin	-	-	-	-	-
Rifamycin SV	-	-	-	-	-
Minocycline	-	-	-	-	-
Lincomycin	-	-	-	-	-
Guanidine HCl	-	-	-	-	-
Niaproof 4	-	-	-	-	-
Vancomycin	-	-	-	-	-
Tetrazolium Violet	-	-	-	-	-
Tetrazolium Blue	-	-	-	-	-
Nalidixic Acid	+	+	+	+	+
Lithium Chloride	w	w	w	w	w
Potassium Tellurite	+	+	+	+	+
Aztreonam	+	+	+	+	+
Sodium Butyrate	-	-	-	-	-
Sodium Bromate	+	+	+	+	+

Table G.6. *Clavibacter michiganensis* subsp. *sepedonicus* strain A2041 BIOLOG data.

Cms Test	Plate 1	Plate 2	Plate 3	Plate 4	*COMBINED*
Carbon Source Utilization:					
Dextrin	-	-	-	-	-
D-Maltose	-	-	-	-	-
D-Trehalose	-	-	-	-	-
D-Cellobiose	+	+	+	+	+
Gentiobiose	-	-	-	-	-
Sucrose	-	-	-	+	-
D-Turanose	-	-	-	-	-
Stachyose	-	-	-	-	-
D-Raffinose	+	+	+	+	+
α-D-Lactose	-	-	-	-	-
D-Melebiose	-	-	-	-	-
β-Methyl-D-Glucoside	-	-	-	-	-
D-Salicin	-	-	-	-	-
N-Acetyl-D-Glucosamine	-	-	-	-	-
N-Acetyl-β-D-Mannosamine	-	-	-	-	-
N-Acetyl-D-Galactosamine	-	-	-	-	-
N-Acetyl-Neuraminic Acid	-	-	-	-	-
α-D-Glucose	+	+	+	+	+
D-Mannose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Galactose	-	-	-	+	-
3-Methyl Glucose	-	-	-	-	-
D-Fucose	-	-	-	-	-
L-Fucose	-	-	-	-	-
L-Rhamnose	-	-	-	-	-
Inosine	-	-	-	-	-
D-Sorbitol	-	-	-	-	-
D-Mannitol	+	+	+	+	+
D-Arabitol	-	-	-	-	-
myo-Inositol	-	-	-	-	-
Glycerol	-	-	-	-	-
D-Glucose-6-Phosphate	-	-	-	-	-
D-Fructose-6-Phosphate	-	-	-	-	-
D-Aspartic Acid	-	-	-	-	-
D-Serine	-	-	-	-	-
Gelatin	-	-	-	-	-
Glycyl-L-Proline	-	-	-	-	-
L-Alanine	-	-	-	-	-
L-Arginine	-	-	-	-	-
L-Aspartic Acid	-	-	-	-	-
L-Glutamic Acid	-	-	-	-	-
L-Histidine	-	-	-	-	-
L-Pyroglutamic Acid	-	-	-	-	-
L-Serine	-	-	-	-	-
Pectin	+	+	+	+	+
D-Galacturonic Acid	-	-	-	-	-
L-Galactonic Acid Lactone	-	-	-	-	-
D-Gluconic Acid	+	+	+	+	+

