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## **Comparative Genomics and Phylogenetic Analyses Suggest Several Novel Species within *Clavibacter* sp. Including Non-Pathogenic Tomato-Associated Strains**

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1 Running Title: Comparative genomics of *Clavibacter* spp.

2

3 **Comparative Genomics and Phylogenetic Analyses Suggest Several Novel Species**  
4 **within *Clavibacter* sp. Including Non-Pathogenic Tomato-Associated Strains**

5

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19

20

21 **Abstract**

22 Members of *Clavibacter* spp. are economically important bacterial plant pathogens infecting a set  
23 of diverse agricultural crops (e.g. alfalfa, corn, potato, tomato, and wheat). Tomato-associated  
24 *Clavibacter* spp. strains occupy a great portion of genetic diversity of the genus, and *C.*  
25 *michiganensis sensu stricto* (formerly *C. michiganensis* subsp. *michiganensis*) causing bacterial  
26 canker disease considered one of the destructive seed-borne agents of the crop worldwide.  
27 However, current taxonomic descriptions of the genus do not reflect the existing diversity of the  
28 strains, resulting in unsatisfactory consequences in quarantine surveys for the pathogens. In this  
29 study, we used all the available genome sequences of *Clavibacter* spp. strains – including the type  
30 strains of newly described subspecies – to provide a precise insight into the diversity of tomato-  
31 associated members of the genus, and further clarify taxonomic status of the strains using  
32 genotypic and phenotypic features. Results of phylogenetic analyses revealed the existence of  
33 nine hypothetical new species among the investigated strains. None of the three new subspecies  
34 (i.e. *C. michiganensis* subsp. *californiensis*, *C. michiganensis* subsp. *chilensis* and *C. michiganensis*  
35 subsp. *phaseoli*) is included within the tomato-pathogenic *C. michiganensis sensu stricto* lineage.  
36 Although comparative genomics revealed the lack of *chp* and *tomA* pathogenicity determinant  
37 gene clusters in the non-pathogenic strains, a number of pathogenicity related genes were noted  
38 to be present in all the strains regardless of their pathogenicity characteristics. Altogether, our  
39 results advocate a need for a formal taxonomic reconsideration of tomato-associated *Clavibacter*  
40 spp. strains to facilitate differentiation of the lineages in quarantine inspections.

41

42 **Importance**

43 *Clavibacter* spp. are economically important bacterial plant pathogens infecting a set of diverse  
44 agricultural crops such as alfalfa, corn, pepper, potato, tomato, and wheat. A number of plant  
45 pathogenic members of the genus (*e.g. C. michiganensis sensu stricto* and *C. sepedonicus* infecting  
46 tomato and potato plants, respectively) are included in the A2 (high risk) list of quarantine  
47 pathogens by the European and Mediterranean Plant Protection Organization (EPPO). Although  
48 tomato-associated members of *Clavibacter* spp. occupy a significant portion of the genetic  
49 diversity in the genus, only the strains belonging to *C. michiganensis sensu stricto* (formerly *C.*  
50 *michiganensis* subsp. *michiganensis*) cause bacterial canker disease of tomato and subjected to  
51 the quarantine inspections. Hence, discrimination of the pathogenic and non-pathogenic  
52 *Clavibacter* spp. strains associated with tomato seeds and transplants plays a pivotal role in the  
53 accurate detection and cost efficiently management of the disease. On the other hand, detailed  
54 information on the genetic contents of different lineages of the genus would lead to the  
55 development of genome-informed specific detection techniques. In this study, we have provided  
56 an overview on the phylogenetic and genomic differences between the pathogenic and non-  
57 pathogenic tomato-associated *Clavibacter* spp. strains. We have also noted that the taxonomic  
58 status of newly introduced subspecies of *C. michiganensis* (*i.e. C. michiganensis* subsp.  
59 *californiensis*, *C. michiganensis* subsp. *chilensis* and *C. michiganensis* subsp. *phaseoli*) should be  
60 reconsidered.

61 **Keywords:** Actinobacteria, Bacterial canker of tomato, Bacterial Taxonomy, *Clavibacter*  
62 *michiganensis sensu stricto*, Quarantine pathogen

63

## 64 **Introduction**

65 A number of plant-pathogenic bacterial species are reported to have non-pathogenic lineages  
66 which usually exist in the same ecological niche with their pathogenic counterparts (1-3). Non-  
67 pathogenic lineages or strains typically have similar genetic contents to their pathogenic relatives,  
68 but lack some of the key pathogenicity determinants (*e.g.* pathogenicity islands, virulence genes,  
69 and plasmids) (4-6). As far as economically important quarantine plant pathogenic bacteria are  
70 concerned, presence of non-pathogenic strains in commercial seeds or propagative parts of plants  
71 will be interfering in the accurate detection of the pathogens, leading to false positive results in  
72 the quarantine inspections, and dissatisfaction of seed producers and traders (7). This is due in  
73 part to the fact that most of the non-pathogenic bacterial strains are phenotypically similar to  
74 their pathogenic relatives; therefore, not differentiable from each other on the culture media (7).  
75 Furthermore, most of the molecular detection protocols (*e.g.* PCR primers, probes, and  
76 antibodies) are designed based on the general features of a given species/subspecies/pathovar  
77 rather than focusing on the pathogenicity determinants of the pathogen (7). As a consequence,  
78 contradictions in the results of quarantine inspections will be leading to economic loses of seed  
79 producers and will have negative impact on transportation of plant materials in a global scale (7,  
80 8).

81 *Clavibacter* spp. are economically important Gram-positive bacterial plant pathogens infecting a  
82 set of diverse agricultural crops *e.g.* alfalfa, corn, pepper, potato, tomato, and wheat (9). Until  
83 recently, the genus *Clavibacter* was considered to include only one species *C. michiganensis*,  
84 comprising five plant pathogenic subspecies *i.e.* *C. michiganensis* subsp. *insidiosus*; *C.*  
85 *michiganensis* subsp. *michiganensis*; *C. michiganensis* subsp. *nebraskensis*; *C. michiganensis* subsp.  
86 *sepedonicus*; and *C. michiganensis* subsp. *tessellarius* (9, 10). Furthermore, all the tomato- and  
87 pepper-associated *Clavibacter* sp. strains were classified as members of *C. michiganensis* subsp.  
88 *michiganensis* regardless of being pathogenic or non-pathogenic on the host of isolation.  
89 However, using the multilocus sequence analysis and typing (MLSA/MLST) Jacques et al. (11)  
90 showed that tomato-associated non-pathogenic *Clavibacter* spp. strains are phylogenetically  
91 distinct from the pathogenic counterparts in the species. Differentiation of the pathogenic and  
92 non-pathogenic strains of *C. michiganensis* has always been an ongoing challenge for official  
93 sanitary agencies, quarantine inspectors and seed providers (12), since the false positive results  
94 would lead to the rejection of seed/seedling lots in an economically significant scale (7, 8). This led  
95 to the assumption that a comprehensive complete genome sequence-based reconsideration in the  
96 *C. michiganensis sensu lato* (all the former members of *C. michiganensis* according to Davis et al.  
97 (9)) is warranted to shed a light on the genetic diversity, genomic repertoires and taxonomic  
98 status of the pathogenic and non-pathogenic tomato-associated strains of the species (11).

99 Following the emergence of high throughput molecular-phylogenetic techniques many *Clavibacter*  
100 spp. strains, which have often previously been misidentified based on phenotypic features, were  
101 assigned into novel taxa. For instance, tomato-associated non-pathogenic members of *C.*  
102 *michiganensis sensu lato* were assigned into two new subspecies *C. michiganensis* subsp.

103 *californiensis* and *C. michiganensis* subsp. *chilensis* (13). Additionally, *C. michiganensis* subsp.  
104 *phaseoli* and *C. michiganensis* subsp. *capsici* were identified as the causal agents of bacterial bean  
105 leaf yellowing on common bean (*Phaseolus vulgaris*) and bacterial canker of pepper (*Capsicum*  
106 *annuum*), respectively (14, 15). Furthermore, non-pathogenic peach-colored strains isolated from  
107 tomato phyllosphere were reported to be distinct from the tomato-pathogenic members of  
108 *Clavibacter* spp. (16).

109 Recently, a re-classification of *Clavibacter* spp. into five new species and a new combination was  
110 proposed based on the genomic information *e.g.* average nucleotide identity (ANI) and digital  
111 DNA-DNA hybridization (dDDH) indices (17-19). The original subspecies of *C. michiganensis sensu*  
112 *lato* were elevated at the species level and designated as *C. michiganensis* (hereafter referred to  
113 as *C. michiganensis sensu stricto*: formerly *C. michiganensis* subsp. *michiganensis*), *C. tessellarius*,  
114 *C. insidiosus*, *C. nebraskensis*, *C. capsici*, as well as *C. sepedonicus* as a new combination (17, 19).  
115 However, due to the lack of genomic information from the newly proposed subspecies (*C.*  
116 *michiganensis* subsp. *californiensis*, *C. michiganensis* subsp. *chilensis*, and *C. michiganensis* subsp.  
117 *phaseoli*) as well as several taxonomically undetermined strains, additional investigations are  
118 warranted to further clarify the taxonomy of the genus. Moreover, strains associated with  
119 solanaceous vegetables contain a large fraction of diversity within the *Clavibacter* spp. members,  
120 and much of the molecular, phylogenetic, and genomic information for these strains remain  
121 unexplored. As for the tomato-associated strains of *Clavibacter* spp., comparative genomics on a  
122 wide collection of non-pathogenic and pathogenic strains would further elucidate the genetic  
123 diversity of these bacteria, resulting in the development of genome-informed specific molecular  
124 markers (*e.g.* specific conventional PCR and real-time PCR primers, as well as loop-mediated

125 isothermal amplification) for the detection and differentiation of the pathogenic and non-  
126 pathogenic strains in the quarantine posts.

127 The objectives of the present study were to I) investigate genetic diversity of tomato-associated  
128 *Clavibacter* spp. strains using the genome sequences of all available non-pathogenic and  
129 pathogenic strains and II) provide a novel taxonomic overview into the status of tomato-  
130 pathogenic and non-pathogenic strains within the genus. For this aim, we used the genome  
131 sequences of 40 *Clavibacter* spp. strains including the type strains of three newly described  
132 subspecies (*C. michiganensis* subsp. *californiensis*, *C. michiganensis* subsp. *chilensis* and *C.*  
133 *michiganensis* subsp. *phaseoli*), as well as additional atypical non-pathogenic strains isolated from  
134 tomato plants around the globe (20). Draft genome sequence-based phylogenetic analyses  
135 revealed a higher diversity among the non-pathogenic strains of *Clavibacter* spp. than that has  
136 previously been reported, delineating them into several new species. On the other hand, our data  
137 revealed that the two individual subspecies *C. michiganensis* subsp. *chilensis* and *C. michiganensis*  
138 subsp. *phaseoli* need to be considered as the members of one species according to the 99%  
139 genome similarity among the type strains. Furthermore, comparative genomics among the  
140 pathogenic and non-pathogenic strains of tomato-associated strains, as well as the type strains of  
141 the remaining species/subspecies within the genus revealed several pathogenicity determinant  
142 genes presenting only in *C. michiganensis sensu stricto*, which could be considered as suitable  
143 genomic targets for the development of specific detection methods for the tomato pathogen.

144

145 **Results**



146 **Pathogenicity and Host Range**

147 Tomato and pepper plants inoculated with the standard strain of *C. michiganensis sensu stricto*  
148 (ICMP 22049) showed the expected disease symptoms 10-12 days post inoculation. Although  
149 tomato plants inoculated with the strain ICMP 22049 showed wilting and plant death (Figure S1a),  
150 pepper plants inoculated using the same strain showed only stem canker symptoms on the site of  
151 inoculation with no wilting nor plant death in the same timeframe (Figure S1b). However, neither  
152 *C. michiganensis* subsp. *phaseoli* nor *C. michiganensis* subsp. *chilensis* did induce symptoms on the  
153 inoculated plant species *i.e.* common bean, cowpea, pepper, mung bean, and tomato (Table 1).  
154 Furthermore, we could not re-isolate *C. michiganensis* subsp. *phaseoli* and *C. michiganensis* subsp.  
155 *chilensis* from the stem, petiole, and leaf tissues 5-10 cm above the inoculation site on the stem.  
156 This could be an indication of the fact that *C. michiganensis* subsp. *phaseoli* and *C. michiganensis*  
157 subsp. *chilensis* were unable to endophytically colonize the evaluated plant species. As for the  
158 orange-pigmented tomato-associated strains CFBP 8615 and CFBP 8616, although no symptoms  
159 were observed on common bean, cowpea, pepper, mung bean, and tomato plants, bacterial  
160 colonies similar to those originally inoculated were consistently re-isolated from the leaf tissues of  
161 common bean cv. Navy plants inoculated with CFBP 8616 (Table 1). Furthermore the standard  
162 strain ICMP 22049 was consistently re-isolated from the symptomatic pepper and tomato plants  
163 on YPGA medium and their identity was confirmed using the genus-specific primer pair  
164 CMR16F1/CMR16R1 (data not shown). Similar results were obtained in both the replications of  
165 the experiments, while the negative control plants remained healthy.