Cms Test	Plate 1	Plate 2	Plate 3	Plate 4	*COMBINED*
D-Glucuronic Acid	-	-	-	-	-
Glucuronamide	-	-	-	-	-
Mucic Acid	-	-	-	-	-
Quinic Acid	-	-	-	-	-
D-Saccharic Acid	-	-	-	-	-
p-Hydroxy-Phenylacetc Acid	-	-	-	-	-
Methyl Pyruvate	-	-	-	-	-
D-Lactic Acid Methyl Ester	-	-	-	-	-
L-Lactic Acid	-	-	-	-	-
Citric Acid	-	-	-	-	-
α-Ketoglutaric Acid	-	-	-	-	-
D-Malic Acid	-	-	-	-	-
L-Malic Acid	-	-	-	w	-
Bromo-Succinic Acid	-	-	-	-	-
Tween 40	-	-	-	-	-
γ-Amino-Butyric Acid	-	-	-	-	-
α-Hydroxy-Butyric Acid	-	-	-	-	-
β-Hydroxy-D,L-Butyric Acid	-	-	-	-	-
α-Keto-Butyric Acid	-	-	-	-	-
Acetoacetic Acid	+	+	+	+	+
Propionic Acid	-	-	-	-	-
Acetic Acid	-	-	-	-	-
Formic Acid	-	-	-	-	-
Chemical Sensitivity:					
pH6	+	+	+	+	+
pH5	-	-	-	-	-
1% NaCl	w	w	w	w	w
4% NaCl	-	-	-	-	-
8% NaCl	-	-	-	-	-
1% Sodium Lactate	-	-	-	-	-
Fusidic Acid	-	-	-	-	-
D-Serine	-	-	-	-	-
Troleandomycin	-	-	-	-	-
Rifamycin SV	-	-	-	-	-
Minocycline	-	-	-	-	-
Lincomycin	-	-	-	-	-
Guanidine HCl	-	-	-	-	-
Niaproof 4	-	-	-	-	-
Vancomycin	-	-	-	-	-
Tetrazolium Violet	w	w	w	-	w
Tetrazolium Blue	-	-	-	-	-
Nalidixic Acid	+	+	+	+	+
Lithium Chloride	-	-	-	-	-
Potassium Tellurite	+	+	+	+	+
Aztreonam	+	+	+	+	+
Sodium Butyrate	-	-	-	-	-
Sodium Bromate	-	-	-	-	-

Table G.7. *Clavibacter michiganensis* subsp. *tessellarius* strain LMG 7294 BIOLOG data.

Cmt Test	Plate 1	Plate 2	Plate 3	Plate 4	*COMBINED*
Carbon Source Utilization:					
Dextrin	+	+	+	+	+
D-Maltose	+	+	+	+	+
D-Trehalose	+	+	+	+	+
D-Cellobiose	+	+	+	+	+
Gentiobiose	+	+	+	+	+
Sucrose	+	+	+	+	+
D-Turanose	+	+	+	+	+
Stachyose	+	+	+	+	+
D-Raffinose	-	w	-	w	w/-
α-D-Lactose	+	+	+	+	+
D-Melebiose	+	+	+	+	+
β-Methyl-D-Glucoside	+	+	+	+	+
D-Salicin	+	+	+	+	+
N-Acetyl-D-Glucosamine	-	-	-	-	-
N-Acetyl-β-D-Mannosamine	-	-	-	-	-
N-Acetyl-D-Galactosamine	-	-	-	-	-
N-Acetyl-Neuraminic Acid	-	-	-	-	-
α-D-Glucose	+	+	+	+	+
D-Mannose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Galactose	+	+	+	+	+
3-Methyl Glucose	-	-	-	-	-
D-Fucose	-	-	-	-	-
L-Fucose	-	-	-	-	-
L-Rhamnose	-	-	-	-	-
Inosine	+	+	+	+	+
D-Sorbitol	+	+	+	+	+
D-Mannitol	+	+	+	+	+
D-Arabitol	-	-	-	-	-
myo-Inositol	+	+	+	+	+
Glycerol	+	+	+	+	+
D-Glucose-6-Phosphate	-	-	-	-	-
D-Fructose-6-Phosphate	-	-	-	-	-
D-Aspartic Acid	-	-	-	-	-
D-Serine	-	-	-	-	-
Gelatin	w	+	-	w	w/v
Glycyl-L-Proline	-	-	-	-	-
L-Alanine	+	+	+	+	+
L-Arginine	-	-	-	-	-
L-Aspartic Acid	-	-	-	-	-
L-Glutamic Acid	+	+	w	w	+/w
L-Histidine	-	-	-	-	-
L-Pyroglutamic Acid	-	-	-	-	-
L-Serine	+	+	+	+	+
Pectin	+	+	+	+	+
D-Galacturonic Acid	-	-	-	-	-
L-Galactonic Acid Lactone	-	-	-	-	-
D-Gluconic Acid	+	+	+	+	+

Cmt Test	Plate 1	Plate 2	Plate 3	Plate 4	*COMBINED*
D-Glucuronic Acid	-	-	-	-	-
Glucuronamide	-	-	-	-	-
Mucic Acid	-	-	-	-	-
Quinic Acid	-	-	-	-	-
D-Saccharic Acid	-	-	-	-	-
p-Hydroxy-Phenylacetc Acid	-	-	-	-	-
Methyl Pyruvate	-	-	-	-	-
D-Lactic Acid Methyl Ester	-	-	-	-	-
L-Lactic Acid	-	-	-	-	-
Citric Acid	+	+	+	+	+
α-Ketoglutaric Acid	-	-	-	-	-
D-Malic Acid	-	-	-	-	-
L-Malic Acid	+	+	+	+	+
Bromo-Succinic Acid	+	+	+	+	+
Tween 40	+	+	+	+	+
γ-Amino-Butyric Acid	-	+	-	-	-
α-Hydroxy-Butyric Acid	-	-	-	-	+
β-Hydroxy-D,L-Butyric Acid	-	-	-	-	+
α-Keto-Butyric Acid	w	+	w	-	w/v
Acetoacetic Acid	+	+	+	+	+
Propionic Acid	w	w	-	-	w/-
Acetic Acid	+	+	w	w	+/w
Formic Acid	-	-	-	-	-
Chemical Sensitivity:					
pH6	+	+	+	+	+
pH5	+	+	+	+	+
1% NaCl	+	+	+	+	+
4% NaCl	+	+	+	+	+
8% NaCl	-	w	+	+	+/v
1% Sodium Lactate	+	+	+	+	+
Fusidic Acid	-	-	-	-	-
D-Serine	-	-	-	-	-
Troleandomycin	-	-	-	-	-
Rifamycin SV	-	-	-	-	-
Minocycline	-	-	-	-	-
Lincomycin	-	-	-	-	-
Guanidine HCl	-	-	-	-	-
Niaproof 4	-	-	-	-	-
Vancomycin	-	-	-	-	-
Tetrazolium Violet	-	-	-	-	-
Tetrazolium Blue	-	-	-	-	-
Nalidixic Acid	+	+	+	+	+
Lithium Chloride	+	+	+	+	+
Potassium Tellurite	+	+	+	+	+
Aztreonam	+	+	+	+	+
Sodium Butyrate	+	+	+	+	+
Sodium Bromate	+	+	+	+	+

Table G.8. Strain PS005 BIOLOG data.

PS005 Test	Plate 1	Plate 2	Plate 3	Plate 4	*COMBINED*
Carbon Source Utilization:					
Dextrin	+	+	+	+	+
D-Maltose	+	+	+	+	+
D-Trehalose	+	+	+	+	+
D-Cellobiose	+	+	+	+	+
Gentiobiose	+	+	+	+	+
Sucrose	+	+	+	+	+
D-Turanose	+	+	+	+	+
Stachyose	+	+	+	+	+
D-Raffinose	-	w	-	w	w/-
α-D-Lactose	+	+	+	+	+
D-Melebiose	+	+	+	+	+
β-Methyl-D-Glucoside	+	+	+	+	+
D-Salicin	+	+	+	+	+
N-Acetyl-D-Glucosamine	-	-	-	-	-
N-Acetyl-β-D-Mannosamine	-	-	-	-	-
N-Acetyl-D-Galactosamine	-	-	-	-	-
N-Acetyl-Neuraminic Acid	-	-	-	-	-
α-D-Glucose	+	+	+	+	+
D-Mannose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Galactose	+	+	+	+	+
3-Methyl Glucose	-	-	-	-	-
D-Fucose	-	-	-	-	-
L-Fucose	-	-	-	-	-
L-Rhamnose	-	-	-	-	-
Inosine	+	+	+	+	+
D-Sorbitol	+	+	+	+	+
D-Mannitol	+	+	+	+	+
D-Arabitol	-	-	-	-	-
myo-Inositol	+	+	+	+	+
Glycerol	+	+	+	+	+
D-Glucose-6-Phosphate	w	w	w	w	w
D-Fructose-6-Phosphate	w	w	w	w	w
D-Aspartic Acid	-	-	-	-	-
D-Serine	-	-	-	-	-
Gelatin	+	+	+	+	+
Glycyl-L-Proline	w	w	+	+	+/w
L-Alanine	+	+	+	+	+
L-Arginine	-	w	-	-	-
L-Aspartic Acid	w	w	w	w	w
L-Glutamic Acid	+	+	+	+	+
L-Histidine	-	-	-	-	-
L-Pyroglutamic Acid	-	-	-	-	-
L-Serine	+	+	+	+	+
Pectin	+	+	+	+	+
D-Galacturonic Acid	-	-	-	-	-
L-Galactonic Acid Lactone	-	-	-	-	-
D-Gluconic Acid	+	+	+	+	+