166

167 **Phylogenetic Analyses**

168 Neighbor joining phylogenetic tree constructed using the genome sequences of 40 *Clavibacter*  
169 spp. strains (Table 2) via ANI Calculator online service with all-vs.-all strategy revealed high genetic  
170 diversity among tomato-associated non-pathogenic strains of the genus (Figure 1). ANI values  
171 between different pairs of strains varied from 87% to 100% among the *Clavibacter* spp. strains  
172 (Table 3). While all tomato-pathogenic strains of *C. michiganensis sensu stricto* clustered in a  
173 monophyletic clade showing 99-100% ANI with one another, non-pathogenic strains isolated from  
174 tomato were scattered in several clades, most of which had <96% ANI values with the other clades  
175 (Table 3). The closest non-pathogenic clade to *C. michiganensis sensu stricto* group consisted of  
176 three strains *i.e.* type strain of *C. michiganensis* subsp. *californiensis* (CFBP 8216<sup>T</sup>), and the non-  
177 pathogenic strains LMG 26808 and CFBP 7493. ANI value between the type strain of *C.*  
178 *michiganensis sensu stricto* (LMG 7333<sup>T</sup>) and the type strain of *C. michiganensis* subsp.  
179 *californiensis* was 95% in all the calculating strategies, retaining them at the threshold of species  
180 definition (21). Nevertheless, the dDDH value (57.70%) between the type strains of *C.*  
181 *michiganensis sensu stricto* and *C. michiganensis* subsp. *californiensis* was far below the threshold  
182 for species delineation (70%) with this method (Table 3). Altogether, given the differences in their  
183 pathogenicity and biochemical characteristics (13) as well as bellow-threshold genomic similarity,  
184 the two taxa *C. michiganensis sensu stricto* and *C. michiganensis* subsp. *californiensis* could be  
185 considered as separate species. Furthermore, ANI between the type strains of *C. michiganensis*  
186 *sensu stricto*, *C. michiganensis* subsp. *californiensis*, and the cluster which included the non-  
187 pathogenic strains LMG 26808 and CFBP 7493 was 94-95% (Table 3). The dDDH values between

188 these type strains and LMG 26808 and CFBP 7493 were also 57-58% indicating them as separate  
189 species (Figure 1).

190 *Clavibacter sepedonicus* strains formed a monophyletic cluster separate from all the other lineages  
191 by ANI values <93%, which is in coherence with its elevation at the species level (17, 18).

192 *Clavibacter insidiosus* and *C. nebraskensis* strains clustered in a monophyletic group showing 95%  
193 ANI between the type strains of the species (Table 3). Here also, the dDDH value (59.90%)

194 between the type strains of these two taxa was far below the threshold for species definition  
195 (70%) with this method (Table 3) supporting their elevation into separate species (17-19). The two

196 taxa are also different in their host of isolation and pathogenicity pattern. While the strain CFBP  
197 7494 showed only 64.60% dDDH value with the type strain of *C. insidiosus*, 96% ANI (on the upper

198 edge of species definition) prevents differentiation of this strain from the *C. insidiosus* species. The  
199 strain CFBP 7494 was isolated from tomato but was non-pathogenic on this plant species, while it

200 has been shown that induces disease symptoms on wheat plants in greenhouse conditions (22).  
201 Further evidences – including a comprehensive field survey and host range assay – are needed to

202 elucidate the prevalence and exact taxonomic status of the strain CFBP 7494. Surprisingly, type  
203 strains of *C. michiganensis* subsp. *phaseoli* and *C. michiganensis* subsp. *chilensis* shared 99% ANI

204 with one another and 98% ANI with CFBP 7491 isolated from tomato seeds. These three strains  
205 had ANIs below 93% with all the remaining clades, suggesting a novel species within the genus.

206 High dDDH value (87.50%) also confirmed the close relationships between the type strains of *C.*  
207 *michiganensis* subsp. *phaseoli* and *C. michiganensis* subsp. *chilensis* (Table 3).

208 Two peach-colored strains CFBP 8615 and CFBP 8616 shared 100% ANI with one another, while  
209 they differed from all the remaining clades with the ANI values <93%. Furthermore, non-

210 pathogenic strain CFBP 8019 was determined as the phylogenetically closest strain to the peach-  
211 colored strains with 93% ANI value between the two clades. These ANI values are far below the  
212 accepted threshold (95-96%) for the definition of prokaryotic species (21), suggesting that the  
213 strains CFBP 8615 and CFBP 8616 could be defined as forming a new species separated from CFBP  
214 8019, while the strain CFBP 8019 itself belong to a new stand-alone specie (Figure 1). Low ANI  
215 values were also confirmed by dDDH which were <48% between the peach-colored strains and all  
216 the remaining clades (Table 3). The strain CF11 – isolated from soil in a tomato growing  
217 greenhouse (23) – as well as the type strain of the pepper pathogen *C. capsici* (PF008<sup>T</sup>) clustered in  
218 a monophyletic clade, while differed from one another with 95% ANI and 58.50% dDDH values.  
219 Hence, CF11 could be proposed as forming a new species within the genus, while the elevation of  
220 *ex-C. michiganensis* subsp. *capsici* into the species level (*C. capsici*) was confirmed as proposed by  
221 Li et al. (17). Type strain of *C. tessellarius* showed <93% ANI with the type strains of all the other  
222 subspecies/species confirming the wheat pathogen as a stand-alone species. However, none of  
223 the two strains CFBP 8017 and DOAB 609, which were clustered in a shared clade with the type  
224 strain of *C. tessellarius* could be included within this species. The ANIs of CFBP 8017 and DOAB 609  
225 with the type strain of *C. tessellarius* were 95% and 93%, respectively, while the dDDH values  
226 between the same strains were 57.70% and 49.00%, respectively (Table 3). Thus, each of the CFBP  
227 8017 and DOAB 609 strains could be defined as representing novel species. The non-pathogenic  
228 strain CASJ009 also had ANI values <90% with all the *Clavibacter* spp. strains evaluated in this  
229 study, indicating that this strain also represents a novel species within the genus (Figure 1; Table  
230 3).

231

232 **Comparative Genomics**

233 Comparative genomics data obtained using the RAST online service revealed that the genome size  
 234 among the *Clavibacter* spp. strains varied between 3,024 kbp in CFBP 8019 and 3,420 kbp in LMG  
 235 26808 with the G+C% content of 72.0% in LMG 26808 to 73.7% in *C. tessellarius* ATCC 33566<sup>T</sup>.  
 236 Furthermore, the number of coding sequences (CDS) varied from 2,629 in *C. michiganensis* subsp.  
 237 *chilensis* (CFBP 8217<sup>T</sup>) to 3,181 in DOAB 609. Genomic characteristics of *Clavibacter* spp. in a panel  
 238 of 20 representative strains, which were selected on the basis of the phylogenetic analyses (as  
 239 detailed above) to cover all lineages/clades of the genus are shown in Table 4. The number of  
 240 subsystems varied from 260 in CF11 to 345 in the reference strain of *C. michiganensis sensu stricto*  
 241 NCPB 382 and type strain of *C. sepedonicus* ATCC 33113<sup>T</sup>. Although the feature counts were  
 242 similar in most of the subsystems among the pathogenic and non-pathogenic *Clavibacter* spp.  
 243 strains, differences in the siderophore producing subsystems were observed, and siderophore  
 244 assembly kit was detected only in the non-pathogenic strain CFBP 8616 (Table 4).

245 One-vs.-one BLASTn- and BLASTp-based explorations using the complete genome sequence of *C.*  
 246 *michiganensis sensu stricto* NCPPB 382 as the reference genome vs. the individual *Clavibacter* spp.  
 247 strains revealed the lack of pathogenicity determinant genes/clusters in all the tomato-associated  
 248 non-pathogenic strains evaluated in this study (Table 5). For the *chp* gene cluster (*i.e.* loci  
 249 CMM\_0034 to CMM\_0077 in NCPPB 382 genome sequence: AM711867.1) only a fraction of the  
 250 genes were detected in the non-pathogenic strains (Figure 2). For instance, a sugar phosphate  
 251 isomerase (CMM\_0034) was present in all the non-pathogenic strains. A putative  
 252 phosphotransferase (CMM\_0065) and ATPase (*parX*=CMM\_0066) were found in CFBP 7491, CFBP  
 253 7493, and LMG 26808. A hypothetical protein produced by CMM\_0054 locus and a transcriptional

254 regulator protein secreted by CMM\_0055 were found only in the type strain of *C. michiganensis*  
 255 subsp. *californiensis*. A serine protease (*ppaD*=CMM\_0075) and a putative ATPase (CMM\_0067)  
 256 were found only in CASJ009 and CFBP 7491, respectively, while a putative DNA invertase  
 257 (CMM\_PS\_07) was found in both the last strains. Among the pathogenicity determinant genes  
 258 inside the *chp* gene cluster, *ppaA* (CMM\_0041), *peIA1* (CMM\_0043), *peIA2* (CMM\_0051), *chpC*  
 259 (CMM\_0052), and *chpG* (CMM\_0059) were found in none of the evaluated non-pathogenic  
 260 strains. Non-*chp* pathogenicity determinant genes *clvG* (CMM\_1963), *clvF* (CMM\_1964), *clvA*  
 261 (*mica*=CMM\_1967), and *perF* (CMM\_2382) were also not detected in the evaluated non-  
 262 pathogenic bacterial strains. Interestingly, a subtilisin-like serine proteases (*sbtA*=CMM\_0070) was  
 263 found in a number of non-pathogenic strains (Table 5), while the nucleotides 1-600 were missing  
 264 in all the non-pathogenic members. The expansin encoding gene *expA* (CMM\_1480) was found in  
 265 CFBP 8017, DOAB 609, CFBP 7493 and LMG 26808.

266 As for the *tomA* gene cluster (CMM\_0078 to CMM\_0112 in the genome sequence of NCPPB 382) a  
 267  $\beta$ -glucosidase related gene (*bglC*=CMM\_0083) was found in the type strains of *C. michiganensis*  
 268 subsp. *chilensis*, *C. capsici*, *C. michiganensis* subsp. *phaseoli*, as well as the strains CFBP 8615,  
 269 CFBP 8616, CFBP 7493, LMG 26808, and CFBP 7494. A putative Alpha-glucosidases gene  
 270 (*aglA*=CMM\_0106) was found in CFBP 7491, CFBP 8019, and CASJ009. Furthermore, a putative  
 271 ABC-type sugar transport permease (CMM\_0108) was found in CFBP 7491 and CASJ009, while the  
 272 *srtA* gene (CMM\_0013) which encodes a putative sortase enzyme was found in all the evaluated  
 273 non-pathogenic strains in this study (Table 5).

274 We have also assessed the presence of a set of eight virulence genes *i.e.* *celB* (CMM\_2443), *peIA1*  
 275 (CMM\_0043), *peIA2* (CMM\_0051), *xysA* (CMM\_1673), *xysB* (CMM\_1674), CMM\_2691,

276 CMM\_2692, and CMM\_2871, which are responsible for cell wall degradation at the later stages of  
277 tomato infection by *C. michiganensis sensu stricto*. The polygalacturonase encoding locus  
278 CMM\_2871 was found in tomato-associated strains CFBP 7493 and LMG 26808, as well as the type  
279 strains of *C. insidiosus* (LMG 3663<sup>T</sup>), *C. nebraskensis* (NCPBP 2581<sup>T</sup>) and *C. tessellarius* (ATCC  
280 33566<sup>T</sup>). Furthermore, *celB* (CMM\_2443) which is a homologue of plasmid-born *celA* gene (24)  
281 was present in tomato-associated non-pathogenic strains CFBP 8017, DOAB 609, CFBP 7493, LMG  
282 26808, and CFBP 7494. The four loci CMM\_1673, CMM\_1674, CMM\_2691, and CMM\_2692 were  
283 present in all tomato-associated strains except for CMM\_2692 which was absent in CASJ009, while  
284 the query coverage varied between 30-100%, and sequence similarity varied from 75-100% among  
285 the strains. Surprisingly, the virulence-associated transcriptional regulator genes *vatr1*  
286 (CMM\_2645) and *vatr2* (CMM\_2969), which regulate *C. michiganensis sensu stricto* virulence  
287 during the infection (25) were present in all the strains evaluated in this study regardless of their  
288 pathogenicity status (Table 5).

289 Orthologous gene clusters were determined using OrthoVenn online service through four-vs.-four  
290 and five-vs.-five designations of the representative strains from different phylogenetic lineages  
291 (Figure 3A-D). Type strains of the five ex-*C. michiganensis sensu lato* subspecies shared 2,157  
292 proteins in their genome sequences (Figure 3A). Although none of the type strains of *C.*  
293 *michiganensis sensu stricto* and *C. nebraskensis* showed unique proteins in their sequences, type  
294 strains of *C. sepedonicus*, *C. tessellarius* and *C. insidiosus* showed four, 12, and 16 unique proteins  
295 among their genome sequences, respectively. Furthermore, when the two phylogenetic  
296 neighboring clades of *C. michiganensis sensu stricto* (i.e. *C. michiganensis* subsp. *californiensis* and  
297 CFBP 7493) were compared with the type/reference strains of *C. michiganensis sensu stricto* (LMG

298 7333<sup>T</sup> and NCPPB 382), seven and 11 unique proteins were detected in the genome sequences of  
299 CFBP 7493 and *C. michiganensis* subsp. *californiensis* strain CFBP 8216<sup>T</sup>, respectively (Figure 3B).  
300 Unique and shared proteins in the type strains *C. michiganensis* subsp. *californiensis*, *C.*  
301 *michiganensis* subsp. *phaseoli* and *C. michiganensis* subsp. *chilensis* as well as the atypical peach-  
302 colored strain CFBP 8615 are depicted in the Figure 3C and D.

303

#### 304 **Plasmids, Phages, and Bacteriocins**

305 No integrative plasmid (episome) was detected using the PlasmidFinder online service in the draft  
306 genome sequences of bacterial strains investigated in this study, except for LMG 26808 in which  
307 two *Enterobacteriaceae* plasmids IncL/M(pOXA-48) and IncR were identified. Surprisingly  
308 homologues sequences to the plasmid-born *celA* gene were detected in the sequences of the type  
309 strains of *C. insidiosus* (LMG 3663<sup>T</sup>) and *C. nebraskensis* (NCPPB 2581<sup>T</sup>) with the query coverage of  
310 respectively 97% and 76%, and sequence similarity of 90% and 84% to the sequence of the  
311 reference strain NCPPB 382 (Table 5). On the other hand, plasmid profiling detected the two  
312 expected plasmids pCM1 (≈27 kb) and pCM2 (≈70 kb) in tomato-pathogenic strain of *C.*  
313 *michiganensis sensu stricto* ICMP 22049 (data not shown). However, type strains of *C.*  
314 *michiganensis* subsp. *phaseoli* and *C. michiganensis* subsp. *chilensis*, as well as the two peach-  
315 colored strains CFBP 8615 and CFBP 8616 did not carry any detectable plasmid (data not shown).

316 The PHASTER online service was used to detect prophage sequences within the bacterial genomes.  
317 Altogether five hypothetical prophage groups *i.e.* Gordon\_Schwabeltier (NC\_031255),  
318 Gordon\_Smoothie (NC\_030696), N15 (NC\_001901), P1 (NC\_005856), and Phi92 (NC\_023693) were



319 detected in the *Clavibacter* spp. strains investigated in this study (Table 6). Prophages were  
320 detected in tomato-associated strains CFBP 8615, CFBP 7491, LMG 26808, and CASJ009 as shown  
321 in Table 6. While the strain LMG 26808 contained three prophages, only one prophage per strain  
322 was detected in the strains CFBP 8615, CFBP 7491, and CASJ009. The phage Phi92, which has  
323 originally been isolated from a pathogenic *Escherichia coli* strain was detected in CFBP 8615, LMG  
324 26808, CASJ009, *C. sepedonicus* (ATCC 33113<sup>T</sup>) and *C. tessellarius* (ATCC 33566<sup>T</sup>) while each of the  
325 remaining four prophages was detected in only one strain (Table 6).

326 *In silico* screening for bacteriocins and antibiotic peptides revealed distinct differences between  
327 the pathogenic and non-pathogenic tomato-associated *Clavibacter* spp. strains. The lantibiotic  
328 “Michiganin A” was detected in all the 12 *C. michiganensis sensu stricto* strains (data not shown)  
329 but was not detected in the non-pathogenic tomato-associated strains nor in the pathogenic  
330 strains on other plant species. Furthermore, sactipeptides (peptides with cysteine sulfur to  $\alpha$ -  
331 carbon crosslinks) were the most common group of bacteriocins among all the *Clavibacter* spp.  
332 strains. Indeed, except for tomato-pathogenic *C. michiganensis sensu stricto* strains, all the strains  
333 which contained bacteriocins has had at least one type of sactipeptides (Table 7). Linear azol(in)e-  
334 containing peptides (LAPs) were detected in both the pathogenic and non-pathogenic strains,  
335 while thiopeptides which are commonly produced by Actinobacteria found in the two  
336 phylogenetically closely related non-pathogenic strains CFBP 7493 and LMG 26808.  
337 Enterocin\_AS\_48, a circular bacteriocin produced by *Enterococcus* sp. was exclusively detected in  
338 the strain CF11 which was originally isolated from soil in a tomato-growing greenhouse (23).

339

340 **Discussion**

341 In this study, using phylogenetic analyses, comparative genomics, and pathogenicity assays, we  
342 provide a novel insight into the diversity of *Clavibacter* spp. strains with a special focus on tomato-  
343 associated members of the genus. Phylogenetic analyses accomplished with ANI and dDDH  
344 calculations revealed a higher genetic diversity of *Clavibacter* spp. strains than that has so far been  
345 assumed (17). We also aim to decipher phylogenetic position of the three newly described  
346 subspecies of *C. michiganensis sensu lato* (i.e. *C. michiganensis* subsp. *californiensis*, *C.*  
347 *michiganensis* subsp. *chilensis* and *C. michiganensis* subsp. *phaseoli*). Although our results confirm  
348 that the three mentioned subspecies are no longer included in the *C. michiganensis sensu stricto*,  
349 we still used their original name in this study to avoid confusions. A formal taxonomic study would  
350 provide appropriate epithet for these taxa. On the other hand, BLAST-based comparative  
351 genomics revealed that several genes (i.e. *vatr1*, *vatr2*, *xysA*, *xysB*, and *srtA*) which had previously  
352 been identified as pathogenicity determinants, were detected in all the pathogenic and non-  
353 pathogenic tomato-associated strains, indicating further complexities in the functions of these  
354 genes (25, 26). Non-pathogenic counterparts of actinobacterial plant pathogens have frequently  
355 been isolated from a set of taxonomically diverse plant species, which were distant from the main  
356 host of the pathogen. For instance, both the pathogenic and non-pathogenic *Curtobacterium*  
357 *flaccumfaciens* strains phylogenetically closely related to the common bean pathogen *C.*  
358 *flaccumfaciens* pv. *flaccumfaciens* were isolated from solanaceous annual crops i.e. eggplant,  
359 pepper, and tomato (2). While the pathogenic and non-pathogenic strains of *C. flaccumfaciens* are  
360 not differentiable using the routing molecular techniques e.g. MLSA (27), all the non-pathogenic

361 members of *C. michiganensis sensu lato* could be differentiated from the tomato-pathogenic *C.*  
362 *michiganensis sensu stricto* strains (11).

363 Non-pathogenic strains of *Clavibacter* spp. were consistently reported to associate with seeds,  
364 transplants, and aerial portions of tomato plants (11, 13, 22, 28). However, until recently, only a  
365 few number of genome sequences from non-pathogenic *Clavibacter* spp. strains were available  
366 (22, 28) limiting our understanding of the putative role of these bacteria on the host plants and  
367 their environment. In a preliminary complete genome sequence-based comparative study, Zaluga  
368 et al. (28) investigated the genome of tomato-associated non-pathogenic strain LMG 26808 and  
369 provided initial insights into the genetic bases of differences between the pathogenic and non-  
370 pathogenic members of *C. michiganensis sensu lato*. However, it has been noted that LMG 26808  
371 is phylogenetically very close to the *C. michiganensis sensu stricto* clade leaving a greater portion  
372 of non-pathogenic *Clavibacter* spp. diversity uninvestigated (28). Our results revealed that the  
373 strain LMG 26808 as well as two other strains *i.e.* CFBP 7493 and CFBP 8216<sup>T</sup> are phylogenetically  
374 closely related, and fall into a monophyletic clade along with the pathogenic members of *C.*  
375 *michiganensis sensu stricto* (Figure 1). Genomic contents of the two clades represented by LMG  
376 26808/CFBP 7493 as “hypothetical new species I” and CFBP 8216<sup>T</sup> as “hypothetical new species II”  
377 varied in the pathogenicity-related genes *sbtA*, *expA*, *celB*, and the locus CMM\_2871, while there  
378 was no difference between the strains LMG 26808 and CFBP 7493 in the evaluated genomic areas  
379 (Table 5). More specifically, the *expA* gene (CMM\_1480) which is responsible for expansin  
380 production (29), as well as a polygalacturonase encoded by CMM\_2871 locus at the final stages of  
381 infection were found in CFBP 7493 and LMG 26808 strains but not in the type strain of *C.*  
382 *michiganensis* subsp. *californiensis* (Table 5).

383 Recently, Li and his colleagues (17) re-evaluated the taxonomy of *C. michiganensis sensu lato* and  
384 proposed the re-classification of each of the ex-*C. michiganensis* subspecies into the species  
385 status. Since only two genome sequences of *C. insidiosus* were available at that time (both isolated  
386 in the USA; 29), we sequenced two further “Old World” strains (*i.e.* CFBP 1195 and CFBP 6488  
387 isolated in the UK and Czech Republic, respectively) to gain a precise vision from the intra-species  
388 diversity of the alfalfa pathogen (20). Our analyses confirm the existence of *C. capsici*, *C.*  
389 *insidiosus*, *C. michiganensis sensu stricto*, *C. nebraskensis*, *C. sepedonicus* and *C. tessellarius* as  
390 stand-alone species. While the 95% ANI did not solely support the separation of the alfalfa and  
391 maize pathogens, 59.90% dDDH between the type strains of the two taxa, as well as their distinct  
392 host plants could be considered as the evidences for the separation of *C. insidiosus* and *C.*  
393 *nebraskensis* (Tables 2, 3). The strain CFBP 7494 which was isolated from tomato seeds and causes  
394 disease symptoms on wheat plants in greenhouse conditions (22) clustered in a monophyletic  
395 clade with the alfalfa pathogenic strains and still fall into *C. insidiosus* species with 96% ANI and  
396 64.60% dDDH. While the phylogenetic position of the strain CFBP 7494 was clarified in these  
397 analyses, only further investigations using a larger collection of strains will shed a light on the  
398 genetic content, biological characteristics and taxonomic status of tomato-associated wheat-  
399 pathogenic members of *Clavibacter* spp.

400 Draft genome sequences of the three new subspecies of *C. michiganensis sensu lato* revealed their  
401 phylogenetic position, highlighting inaccuracy in the nomenclature of *C. michiganensis* subsp.  
402 *chilensis* and *C. michiganensis* subsp. *phaseoli*. Type strains of these two subspecies shared 99%  
403 ANI and 87.50% dDDH with one another, indicating a synonymy and orientating to the proposal of  
404 a novel unique new species (Figure 1, Table 3). Type strain of *C. michiganensis* subsp. *phaseoli*

405 (CFBP 8627<sup>T</sup>) was isolated from common bean seeds in Spain and reported to cause bacterial  
406 bean leaf yellowing disease in greenhouse assays (14). However, we could not observe any  
407 symptoms on the inoculated common bean plants even when three different cultivars were  
408 evaluated in the pathogenicity tests (Table 1). This could be attributed to differences in the  
409 environmental conditions between the two assays, and probably also differences in the  
410 susceptibility of common bean cultivars used in the two studies. Further field surveys are needed  
411 to decipher potential natural occurrence in the field conditions, and putative frequency and  
412 prevalence of the common bean-associated *Clavibacter* spp. strains.