PS005 Test	Plate 1	Plate 2	Plate 3	Plate 4	*COMBINED*
D-Glucuronic Acid	-	-	-	-	-
Glucuronamide	-	-	-	-	-
Mucic Acid	w	w	w	+	w
Quinic Acid	-	-	-	-	-
D-Saccharic Acid	-	-	-	-	-
p-Hydroxy-Phenylacetc Acid	-	-	-	-	-
Methyl Pyruvate	+	+	+	+	+
D-Lactic Acid Methyl Ester	-	-	-	-	-
L-Lactic Acid	-	-	-	-	-
Citric Acid	+	+	+	+	+
α-Ketoglutaric Acid	w	w	w	+	w
D-Malic Acid	-	-	-	-	-
L-Malic Acid	+	+	+	+	+
Bromo-Succinic Acid	+	+	+	+	+
Tween 40	+	+	+	+	+
γ-Amino-Butyric Acid	-	-	-	-	-
α-Hydroxy-Butyric Acid	-	-	-	-	-
β-Hydroxy-D,L-Butyric Acid	-	-	-	-	-
α-Keto-Butyric Acid	-	-	-	-	-
Acetoacetic Acid	+	+	+	+	+
Propionic Acid	-	-	-	-	-
Acetic Acid	+	+	+	+	+
Formic Acid	-	-	-	-	-
Chemical Sensitivity:					
pH6	+	+	+	+	+
pH5	+	+	+	+	+
1% NaCl	+	+	+	+	+
4% NaCl	+	+	+	+	+
8% NaCl	w	+	w	w	w
1% Sodium Lactate	+	+	+	+	+
Fusidic Acid	-	-	-	-	-
D-Serine	-	-	-	-	-
Troleandomycin	-	-	-	-	-
Rifamycin SV	-	-	-	-	-
Minocycline	-	-	-	-	-
Lincomycin	-	-	-	-	-
Guanidine HCl	-	-	-	-	-
Niaproof 4	-	-	-	-	-
Vancomycin	-	-	-	-	-
Tetrazolium Violet	-	-	-	-	-
Tetrazolium Blue	-	-	-	-	-
Nalidixic Acid	+	+	+	+	+
Lithium Chloride	+	+	+	+	+
Potassium Tellurite	+	+	+	+	+
Aztreonam	+	+	+	+	+
Sodium Butyrate	-	w	w	-	w/-
Sodium Bromate	+	+	+	+	+

* Symbols: +, positive; -, negative; w, weak positive.

APPENDIX H: BIOLOG Database.

Table H.1. *Clavibacter* profiles from BIOLOG database.[*$]