413 Comparative genomics revealed that tomato-associated *C. michiganensis sensu lato* strains are  
414 adapted to a non-pathogenic lifestyle, which is reflected by the lack of pathogenicity gene clusters  
415 present in the pathogenic members (Table 5; Figure 2). Although the absence of almost all 129-kb  
416 *chp/tomA* region was common among all the non-pathogenic strains, some of the putative  
417 virulence factors were present in the non-pathogenic strains, suggesting contribution of these  
418 genes in the endophytic lifestyle of the bacteria. An in-depth comparative analysis with newly  
419 sequenced *Clavibacter* spp. genomes allowed us to illustrate a more precise insight underlying  
420 genetic contents of these bacteria. For instance the *expA* gene was detected in the non-  
421 pathogenic strains CFBP 7493 and LMG 26808 as well as the wheat-pathogenic strains CFBP 8017  
422 and DOAB 609, but not in the type strain of *C. tessellarius* (Table 5). Microbial expansins are found  
423 in the genomes of several plant pathogenic bacteria, and it is assumed that they provide particular  
424 advantages to xylem-dwelling phytopathogens (29, 30). Expansin enhances cellulose breakdown  
425 by cellulase enzymes in the later stages of pathogen invasion (31). These observations correlate  
426 with the initial assumptions that non-pathogenic *Clavibacter* spp. strains must have lost or never

427 contained prominent virulence determinants (e.g. 129-kb *chp/tomA* region) responsible for  
428 disease induction in tomato plants. With the availability of genome sequences covering a broader  
429 diversity of non-pathogenic *Clavibacter* spp. strains one would assume the gene flow and  
430 evolutionary pathways of pathogenicity determinants in the genus similar to that has previously  
431 been estimated for plant pathogenic xanthomonads (4).

432 In conclusion, our results obtained from the analyses of 40 genome sequences provide a  
433 comprehensive insight into the genetic diversity of *Clavibacter* spp., and confirm the recent  
434 taxonomic revision of the genus. However, phylogenetic analyses suggest that the recently  
435 described subspecies *C. michiganensis* subsp. *chilensis* and *C. michiganensis* subsp. *phaseoli* should  
436 be classified as members of the same novel species (13, 14). Taking together all the phylogenetic,  
437 genomics and pathogenicity data, nine hypothetical novel species could be identified within  
438 *Clavibacter* spp., seven of which (i.e. hypothetical new species I, II, III, IV, V, VIII, and IX as shown in  
439 Figure 1) were isolated from asymptomatic tomato tissues or seed lots. These findings raise a  
440 question whether current taxonomy of tomato-associated *Clavibacter* spp. strains is technically  
441 applicable to the quarantine purposes, and emphasize at the same time the need for more  
442 detailed taxonomic investigations among the phylogenetically diverse tomato-associated  
443 *Clavibacter* spp. strains. Indeed, the only pathogenic lineage of tomato-associated strains is *C.*  
444 *michiganensis sensu stricto* while the seven non-pathogenic lineages need to designate into novel  
445 formal taxa. This would help the plant pathology agencies and tomato seed industry inspectors to  
446 specifically target the enemy and neglect the non-pathogenic lineages. Only a formal taxonomic  
447 study would address this issue with delineation of appropriate epithet and species description for  
448 these new taxa. On the other hand, the nine pathogenicity determinant genes (Table 5) would be

449 appropriate targets for the development of novel genome-informed detection methods for  
450 differentiation of tomato-pathogenic and non-pathogenic strains.

451

## 452 **Materials and Methods**

### 453 **Bacterial Strains and Genome Sequences**

454 Draft genome sequences of 10 *Clavibacter* spp. strains (Table 2) were prepared using the shotgun  
455 genome sequencing facility of Illumina HiSeq X platform. Culture media, bacterial growth  
456 conditions, genomic DNA preparation, sequencing procedure and genome annotation were  
457 described previously (20). In this framework, we investigated the type strains of *C. michiganensis*  
458 subsp. *californiensis* (CFBP 8216<sup>T</sup>), *C. michiganensis* subsp. *chilensis* (CFBP 8217<sup>T</sup>) and *C.*  
459 *michiganensis* subsp. *phaseoli* (CFBP 8627<sup>T</sup>), two *C. insidiosus* (CFBP 1195 and CFBP 6488), one *C.*  
460 *nebraskensis* (CFBP 7577), as well as the non-pathogenic peach colored (*i.e.* CFBP 8615 and CFBP  
461 8616) and yellow-pigmented (*i.e.* CFBP 7491 and CFBP 7493) strains. Furthermore, all the publicly  
462 available genome sequences of *Clavibacter* spp. – until April 2019 – were retrieved from the NCBI  
463 GenBank database and included in the phylogenetic analysis and comparative genomics. Table 2  
464 describes the 40 *Clavibacter* spp. strains used in this study, their origin of isolation and  
465 pathogenicity features.

466

### 467 **Pathogenicity Tests and Host Range**

468 Due to the close phylogenetic relationships between the type strains of *C. michiganensis* subsp.  
469 *phaseoli* (CFBP 8627<sup>T</sup>) and *C. michiganensis* subsp. *chilensis* (CFBP 8217<sup>T</sup>), these two strains as well  
470 as the atypical peach-colored strains recently isolated from tomato (16) were subjected to the  
471 pathogenicity tests and host range assays in greenhouse conditions. Pathogenicity tests were  
472 performed on bell pepper (cv. Sereno), chili pepper (cv. Aziz), common bean (cvs. Red kidney,  
473 Pinto, and Navy), cowpea (*Vigna unguiculata* cv. Partow), mung bean (*Vigna radiata* cv. Mashhad),  
474 and tomato (cv. Sunseed 6189) plants. Plant growth conditions, inoculation procedure, and  
475 incubation environment were the same as detailed previously (32, 33). Inoculated plants were  
476 periodically monitored for the appearance of disease symptoms up to 30 days post inoculation.  
477 Positive and negative control plants were treated in the same manner using the standard strain of  
478 *C. michiganensis sensu stricto* (ICMP 22049; isolated from symptomatic tomato plant in Iran in  
479 2015; 16) and sterile distilled water, respectively. Koch's postulates were accomplished by re-  
480 isolating the inoculated strains on yeast-extract peptone glucose agar (YPGA) medium from all  
481 inoculated plants. Confirmation of the identity of the re-isolated bacteria was made by  
482 determining Gram reaction and colony characteristics on yeast extract-dextrose-calcium  
483 carbonate (YDC) agar medium as well as by using the genus-specific primer pair  
484 CMR16F1/CMR16R1 (34) as described previously (16). The pathogenicity tests were conducted  
485 twice.

486

#### 487 **Phylogenetic Analyses**



488 Average Nucleotide Identity (ANI) was calculated among all the *Clavibacter* spp. genome  
489 sequences included in this study. The ANI was estimated using both one-vs.-one and all-vs.-all  
490 strategies via different algorithms *i.e.* JSpeciesWS (<http://jspecies.ribohost.com/jspeciesws/>; 35),  
491 ANI calculator (<http://enve-omics.ce.gatech.edu/g-matrix/>; 36), and OrthoANIu  
492 (<https://www.ezbiocloud.net/tools/orthoaniu>; 37). An ANI-based Neighbor Joining phylogenetic  
493 tree was constructed using the ANI calculator online service, and genome sequence of *Leifsonia*  
494 *xyli* subsp. *cynodontis* (DSM 46306; NC\_022438.1) was used as an out-group in the tree.  
495 Additionally, Genome-to-Genome Distance Calculator online service  
496 (<http://ggdc.dsmz.de/distcalc2.php>) was used to calculate digital DNA-DNA hybridization (dDDH)  
497 value which infers to the genome-to-genome distances between pairs of genomes based on the  
498 Genome Blast Distance Phylogeny (38). A combination of ANI and dDDH indices was used to  
499 designate a taxonomic status to a given phylogenetic clade, where the “new species” status was  
500 assigned to a clade only when both ANI and dDDH values were below the accepted threshold  
501 ( $\leq 95\%$  and  $\leq 70\%$  for ANI and dDDH, respectively, 21)

502

### 503 **Comparative Genomics**

504 Twenty strains representing the entire genetic diversity of *Clavibacter* spp. based on the  
505 ANI/dDDH data, host of isolation and pathogenicity characteristics were subjected to the  
506 comparative genomics analyses. Type strains of all the *C. michiganensis sensu lato*  
507 species/subspecies, as well as all the individual strains sharing  $\leq 95\%$  and  $\leq 70\%$  ANI and dDDH  
508 values, respectively, with the other taxa were selected for comparative genomics analyses.

509 Genome length (bp), G + C content (%), total number of protein-coding sequences (CDS), RNA  
510 genes, and pseudogenes were determined for all the genomes.

511 The online annotating service RAST (Rapid Annotations using Subsystems Technology;  
512 <http://rast.nmpdr.org/>; 39) was used for fully automated annotation of the bacterial genomes and  
513 the obtained information was used to reconstruct metabolic networks and subsystems. A  
514 subsystem is a set of functional roles that the annotator considers as related categories.  
515 Subsystems represent a collection of functionally related protein families that make up a  
516 metabolic pathway (*e.g.* Iron acquisition and metabolism), a complex (*e.g.* the ribosome), or a  
517 class of proteins (*e.g.* bacteriocins) (40). Subsequently, the genomes were transferred to the  
518 comparative environment of the SEED-Viewer ([http://www.theseed.org/wiki/Main\\_Page](http://www.theseed.org/wiki/Main_Page); 41) for  
519 comparative genomics analyses. The SEED-Viewer was used for the identification of protein-  
520 encoding sequences (CDS), assigning functions to the genes, and prediction of represented gene  
521 clusters in the genomes. Distribution of the genes among various clusters, and specific protein  
522 encoding genes within each cluster was estimated using the same service. Furthermore,  
523 BLASTn/BLASTp-based investigation was performed to decipher whether the pathogenicity  
524 determinant genes/clusters are present in the genomes (26). Using the complete genome of *C.*  
525 *michiganensis sensu stricto* NCPPB 382, one-vs.-one BLASTn/BLASTp search was accomplished  
526 against the sequences of the pathogenicity island (a 129-kb low G+C region which includes *chp* and  
527 *tomA* clusters) as well as several individual genes proposed to have effective contribution to the  
528 virulence of *C. michiganensis sensu stricto* (22, 25, 26, 28, 42, 43). Proteins with amino acid  
529 sequence similarities higher than 50% and with a query coverage higher than 70% were  
530 considered homologs (28).

531 On the other hand, we screened the genome sequences for the presence of hypothetical  
532 bacteriocin-encoding genes/clusters using the web-based tool BAGEL4  
533 (<http://bagel4.molgenrug.nl/>, 44). BAGEL4 combines direct mining for the structural genes with  
534 indirect mining for bacteriocin-associated genes. Furthermore, the online service PlasmidFinder  
535 2.0 (<https://cge.cbs.dtu.dk/services/PlasmidFinder/>; 45) was used for the screening of all the  
536 genomic sequences for presence of integrative plasmids/ episomes. Identification and annotation  
537 of prophage sequences within bacterial genomes were performed using the online service  
538 PHASTER (PHAge Search Tool Enhanced Release; <http://phaster.ca/>; 46). Given to the fact that  
539 identification of overlaps among the orthologous clusters can enable us to elucidate the function  
540 and evolution of proteins across multiple species, genome wide comparisons and visualization of  
541 orthologous clusters were performed using the online service OrthoVenn  
542 (<http://www.bioinfogenome.net/OrthoVenn/>; 47). The analyses were conducted on the “bacteria”  
543 section of the platform using default settings (E-value: 1e-5 and inflation value: 1.5). Regarding the  
544 numeric limitation of OrthoVenn in the handling of bacterial genomes (up to six genomes per run)  
545 different series of the strains were evaluated using the same parameters.

546

#### 547 **Plasmid Profiling**

548 In order to further investigate the genetic contents of non-pathogenic tomato-associated  
549 *Clavibacter* spp. strains, we evaluated the plasmid profile of the type strains of *C. michiganensis*  
550 subsp. *chilensis* (CFBP 8217<sup>T</sup>) and *C. michiganensis* subsp. *phaseoli* (CFBP 8627<sup>T</sup>) as well as the two  
551 peach-colored strains CFBP 8615 and CFBP 8616 isolated from tomato. Plasmids were isolated

552 according to the procedure described by Kotchoni et al. (48) with minor modifications. Tomato-  
553 pathogenic strain of *C. michiganensis sensu stricto* ICMP 22049 was used as positive control (49).  
554 Bacterial strains were grown in 50 ml Luria-Bertani (LB) medium on a 150 rpm shaker at 27 °C for  
555 48 h. Bacterial cells were harvested by centrifugation at 13,000 g for 1 min at room temperature,  
556 the pellet was re-suspended in 200 µl of “solution I” of Kotchoni et al. (48) protocol, mixed well,  
557 and incubated at 37 °C for 20 min. Subsequently, 400 µl of freshly prepared “solution II” was  
558 added into the microtubes and mixed well by inverting gently four to six times to avoid breaking  
559 the plasmid(s). Immediately 200 µl of “solution III” was added, mixed very gently, and incubated  
560 at 4 °C for 15 min without any intervention. The mixture was centrifuged at 10,000 g for 5 min,  
561 supernatant was transferred to a new microtube, 0.6 volume of isopropanol was added to the  
562 supernatant, mixed gently by inverting four to six times, and kept at room temperature for 10 min.  
563 Then, centrifuged at 10,000 g for 5 min and the supernatant was discarded. The pellet containing  
564 precipitated plasmid DNA was washed with 400 µl of 70 % (v/v) ethanol and centrifuged at 10,000  
565 g for 3 min at room temperature. Supernatant was removed and the pellet was air-dried. Finally,  
566 plasmid DNA was re-suspend in 50 µl sterile distilled water containing 10 mg/ml RNase A.  
567 Presence of plasmids was analyzed on 0.6 % agarose gel as described previously (27).

568

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575

#### 576 **Conflict of Interest Statement**

577 The authors declare that the research was conducted in the absence of any commercial or  
578 financial relationships that could be construed as a potential conflict of interest.

579

#### 580 **Data availability**

581 The datasets generated for this study can be found in the NCBI GenBank Database.

582

#### 583 **Author Contribution**

584 EO and MAJ conceived and designed the study, with assistance from TR. EO, MA, SMT, and SZ  
585 carried out the experiments. MB and PP performed the genome sequencing and annotation. EO  
586 analyzed and interpreted the data with assistance from MAJ, TR, and MB. EO prepared the  
587 manuscript with assistance from MAJ. All the co-authors revised the final manuscript, while EO  
588 and MAJ acted as the corresponding authors.

589

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593

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781

## 782 Legend of Figures and Tables

783 **Figure 1:** Average Nucleotide Identity (ANI)-based Neighbor Joining phylogenetic tree of 40  
 784 *Clavibacter* spp. strains constructed using the ANI calculator online service. Different colors  
 785 represent hypothetical new species (I-IX). Seven hypothetical novel species were  
 786 determined among tomato-associated non-pathogenic *Clavibacter* spp. strains.  
 787 Furthermore, based on the ANI/dddH indices, type strains of *C. michiganensis* subsp.  
 788 *chilensis* and *C. michiganensis* subsp. *phaseoli* belong to the same novel species. \**C.*  
 789 *michiganensis* subsp. *californiensis*

790

791 **Figure 2:** Results of comparative genomics on the two main pathogenicity determinant regions of  
 792 *Clavibacter michiganensis sensu stricto* NCPPB 382 (*i.e.* *chp* and *tomA*) against the non-  
 793 pathogenic tomato-associated strains, as well as the type strains of five species pathogenic

794 on other plants. While only a fraction (<10%) of the clusters were detected in the non-  
795 pathogenic strains (A), variations were observed in the patterns of the genes (B).

796

797 **Figure 3:** Venn diagrams constructed using the OrthoVenn online service showing the distribution  
798 of shared gene families (orthologous clusters) among different sets of *Clavibacter* spp.  
799 strains.

800

801 **Table 1:** Results of pathogenicity tests and host range assays of the type strains of *Clavibacter*  
802 *michiganensis* subsp. *phaseoli* and *C. michiganensis* subsp. *chilensis* as well as two atypical  
803 peach-colored *Clavibacter* sp. strains on different annual crops including their host of  
804 isolation in greenhouse conditions. None of the evaluated bacterial strains was pathogenic  
805 on the tested plants, while the non-pathogenic strain CFBP 8616 was re-isolated from  
806 common bean cv. Navy tissues.

807 **Table 2:** *Clavibacter* spp. genome sequences used for the comparative genomics and phylogenetic  
808 analyses. The first ten sequences were obtained in this study and announced previously  
809 (20), while the remaining ones were retrieved from the NCBI GenBank database. The  
810 leftmost column represents the original nomenclature of the taxa, while the second  
811 column indicates either revised taxonomy of the strains (17) or their new taxonomic status  
812 as proposed in this study.

813



814 **Table 3:** Average nucleotide identity (ANI; lower diagonal) and digital DNA–DNA hybridization  
815 (dDDH; upper diagonal) values among the type and/or representative strains of different  
816 lineages defined within the genus *Clavibacter* spp. ANI values were calculated using three  
817 different algorithms, *i.e.* JSpeciesWS, ANI calculator, and OrthoANlu, and presented  
818 respectively in a left to right order. A combination of ANI and dDDH indices was used to  
819 designate a taxonomic status to a given phylogenetic clade, where the “new species”  
820 status was assigned to a clade only when both the ANI and dDDH values were below the  
821 accepted threshold ( $\leq 95\%$  and  $\leq 70\%$  for ANI and dDDH, respectively; 21).

822

823 **Table 4:** Genomic characteristics of *Clavibacter* spp. strains used in this study. Individual genomes  
824 were analyzed using the online annotating service RAST, and protein-encoding sequences  
825 (CDS), functions of the genes, and represented subsystems in the genomes were  
826 determined for each genome using the SEED-Viewer comparative environment. Feature  
827 corresponding to cell wall and capsule, DNA metabolism, dormancy and sporulation,  
828 membrane transport, respiration, RNA metabolism, as well as miscellaneous groups have  
829 not been included in the table since they were common among all the evaluated taxa.

830

831 **Table 5:** Results of one-vs.-one BLASTn/BLASTp searches using the genome sequence of  
832 *Clavibacter michiganensis sensu stricto* NCPPB 382 (AM711867.1) against all other genome  
833 sequences shown in Table 2. Putative pathogenicity determinant genes/regions described  
834 in the literature were subjected to the analyses. While nine chromosomal genes (*i.e.* *chpC*,

835 *chpG*, *clvA* (*micA*), *clvF*, *clvG*, *pelA1*, *pelA2*, *perF*, and *ppaA*) as well as the pCM2 plasmid-  
 836 borne pathogenicity-associated gene *pat-1* were determined as exclusively present in *C.*  
 837 *michiganensis sensu stricto*, the remaining genes were detected in different phylogenetic  
 838 lineages regardless of their pathogenicity and host range.

839

840 **Table 6:** Prophages within the genome sequences of *Clavibacter* spp. strains detected using the  
 841 online service PHASTER. Five prophage groups *i.e.* Gordon\_Schwabeltier,  
 842 Gordon\_Smoothie, N15, P1, and Phi92 were detected in CFBP 8615, CFBP 7491, LMG  
 843 26808, CASJ009, LMG 3663, and ATCC 33113 strains. No prophage was detected within the  
 844 genome sequences of the strains ATCC 33566T, CF11, CFBP 7493, CFBP 7494, CFBP 8017,  
 845 CFBP 8019, CFBP 8216T, CFBP 8217T, CFBP 8616, CFBP 8627T, DOAB 609, NCPPB 2581T,  
 846 NCPPB 382, and PF008T.

847

848 **Table 7:** In silico screening for bacteriocins and antibiotic peptides among the *Clavibacter* spp.  
 849 genome sequences analyzed in this study. The lantibiotic “Michiganin A” was detected in  
 850 all the 12 *C. michiganensis sensu stricto* strains (Figure 1) but was not found in non-  
 851 pathogenic tomato-associated strains nor in the pathogenic strains on other plant species.

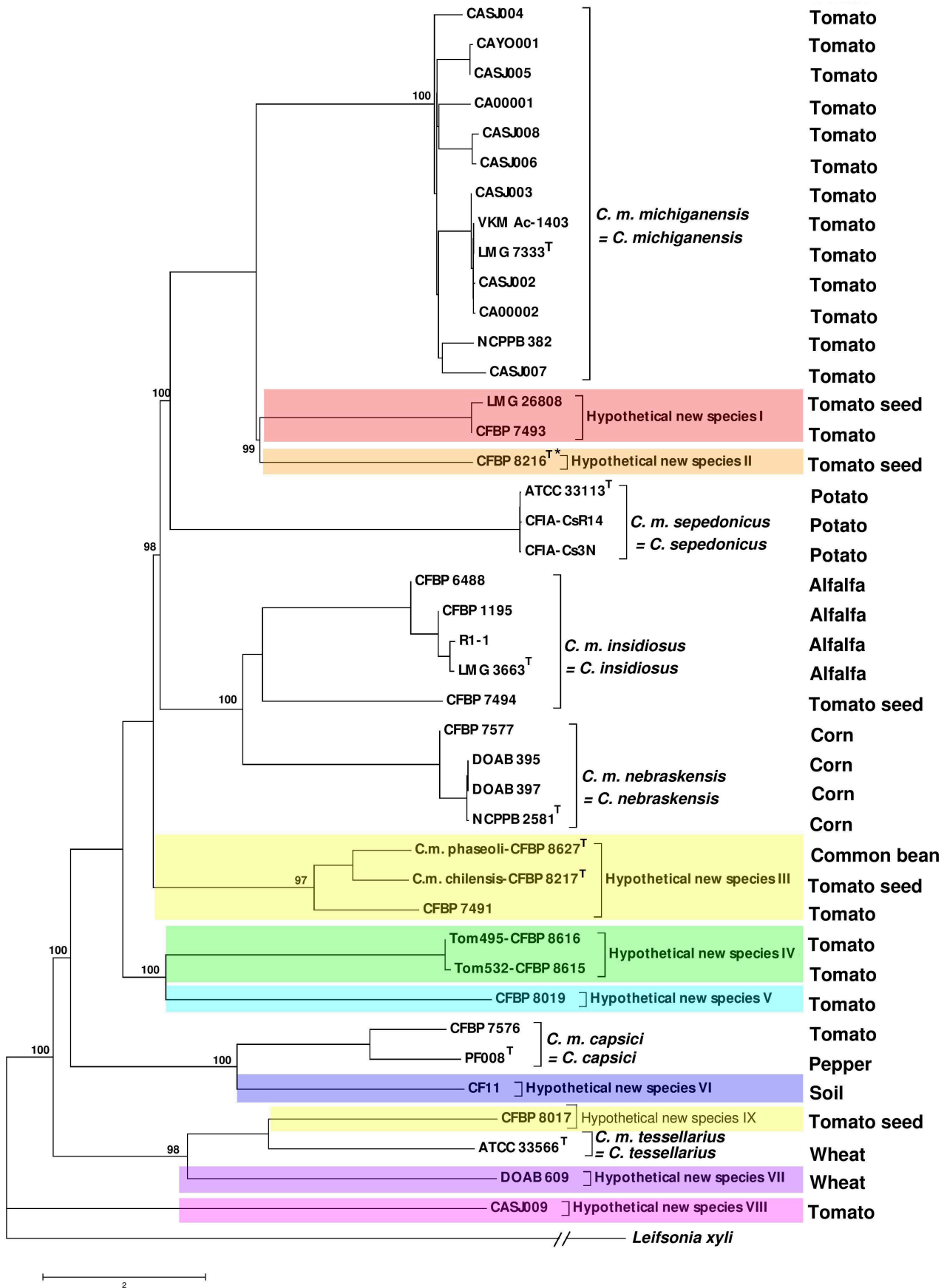
852

853 **Figure S1:** Tomato and pepper plants inoculated with *Clavibacter michiganensis sensu stricto*  
 854 (ICMP 22049). Tomato plants inoculated with the strain ICMP 22049 showed wilting and  
 855 plant death 10-12 days post inoculation (a, right side plant), while pepper plants inoculated

856 using the same strain showed only stem canker symptoms on the site of inoculation with  
857 no wilting nor plant death in the same timeframe (b). Control tomato plant remained  
858 healthy (a, left side plant).