Test	*Cm.* subsp. *insidiosus*	*Cm.* subsp. *michiganensis*	*Cm.* subsp. *nebraskensis*	*Cm.* subsp. *sepedonicus*	*Cm.* subsp. *tesselarius*
Carbon Source Utilization:					
Dextrin	-	-	w	-	+
D-Maltose	w	+	w	-	+
D-Trehalose	-	+	w	-	+
D-Cellobiose	-	+	+	-	+
Gentiobiose	-	+	+	-	+
Sucrose	+	+	+	-	+
D-Turanose	-	+	+	-	+
Stachyose	-	+	-	-	w
D-Raffinose	-	+	-	-	w
α-D-Lactose	-	+	-	-	+
D-Melebiose	-	w	-	-	w
β-Methyl-D-Glucoside	-	-	-	-	-
D-Salicin	-	+	-	-	+
N-Acetyl-D-Glucosamine	-	-	-	-	-
N-Acetyl-β-D-Mannosamine	-	-	-	-	-
N-Acetyl-D-Galactosamine	-	-	-	-	-
N-Acetyl-Neuraminic Acid	-	-	-	-	-
α-D-Glucose	+	+	+	w	+
D-Mannose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Galactose	+	+	+	w	+
3-Methyl Glucose	-	-	-	-	-
D-Fucose	-	-	-	-	-
L-Fucose	-	-	-	-	-
L-Rhamnose	-	-	-	-	-
Inosine	-	-	-	-	-
D-Sorbitol	-	-	-	-	-
D-Mannitol	+	+	+	+	+
D-Arabitol	-	-	-	-	-
myo-Inositol	-	+	+	-	w
Glycerol	+	+	+	-	+
D-Glucose-6-Phosphate	-	-	-	-	-
D-Fructose-6-Phosphate	-	-	-	-	-
D-Aspartic Acid	-	-	-	-	-
D-Serine	-	-	-	-	-
Gelatin	-	-	-	-	-
Glycyl-L-Proline	-	-	-	-	-
L-Alanine	-	-	-	-	-
L-Arginine	-	-	-	-	-
L-Aspartic Acid	-	-	-	-	-
L-Glutamic Acid	-	-	-	-	-
L-Histidine	-	-	-	-	-
L-Pyroglutamic Acid	-	-	-	-	-
L-Serine	-	-	-	-	-
Pectin	+	+	+	+	+
D-Galacturonic Acid	-	-	-	-	-
L-Galactonic Acid Lactone	-	-	-	-	-
D-Gluconic Acid	-	+	+	w	+

Test	*Cm.* subsp. *insidiosus*	*Cm.* subsp. *michiganensis*	*Cm.* subsp. *nebraskensis*	*Cm.* subsp. *sepedonicus*	*Cm.* subsp. *tesselarius*
D-Glucuronic Acid	-	-	-	-	-
Glucuronamide	-	-	-	-	-
Mucic Acid	-	-	-	-	-
Quinic Acid	-	-	-	-	-
D-Saccharic Acid	-	-	-	-	-
p-Hydroxy-Phenylacetc Acid	-	-	-	-	-
Methyl Pyruvate	-	-	-	-	-
D-Lactic Acid Methyl Ester	-	-	-	-	-
L-Lactic Acid	-	-	-	-	-
Cirtic Acid	-	-	-	-	-
α-Ketoglutaric Acid	-	-	-	-	-
D-Malic Acid	-	-	-	-	-
L-Malic Acid	-	-	-	-	+
Bromo-Succinic Acid	-	-	-	-	-
Tween 40	-	-	-	-	-
γ-Amino-Butyric Acid	-	-	-	-	-
α-Hydroxy-Butyric Acid	-	-	-	-	-
β-Hydroxy-D,L-Butyric Acid	-	-	-	-	-
α-Keto-Butyric Acid	-	-	-	-	-
Acetoacetic Acid	+	+	+	+	+
Propionic Acid	-	-	-	-	-
Acetic Acid	+	-	w	+	+
Formic Acid	-	-	-	-	-
Chemical Sensitivity:					
pH6	+	+	+	+	+
pH5	+	w	w	-	+
1% NaCl	+	+	+	+	+
4% NaCl	-	+	w	-	+
8% NaCl	-	-	-	-	-
1% Sodium Lactate	+	-	+	+	+
Fusidic Acid	-	-	-	-	-
D-Serine	-	-	-	-	-
Troleandomycin	-	-	-	-	-
Rifamycin SV	-	-	-	-	-
Minocycline	-	-	-	-	-
Lincomycin	-	-	-	-	-
Guanidine HCl	-	-	-	-	-
Niaproof 4	-	-	-	-	-
Vancomycin	-	-	-	-	-
Tetrazolium Violet	-	-	-	-	-
Tetrazolium Blue	-	-	-	-	-
Nalidixic Acid	+	+	+	+	+
Lithium Chloride	+	+	+	+	+
Potassium Tellurite	+	+	+	+	+
Aztreonam	+	+	+	+	+
Sodium Butyrate	+	-	+	-	w
Sodium Bromate	w	+	+	+	+

[*] Symbols: +, positive; -, negative; w, weak positive.
[$] Data obtained from GEN III Database version 1.1.21 (BIOLOG, Hayward, CA, Cat. No. 22730D).

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