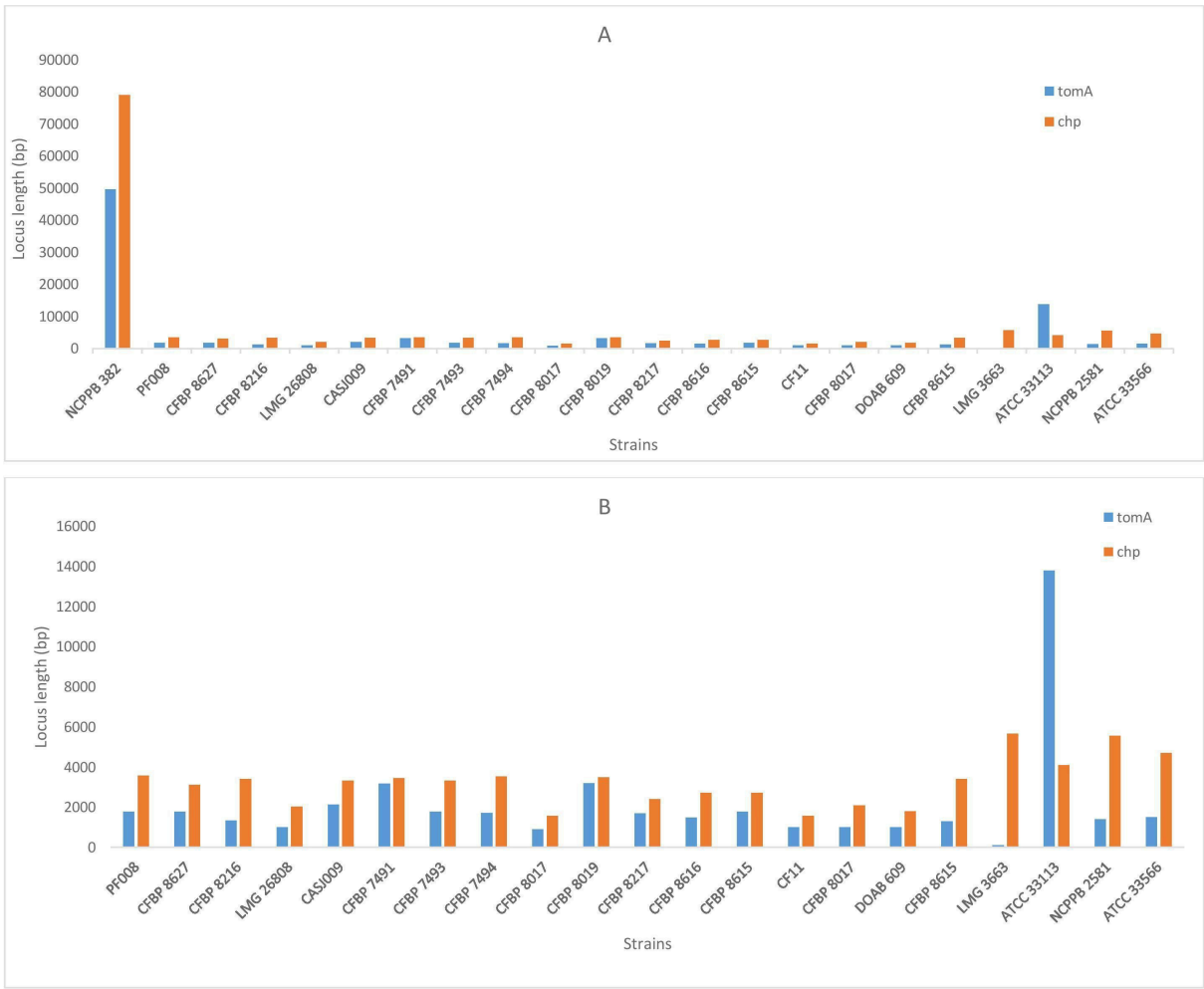
859

860



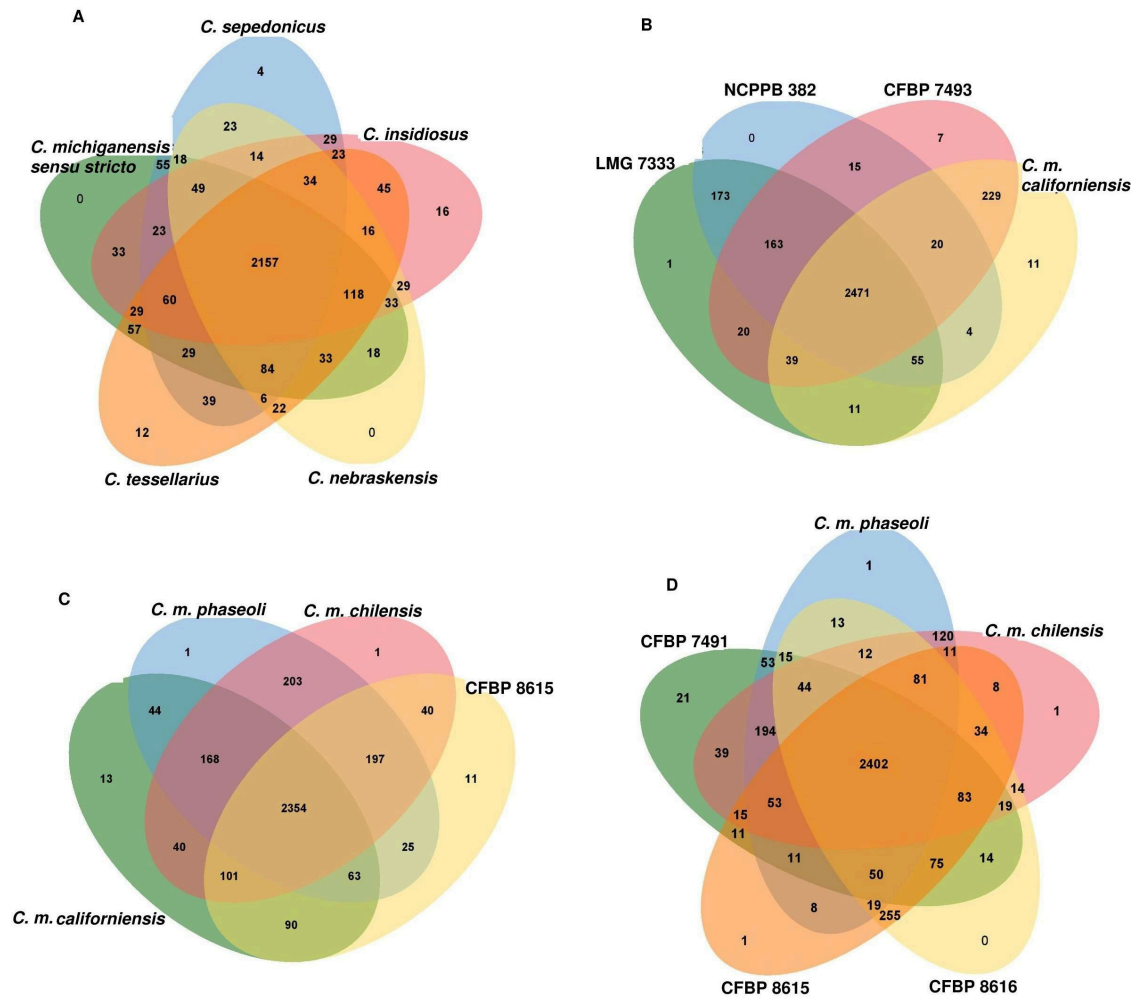
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**Table 1:** Results of pathogenicity tests and host range assays of the type strains of *Clavibacter michiganensis* subsp. *phaseoli* and *C. michiganensis* subsp. *chilensis* as well as two atypical peach-colored *Clavibacter* sp. strains on different annual crops including their host of isolation in greenhouse conditions. None of the evaluated bacterial strains was pathogenic on the tested plants, while the non-pathogenic strain CFBP 8616 was re-isolated from common bean cv. Navy tissues.

Taxon	Strain	Pepper		Common bean				Tomato
		cv. Sereno	cv. Aziz	cv. Red Kidney	cv. Pinto	cv. Navy	Cowpea Mung bean	
<i>C. m. phaseoli</i>	CFBP 8627 <sup>T</sup>	-	-	-	-	-	-	-
<i>C. m. chilensis</i>	CFBP 8217 <sup>T</sup>	-	-	-	-	-	-	-
<i>Clavibacter</i> sp.	CFBP 8615	-	-	-	-	-	-	-
<i>Clavibacter</i> sp.	CFBP 8616	-	-	-	-	- <sup>a</sup>	-	-
<i>C. michiganensis</i> <i>sensu stricto</i>	ICMP 22049	+ <sup>b</sup>	+ <sup>b</sup>	-	-	-	-	+

*C. m.*: *Clavibacter michiganensis* subsp.

cv.: Cultivar

-: Negative

+: Positive

T: Type strain

a: The inoculated bacterial strain was re-isolated from the asymptomatic leaf tissues of the test plants.

b: These plants showed only stem canker symptoms on the site of inoculation with no systemic wilting and plant death.

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**Table 2:** *Clavibacter* spp. genome sequences used for the comparative genomics and phylogenetic analyses. The first ten sequences were obtained in this study and announced previously (20), while the remaining ones were retrieved from the NCBI GenBank database. The leftmost column represents the original nomenclature of the taxa, while the second column indicates either revised taxonomy of the strains (17) or their new taxonomic status as proposed in this study.

Taxonomic position		Strain	Host of Isolation	Pathogenic	Date	Country of Isolation	GenBank Accession No.	Reference
Previous name	New name <sup>1</sup>							
<i>Clavibacter</i> sp.	new species <sup>2</sup> IV	CFBP 8615*	<i>Solanum lycopersicum</i>	NP	2015	Iran	QWGT01000000	20
<i>Clavibacter</i> sp.	new species IV	CFBP 8616*	<i>Solanum lycopersicum</i>	NP	2015	Iran	QWGU01000000	20
<i>C. m. californiensis</i>	new species II	CFBP 8216 <sup>†</sup> *	<i>Solanum lycopersicum</i>	NP	2000	USA	QWEE01000000	20
<i>C. m. chilensis</i>	new species III	CFBP 8217 <sup>†</sup> *	<i>Solanum lycopersicum</i>	NP	2007	Netherlands	QWGS01000000	20
<i>Clavibacter</i> sp.	new species III	CFBP 7491*	<i>Solanum lycopersicum</i>	NP	ND	ND	QWEB01000000	20
<i>Clavibacter</i> sp.	new species I	CFBP 7493*	<i>Solanum lycopersicum</i>	NP	ND	ND	QWEC01000000	20
<i>C. m. insidiosus</i>	<i>C. insidiosus</i>	CFBP 1195	<i>Medicago sativa</i>	alfalfa	1964	UK	QWDZ01000000	20
<i>C. m. insidiosus</i>	<i>C. insidiosus</i>	CFBP 6488	<i>Medicago sativa</i>	alfalfa	1998	Czech Republic	QWEA01000000	20
<i>C. m. nebraskensis</i>	<i>C. nebraskensis</i>	CFBP 7577	<i>Zea mays</i>	corn	ND	ND	QWED01000000	20
<i>C. m. phaseoli</i>	new species III	CFBP 8627 <sup>†</sup>	<i>Phaseolus vulgaris</i>	NP	2009	Spain	QWGV01000000	20
<i>Clavibacter</i> sp.	new species VIII	CASJ009*	<i>Solanum lycopersicum</i>	NP	2011	USA	MDHJ01000000	22
<i>Clavibacter</i> sp.	new species V	CFBP 8019*	<i>Solanum lycopersicum</i>	NP	2011	USA	MDHJ01000000	22
<i>Clavibacter</i> sp.	new species IX	CFBP 8017*	<i>Solanum lycopersicum</i>	wheat	2006	Netherlands	MDJY00000000	22
<i>Clavibacter</i> sp.	new species VI	CF11	soil	NP	2011	China	JROD00000000	23
<i>Clavibacter</i> sp.	new species I	LMG 26808*	<i>Solanum lycopersicum</i>	NP	ND	Netherlands	AZQZ00000000	28
<i>Clavibacter</i> sp.	<i>C. insidiosus</i>	CFBP 7494*	<i>Solanum lycopersicum</i>	wheat	1999	Chile	MDJW00000000	22
<i>Clavibacter</i> sp.	new species VII	DOAB 609	<i>Triticum aestivum</i>	ND	1976	USA	LQXA00000000	50
<i>C. m. michiganensis</i>	<i>C. michiganensis</i>	LMG 7333 <sup>†</sup>	<i>Solanum lycopersicum</i>	tomato	1957	Hungary	NZ_MZMP00000000	51
<i>C. m. michiganensis</i>	<i>C. michiganensis</i>	NCPBP 382	<i>Solanum lycopersicum</i>	tomato	1956	UK	AM711867.1	26
<i>C. m. michiganensis</i>	<i>C. michiganensis</i>	CASJ004	<i>Solanum lycopersicum</i>	tomato	1999	USA	MDHE00000000	22

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<i>C. m. michiganensis</i>	<i>C. michiganensis</i>	CAY001	<i>Solanum lycopersicum</i>	tomato	2001	USA	MDHL00000000	22
<i>C. m. michiganensis</i>	<i>C. michiganensis</i>	CASJ005	<i>Solanum lycopersicum</i>	tomato	2001	USA	MDHF00000000	22
<i>C. m. michiganensis</i>	<i>C. michiganensis</i>	CA00001	<i>Solanum lycopersicum</i>	tomato	2000	USA	MDHK00000000	22
<i>C. m. michiganensis</i>	<i>C. michiganensis</i>	CASJ008	<i>Solanum lycopersicum</i>	tomato	2002	USA	MDHI00000000	22
<i>C. m. michiganensis</i>	<i>C. michiganensis</i>	CASJ006	<i>Solanum lycopersicum</i>	tomato	2002	USA	MDHG00000000	22
<i>C. m. michiganensis</i>	<i>C. michiganensis</i>	CASJ003	<i>Solanum lycopersicum</i>	tomato	1999	USA	MDHD00000000	22
<i>C. m. michiganensis</i>	<i>C. michiganensis</i>	VKM Ac-1403	<i>Solanum lycopersicum</i>	tomato	2017	USA	FVZG00000000	52
<i>C. m. michiganensis</i>	<i>C. michiganensis</i>	CASJ002	<i>Solanum lycopersicum</i>	tomato	1999	USA	MDHC00000000	22
<i>C. m. michiganensis</i>	<i>C. michiganensis</i>	CA00002	<i>Solanum lycopersicum</i>	tomato	2000	USA	MDHM00000000	22
<i>C. m. michiganensis</i>	<i>C. michiganensis</i>	CASJ007	<i>Solanum lycopersicum</i>	tomato	2011	USA	MDHH00000000	22
<i>C. m. capsici</i>	<i>C. capsici</i>	PF008 <sup>†</sup>	<i>Capsicum</i> sp.	pepper	ND	Korea	NZ_CP012573	15
<i>C. m. capsici</i>	<i>C. capsici</i>	CFBP 7576*	<i>Solanum lycopersicum</i>	pepper	1997	ND	MDJX00000000	22
<i>C. m. insidiosus</i>	<i>C. insidiosus</i>	LMG 3663 <sup>†</sup>	<i>Medicago sativa</i>	alfalfa	1955	USA	MZMO00000000	51
<i>C. m. insidiosus</i>	<i>C. insidiosus</i>	R1-1	<i>Medicago truncatula</i>	alfalfa	2009	USA	NZ_CP011043	29
<i>C. m. nebraskensis</i>	<i>C. nebraskensis</i>	NCPBP 2581 <sup>†</sup>	<i>Zea mays</i>	corn	1971	USA	NC_020891.1	50
<i>C. m. nebraskensis</i>	<i>C. nebraskensis</i>	DOAB 395	<i>Zea mays</i>	corn	2014	Canada	LSOE00000000	50
<i>C. m. nebraskensis</i>	<i>C. nebraskensis</i>	DOAB 397	<i>Zea mays</i>	corn	2014	Canada	LAKL00000000	53
<i>C. m. sepedonicus</i>	<i>C. sepedonicus</i>	ATCC 33113 <sup>†</sup>	<i>Solanum tuberosum</i>	potato	ND	Canada	NC_010407.1	54
<i>C. m. sepedonicus</i>	<i>C. sepedonicus</i>	CFIA-CsR14	<i>Solanum tuberosum</i>	potato	ND	Canada	MZMN00000000	51
<i>C. m. sepedonicus</i>	<i>C. sepedonicus</i>	CFIA-Cs3N	<i>Solanum tuberosum</i>	potato	ND	Canada	MZMM00000000	51
<i>C. m. tessellarius</i>	<i>C. tessellarius</i>	ATCC 33566 <sup>†</sup>	<i>Triticum aestivum</i>	wheat	1978	USA	MZMQ01000000	51

1: Although new names of six species (*i.e.* *C. capsici*, *C. insidiosus*, *C. michiganensis*, *C. nebraskensis*, *C. sepedonicus* and *C. tessellarius*) were formally described previously (Li et al. 2018), a formal taxonomic description is needed for the hypothetical new species I to IX.

2: Hypothetical new species based on the ANI/dDDH values (Table 3) and comparative genomics (Table 4).

\*These strains were isolated from tomato plant but were non-pathogenic on the host of isolation.

*C. m.*: *Clavibacter michiganensis* subsp.

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T: Type strain

ND: Not determined

NP: Non-pathogenic

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**Table 3:** Average nucleotide identity (ANI; lower diagonal) and digital DNA–DNA hybridization (dDDH; upper diagonal) values among the type and/or representative strains of different lineages defined within the genus *Clavibacter* spp. ANI values were calculated using three different algorithms, *i.e.* JSpeciesWS, ANI calculator, and OrthoANIu, and presented respectively in a left to right order. A combination of ANI and dDDH indices was used to designate a taxonomic status to a given phylogenetic clade, where the “new species” status was assigned to a clade only when both the ANI and dDDH values were below the accepted threshold ( $\leq 95\%$  and  $\leq 70\%$  for ANI and dDDH, respectively; 21).

Taxon	Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	<i>C. michiganensis sensu stricto</i>	LMG 7333 <sup>T</sup>	57.70	58.70	48.00	48.60	49.70	48.00	49.80	49.50	44.80	43.10	40.20	40.00	37.50	37.20	36.80	34.60
2	<i>C. m. californiensis</i>	CFBP 8216 <sup>T</sup>	95/95/9	57.80	46.40	47.90	48.60	47.40	48.10	47.90	44.80	43.70	40.50	40.40	38.50	38.40	38.00	35.70
3	<i>Clavibacter</i> sp.	LMG 26808	95/94/9	94/95/9	45.80	47.80	48.40	47.10	47.90	47.70	44.30	42.60	39.60	39.50	37.10	37.00	36.40	34.50
4	<i>C. sepedanicus</i>	ATCC 33113 <sup>T</sup>	92/92/9	92/92/9	92/92/9	45.10	46.20	45.20	47.00	46.60	43.90	42.30	39.10	39.10	36.40	36.20	36.00	34.00
5	<i>C. insidiosus</i>	LMG 3663 <sup>T</sup>	93/93/9	93/93/9	92/92/9	92/92/9	64.60	59.90	51.20	51.00	47.10	43.30	40.50	40.20	37.90	37.40	37.20	34.80
6	<i>Clavibacter</i> sp.	CFBP 7494	93/93/9	92/93/9	93/92/9	92/92/9	96/96/9	60.20	52.20	52.10	46.00	44.10	40.70	40.60	38.10	37.60	37.50	34.90
7	<i>C. nebraskensis</i>	NCPPB 2581 <sup>T</sup>	92/93/9	92/93/9	92/92/9	92/92/9	95/95/9	95/95/9	51.40	51.10	45.30	43.30	40.70	40.70	37.70	37.20	36.90	34.90
8	<i>C. m. chilensis</i>	CFBP 8217 <sup>T</sup>	93/93/9	93/93/9	92/93/9	92/93/9	93/93/9	93/93/9	93/93/9	87.50	47.00	45.80	43.40	42.90	40.20	39.50	39.20	37.10
9	<i>C. m. phaseoli</i>	CFBP 8627 <sup>T</sup>	93/93/9	93/93/9	92/92/9	92/93/9	93/93/9	93/93/9	93/93/9	99/99/9	47.10	45.70	43.30	42.90	40.00	39.50	39.10	37.00
10	<i>Clavibacter</i> sp.	CFBP 8615	92/92/9	92/92/9	91/92/9	91/92/9	92/92/9	92/92/9	92/92/9	92/92/9	92/93/9	48.00	40.90	40.50	38.20	37.60	37.70	35.80
11	<i>Clavibacter</i> sp.	CFBP 8019	91/92/9	91/92/9	91/91/9	91/92/9	91/92/9	91/91/9	91/91/9	92/92/9	92/92/9	93/93/9	40.10	40.00	36.90	36.80	36.80	34.40
12	<i>C. capsici</i>	PF008 <sup>T</sup>	90/91/9	90/91/9	90/90/9	90/91/9	90/91/9	90/90/9	90/91/9	91/92/9	91/92/9	90/91/9	90/91/9	58.50	38.40	38.50	37.70	34.50

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		0	1	0	0	0	0	0	1	1	1	0						
13	<i>Clavibacter</i> sp.	CF11	90/90/9	90/90/9	90/90/9	90/90/9	90/90/9	91/90/9	91/90/9	91/91/9	91/91/9	90/90/9	90/90/9	95/95/9	38.10	38.50	37.60	34.40
			0	0	0	0	0	0	1	1	0	0	5					
14	<i>C. tessellarius</i>	ATCC 33566 <sup>T</sup>	90/90/8	90/91/9	89/89/8	89/90/8	90/90/8	90/90/9	90/90/8	91/91/9	91/91/9	90/90/9	89/90/8	90/91/9		49.00	57.70	33.50
			9	0	9	9	9	0	9	0	0	9	0	90/90/9				
15	<i>Clavibacter</i> sp.	DOAB 609	89/89/8	89/90/9	89/89/8	89/89/8	89/89/8	90/90/8	89/89/8	90/90/9	90/90/9	89/90/8	89/89/8	90/90/9	90/90/9	93/93/9	47.20	33.60
			9	0	9	9	9	9	0	0	9	9	0	0	3			
16	<i>Clavibacter</i> sp.	CFBP 8017	89/89/8	89/90/9	89/89/8	89/89/8	89/89/8	90/90/8	89/89/8	90/90/9	90/90/9	89/90/8	89/89/8	90/90/8	90/90/8	95/95/9	92/92/9	33.30
			9	0	9	9	9	9	0	0	9	9	9	9	5	2		
17	<i>Clavibacter</i> sp.	CASJ009	88/89/8	89/90/8	88/88/8	88/89/8	88/89/8	88/88/8	88/89/8	89/90/8	89/90/9	89/90/8	88/89/8	88/89/8	88/88/8	88/89/8	88/88/8	87/88/8
			9	8	8	8	8	8	9	0	9	8	9	8	8	7	7	

*C. m.*: *Clavibacter michiganensis* subsp.

T: Type strain

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**Table 4:** Genomic characteristics of *Clavibacter* spp. strains used in this study. Individual genomes were analyzed using the online annotating service RAST, and protein-encoding sequences (CDS), functions of the genes, and represented subsystems in the genomes were determined for each genome using the SEED-Viewer comparative environment. Feature corresponding to cell wall and capsule, DNA metabolism, dormancy and sporulation, membrane transport, respiration, RNA metabolism, as well as miscellaneous groups have not been included in the table since they were common among all the evaluated taxa.

Subsystem feature	<i>C. michiganensis sensu stricto</i> (NCPBP 382)	<i>C. capsici</i> (PF008 <sup>†</sup> )	<i>Clavibacter</i> sp. (CFBP 8615)	<i>Clavibacter</i> sp. (CFBP 8616)	<i>C. m. californiensis</i> (CFBP 8216 <sup>†</sup> )	<i>Clavibacter</i> sp. (CFBP 7491)	<i>Clavibacter</i> sp. (CFBP 7493)	<i>Clavibacter</i> sp. (CFBP 7494)	<i>Clavibacter</i> sp. (CFBP 8019)	<i>Clavibacter</i> sp. (CFBP 8017)	<i>Clavibacter</i> sp. (DOAB 609)	<i>Clavibacter</i> sp. (CF11)	<i>Clavibacter</i> sp. (LMG 26808)	<i>C. m. phaseoli</i> (CFBP 8627 <sup>†</sup> )	<i>C. m. chilensis</i> (CFBP 8217 <sup>†</sup> )	<i>Clavibacter</i> sp. (CASI009)	<i>C. insidiosus</i> (LMG 3663 <sup>†</sup> )	<i>C. nebraskensis</i> (NCPBP 2581 <sup>†</sup> )	<i>C. sepedanicus</i> (ATCC 33113 <sup>†</sup> )	<i>C. tessellarius</i> (ATCC 33566 <sup>†</sup> )
Genome size (bp)	3,297	3,056	3,129	3,094	3,193	3,288	3,275	3,313	3,024	3,172	3,296	3,118	3,420	3,052	3,044	3,268	3,387	3,063	3,258	3,318
GC content (%)	72.7	73.6	73.2	73.2	72.7	73.0	72.9	73.3	73.5	73.5	73.2	73.6	72.0	73.5	73.5	73.6	72.7	73.0	72.6	73.7
Number of coding sequences (CDS)	2979	2725	2807	2730	2784	2917	2897	2956	2676	3014	3181	3002	3097	2642	2629	3054	3091	2739	3047	2956
Number of subsystems	345	326	323	319	338	316	342	330	326	263	266	260	340	315	311	341	332	325	345	317
RNAs	51	51	47	47	48	50	48	50	48	48	53	49	57	50	49	51	52	51	51	52
Cofactors, vitamins, pigments	177	158	169	157	165	139	173	161	158	107	116	99	175	167	130	181	165	139	169	176
Virulence, disease and defense	34	38	37	36	38	36	36	32	38	26	25	25	40	35	34	42	36	30	33	22
Resistance to antibiotics/toxic compounds	19	23	22	21	23	21	21	17	24	17	16	15	25	21	19	27	21	17	20	18
Invasion and intracellular resistance	15	15	15	15	15	15	15	15	14	9	9	10	15	14	15	15	15	13	13	4
Potassium metabolism	13	12	12	12	13	13	13	16	5	5	5	9	9	9	13	8	13	9	10	11
Phages, prophages, transposable elements	0	0	0	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
Iron acquisition and metabolism	16	17	13	25	16	8	19	18	13	5	10	5	17	10	10	12	15	14	17	16
Siderophores*:																				

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Siderophore Yersiniabactin	2	0	0	0	2	0	2	0	0	0	5	0	2	0	0	0	2	2	0	0
Siderophore Aerobactin	3	3	3	3	3	3	4	3	3	0	0	0	3	3	3	3	3	3	3	3
Nucleosides and nucleotides	87	72	93	92	89	76	100	76	81	80	79	75	74	98	68	90	79	80	99	65
Protein metabolism	211	201	209	192	189	185	209	204	208	166	162	167	205	178	198	192	205	178	213	185
Cell division and cell cycle	26	21	8	8	23	24	24	23	21	0	0	0	8	23	8	22	24	20	22	23
Motility and chemotaxis	1	2	2	2	2	3	1	2	2	0	0	0	3	3	3	3	2	1	2	2
Regulation and cell signaling	33	30	19	20	22	22	25	30	33	14	17	18	36	28	17	29	31	33	35	24
Secondary metabolism	6	18	5	5	4	5	11	12	11	9	9	9	12	0	1	11	5	5	13	11
Fatty acids, lipids, and isoprenoids	88	78	84	103	82	108	78	88	59	44	44	42	79	85	76	82	65	65	80	101
Nitrogen metabolism	7	10	6	6	10	6	7	6	6	6	6	6	7	6	6	11	7	7	6	11
Stress response	67	73	76	69	74	67	64	70	78	27	27	28	68	71	61	82	74	71	78	64
Metabolism of aromatic compounds	10	8	9	8	9	14	10	10	8	4	3	3	10	10	10	18	10	9	10	10
Amino acids and derivatives	224	190	223	212	233	221	210	192	199	217	221	203	236	193	195	224	219	229	232	220
Sulfur metabolism	20	16	16	11	9	12	10	16	17	6	5	5	13	10	11	26	17	9	22	18
Phosphorus metabolism	38	26	25	27	28	26	27	23	25	28	27	27	36	25	26	25	23	23	25	24
Carbohydrates	285	266	255	268	267	283	247	268	237	181	194	175	278	263	261	382	266	266	269	281

*C. m.*: *Clavibacter michiganensis* subsp.

T: Type strain

\*Siderophore assembly kit was detected only in *Clavibacter* sp. CFBP 8616.

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**Table 5:** Results of one-vs.-one BLASTn/BLASTp searches using the genome sequence of *Clavibacter michiganensis sensu stricto* NCPPB 382 (AM711867.1) against all other genome sequences shown in Table 2. Putative pathogenicity determinant genes/regions described in the literature were subjected to the analyses. While nine chromosomal genes (*i.e.* *chpC*, *chpG*, *clvA* (*micA*), *clvF*, *clvG*, *pelA1*, *pelA2*, *perF*, and *ppaA*) as well as the pCM2 plasmid-borne pathogenicity-associated gene *pat-1* were determined as exclusively present in *C. michiganensis sensu stricto*, the remaining genes were detected in different phylogenetic lineages regardless of their pathogenicity and host range.

Locus tag on NCPPB 382 genome	Gene/cluster	Length (bp)	<i>C. michiganensis sensu stricto</i> (NCPPB 382)	<i>Clavibacter</i> sp. (CFBP 8615)	<i>Clavibacter</i> sp. (CF11)	<i>Clavibacter</i> sp. (CFBP 8017)	<i>Clavibacter</i> sp. (DOAB 609)	<i>C. m. californiensis</i> (CFBP 8216 <sup>1</sup> )	<i>C. m. chilensis</i> (CFBP 8217)	<i>C. capsici</i> (PF008 <sup>1</sup> )	<i>Clavibacter</i> sp. (CFBP 7491)	<i>Clavibacter</i> sp. (CFBP 7493)	<i>Clavibacter</i> sp. (LMG 26808)	<i>Clavibacter</i> sp. (CFBP 7494)	<i>Clavibacter</i> sp. (CFBP 8019)	<i>Clavibacter</i> sp. (CAS/009)	<i>C. m. phaseoli</i> (CFBP 8627)	<i>C. insidiosus</i> (LMG 3663 <sup>1</sup> )	<i>C. nebraskensis</i> (NCPBP 2581 <sup>1</sup> )	<i>C. sepedonicus</i> (ATCC 33113 <sup>1</sup> )	<i>C. tessellarius</i> (ATCC 33566 <sup>1</sup> )	Reference	
MMM_0070	<i>sbtA</i>	3102	[100]100	[75]75	[98]82	ND	[97]81	[82]82	[76]94	[97]82	[97]89	ND	ND	ND	[98]82	[77]82	[96]89	ND	[99]80	ND	ND	43	
MMM_0013	<i>srtA</i>	801	[100]100	[100]92	[100]87	[100]87	[100]87	[100]97	[100]91	[100]87	[100]91	[100]97	[100]97	[100]90	[100]89	[100]89	[100]89	[100]91	[100]92	[100]92	[100]93	[100]88	43
MMM_1480	<i>expA</i>	762	[100]100	ND	ND	[77]81	[77]82	ND	ND	ND	ND	[100]99	[100]99	ND	ND	ND	ND	ND	ND	ND	ND*	26	
MMM_1673	<i>yxsA</i>	1296	[100]100	[98]90	[99]87	[99]92	[97]91	[99]95	[81]76	[100]88	[83]76	[99]94	[99]94	[99]93	[99]89	[48]80	[81]75	[99]95	[99]92	ND	[99]92	43	
MMM_1674	<i>yxsB</i>	2010	[100]100	[92]93	[100]89	[100]89	[54]75	[100]93	[100]93	[100]90	[100]93	[94]95	[99]94	[100]92	[100]92	[95]90	[93]93	[100]90	[100]91	ND	[99]89	43	
MMM_2443	<i>ceiB</i>	1608	[100]100	ND	ND	[100]89	[100]87	ND	ND	[100]88	ND	[100]91	[100]91	[100]91	ND	ND	ND	[100]92	[64]85	[100]91	[100]85	26	

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CMM_2691	NA	1002	[100]100	[99]89	[100]89	[100]89	[99]90	[100]91	[100]89	[99]89	[100]89	[100]94	[100]94	[89]92	[99]90	[97]83	[100]88	[100]94	[98]92	[99]89	[100]90	26
CMM_2692	NA	1410	[100]100	[90]93	[100]86	[100]86	[100]86	[91]96	[77]90	[100]89	[30]86	[100]91	[100]91	[100]90	[100]87	ND	[90]90	[100]92	[100]91	[100]86	[100]88	26
CMM_2871	NA	1491	[100]100	ND	ND	ND	ND	ND	ND	ND	ND	[100]90	[100]90	ND	ND	ND	ND	[92]88	[98]92	ND	[100]92	26
CMM_2645	<i>vatr1</i>	624	[100]100	[100]94	[100]92	[100]92	[100]91	[85]98	[100]93	[100]92	[100]94	[100]98	[100]98	[100]96	[100]93	[100]89	[100]94	[100]96	[100]96	[100]94	[100]92	25
CMM_2969	<i>vatr2</i>	1323	[100]100	[100]92	[100]92	[99]92	[100]92	[100]96	[100]94	[100]92	[100]94	[100]95	[100]95	[100]94	[100]93	[100]90	[100]94	[100]93	[100]93	[100]93	[100]91	25
pCM1_0020	<i>celA</i>	2241	ND	ND	[46]84	[46]75	[47]75	ND	ND	[45]77	ND	ND	ND	ND	ND	ND	ND	[97]90	[76]84	ND	[45]75	26

1: The values in brackets refer to the query coverage (%) of the locus while the values at the right side of the brackets refer to the sequence similarity (%) between the reference strain (NCPFB 382<sup>T</sup>) and the strain in question. The query coverage of <50% were not considered as reliable data.

NA: Not Assigned

ND: Not Detected (Absence of the target gene/cluster)

*C. m.*: *Clavibacter michiganensis* subsp.

T: Type strain

\*: A putative expansin protein was found in this species (GenBank: OQJ63896.1) in which the nucleotide sequence was different from CMM\_1480: *expA*.

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**Table 6:** Prophages within the genome sequences of *Clavibacter* spp. strains detected using the online service PHASTER. Five prophage groups *i.e.* Gordon\_Schwabeltier, Gordon\_Smoothie, N15, P1, and Phi92 were detected in CFBP 8615, CFBP 7491, LMG 26808, CASJ009, LMG 3663, and ATCC 33113 strains. No prophage was detected within the genome sequences of the strains ATCC 33566T, CF11, CFBP 7493, CFBP 7494, CFBP 8017, CFBP 8019, CFBP 8216T, CFBP 8217T, CFBP 8616, CFBP 8627T, DOAB 609, NCPPB 2581T, NCPPB 382, and PF008T.

Taxon	Strain	Region Length (kb)	Completeness	Number of proteins	Position	phage (GenBank accession number)	GC%
<i>Clavibacter</i> sp.	CFBP 8615	6.9	incomplete	8	38: 3643-10586	Phi92 (NC_023693)	71.28
<i>Clavibacter</i> sp.	CFBP 7491	6.8	incomplete	14	4: 13543-20391	Gordon_Schwabeltier (NC_031255)	66.29
<i>Clavibacter</i> sp.	LMG 26808	9.9	incomplete	10	13: 128032-137979	Phi92 (NC_023693)	71.79
		7	incomplete	11	15: 712-7762	N15 (NC_001901)	46.49
		23.7	incomplete	10	15: 5546-29310	P1 (NC_005856)	48.19
<i>Clavibacter</i> sp.	CASJ009	7.7	incomplete	9	1009722-1017492	Phi92 (NC_023693)	71.29
<i>C. insidiosus</i>	LMG 3663 <sup>T</sup>	6.7	incomplete	16	scaffold2: 5055-11844	Gordon_Smoothie (NC_030696)	66.95
<i>C. sepedonicus</i>	ATCC 33113 <sup>T</sup>	7.1	incomplete	10	42745-49937	Phi92 (NC_023693)	70.71
		7.7	incomplete	8	660037-667813	Phi92 (NC_023693)	71.20

*C. m.*: *Clavibacter michiganensis* subsp.

ND: Not detected

T: Type strain

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**Table 7:** In silico screening for bacteriocins and antibiotic peptides among the *Clavibacter* spp. genome sequences analyzed in this study. The lantibiotic “Michiganin A” was detected in all the 12 *C. michiganensis sensu stricto* strains (Figure 1) but was not found in non-pathogenic tomato-associated strains nor in the pathogenic strains on other plant species.

Taxon	Strain	#Contig	Start codon	End codon	Class
<i>C. michiganensis sensu stricto</i>	NCPBP 382	1	2211104	2233501	63.1; Michiganin A (Lantibiotic)
<i>C. capsici</i>	PF008 <sup>T</sup>	1	2406476	2426476	Sactipeptides
<i>Clavibacter</i> sp.	CFBP 8615	14	7079	27079	LAPs
		91	1570	18430	Sactipeptides
<i>Clavibacter</i> sp.	CFBP 8616	166	4888	15112	Sactipeptides
<i>C. m. californiensis</i>	CFBP 8216 <sup>T</sup>	201	9178	10822	Sactipeptides
<i>Clavibacter</i> sp.	CFBP 7491	224	8131	11869	Sactipeptides
<i>Clavibacter</i> sp.	CFBP 7493	1	25925	45925	Thiopeptide; LAPs
		226	-8455	11545	Sactipeptides
<i>Clavibacter</i> sp.	CFBP 7494	9	267758	287758	LAPs
		15	89075	109075	Sactipeptides
<i>Clavibacter</i> sp.	CFBP 8019	11	186443	206443	Sactipeptides
		17	190604	210604	LAPs
<i>Clavibacter</i> sp.	LMG 26808	6	43154	63154	Thiopeptide; LAPs
		10	130865	150865	Sactipeptides
<i>Clavibacter</i> sp.	CF11	17	47765	67765	Sactipeptides
		2	247508	267508	LAPs
		13	138056	158368	150.1;Enterocin_AS_48
<i>Clavibacter</i> sp.	DOAB 609	20	4781	24781	Sactipeptides
		30	31400	51400	LAPs
<i>Clavibacter</i> sp.	CFBP 8017	33	39236	59236	LAPs
		46	11546	31546	Sactipeptides
<i>C. m. phaseoli</i>	CFBP 8627 <sup>T</sup>	212	-9916	10084	Sactipeptides
<i>C. m. chilensis</i>	CFBP 8217 <sup>T</sup>	ND	ND	ND	ND
<i>Clavibacter</i> sp.	CASJ009	ND	ND	ND	ND
<i>C. insidiosus</i>	LMG 3663 <sup>T</sup>	1	2292719	2312719	Sactipeptides

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<i>C. nebraskensis</i>	NCPBPB 2581 <sup>T</sup>	1	2422475	2442475	Sactipeptides
<i>C. sepedonicus</i>	ATCC 33113 <sup>T</sup>	1	2663669	2683669	Sactipeptides
			3119744	3139861	294.1; Plantathiazolicin (Plantazolicin)
<i>C. tessellarius</i>	ATCC 33566 <sup>T</sup>	1	1820699	1840699	Sactipeptides

*C. m.*: *Clavibacter michiganensis* subsp.

ND: Not detected

T: Type strain

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