Seed-associated subspecies of the genus *Clavibacter* are clearly distinguishable from *Clavibacter michiganensis* subsp. *michiganensis*

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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* subsp. *michiganensis* causing bacterial canker of tomato. *Clavibacter michiganensis* subsp. *michiganensis* is often spread through contaminated seed leading to outbreaks of bacterial canker in tomato production areas worldwide. The frequent occurrence of non-pathogenic *Clavibacter michiganensis* subsp. *michiganensis*-like bacteria (CMB) is a concern for seed producers because *Clavibacter michiganensis* subsp. *michiganensis* 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 CMB strains was performed using standard biochemical tests, cell wall analyses, metabolic profiling using Biolog, and single-gene and multilocus sequence analyses. Combined, these tests revealed two distinct populations of seed-associated members of the genus *Clavibacter* that differed from each other, as well as from all other described subspecies of *Clavibacter michiganensis*. 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, strains belonging to the genus *Clavibacter* 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 non-pathogenic subpopulations of *Clavibacter michiganensis*: *Clavibacter michiganensis* subsp. *californiensis* subsp. nov. and *Clavibacter michiganensis* subsp. *chilensis* subsp. nov. for seed-associated strains represented by C55⁴ (ATCC BAA-2691⁴ = CFPB 8216⁴) and ZUM3936⁴ (ATCC BAA-2690⁴ = CFPB 8217⁴), respectively. Recognition of separate subspecies is essential for improved international seed testing operations.

Abbreviations: CMB, *Clavibacter michiganensis* subsp. *michiganensis*-like bacteria; ddH₂O, double-distilled H₂O; LAMP, loop-mediated amplification; ML, maximum-likelihood; MLSA, multi-locus sequence analysis; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the gene sequences of CMB strains C55⁴, C59-A, C63, C6-A, C74-A, C91-A, *Clavibacter michiganensis* subsp. *insidiosus* strain A114⁹, *Clavibacter michiganensis* subsp. *michiganensis* strain K73, *Clavibacter michiganensis* subsp. *nebraskensis* strain NCPPB 2579, *Clavibacter michiganensis* subsp. *sepedonicus* strain A204¹ and *Clavibacter michiganensis* subsp. *tessellarius* strain LMG 729⁴¹, *Clavibacter* strains PF007, PF008, PS003, PS005 and PS006 and CMB strains ZUM3064, ZUM3065, ZUM3936⁶, ZUM4206, ZUM4207, ZUM4209, ZUM4210 and ZUM4211 are KF663873–KF663896 (atpD), KF663907–KF663930 (dnaK), KF663931–KF663954 (gyrB), KF663955–KF663978 (ppK), KF663979–KF664002 (recA) and KF664003–KF664026 (rpoB), respectively. The accession numbers for the 16S rRNA gene sequences of strains C55⁴ and ZUM3936⁶ are KF663871 and KF663872. Those for the dnaA gene sequences of *Clavibacter michiganensis* subsp. *nebraskensis* strains 20037, 200800460 and NCPPB 2579, and strains A2041, LMG 729⁴¹, PF007, PF008, PS003, PS005 and PS006 are KF663897–KF663906, respectively.

Six supplementary tables and two supplementary figures are available with the online Supplementary Material.
The genus *Clavibacter*, which belongs to the plant pathogenic actinomycetes (family *Microbacteriaceae*) (Stackebrandt et al., 1997), was first defined by Davis et al. (1984). Members of the genus *Clavibacter* are Gram-positive, aerobic, non-spore-forming, coryneform bacteria that were previously grouped within the genus *Corynebacterium* (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 the genus *Clavibacter*, *Clavibacter 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). Subspecies of the genus *Clavibacter* are generally pigmented yellow to orange, with the exception of *Clavibacter michiganensis* subsp. *sepedonicus*, which is non-pigmented. The known subspecies of the genus *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 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* subsp. *michiganensis* (Davis et al., 1984; Strider, 1969) and is considered one of the most significant bacterial diseases of tomato (Strider, 1969). *Clavibacter michiganensis* subsp. *sepedonicus* is the causal agent of ring rot of potato (*Solanum tuberosum*) (Manzer & Generex, 1981). *Clavibacter michiganensis* subsp. *insidiosus* causes wilting and stunting in alfalfa (*Medicago sativa*) (McCulloch, 1925). The remaining two subspecies infect monocotyledonous plants, with *Clavibacter michiganensis* subsp. *nebraskensis* causing wilt and blight of maize (*Zea mays*) (Schuster, 1975; Vidaver & Mandel, 1974) and *Clavibacter michiganensis* subsp. *tessellarius* causing leaf freckles and leaf spots in wheat (*Triticum aestivum*) (Carlson & Vidaver, 1982a, b). Recently, a new subspecies was described (González & 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, strains representing the genus *Clavibacter* have been 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, *Clavibacter michiganensis* subsp. *michiganensis* is classified as a quarantine organism in many countries, with cuttings and seeds of tomato requiring certification as *Clavibacter michiganensis* subsp. *michiganensis*-free. The European Plant Protection Organization (EPPO) has released minimal standards for the identification of *Clavibacter michiganensis* subsp. *michiganensis* in plants and seeds (EPPO, 2013). Immunodiagnostic and molecular methods based on primers developed from rRNA genes, repetitive sequences or known virulence genes are available to confirm the identity of *Clavibacter michiganensis* subsp. *michiganensis*, 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 CMB. These bacteria are Gram-positive, coryneform and pigmented, but non-pathogenic on tomato (Jacques et al., 2012; Kaneshiro et al., 2006; Nazina et al., 2002; Yasuhara-Bell et al., 2013; Zaluga et al., 2011, 2013b; Zinniel et al., 2002). CMB do not appear to colonize vascular tissues of tomato (Zaluga et al., 2013b) and do not induce a hypersensitivity reaction in *Nicotiana tabacum* or *Nicotiana benthamiana* (Jacques et al., 2012), as well as Mirabilis jalapa (Zaluga et al., 2014). These bacteria react to tests designed to detect *Clavibacter michiganensis* subsp. *michiganensis* such as the standard field test, the Agdia ImmunoStrip, leading to false diagnosis of *Clavibacter michiganensis* subsp. *michiganensis* infection (Jacques et al., 2012; Kaneshiro & Alvarez, 2001; Kaneshiro et al., 2006; Yasuhara-Bell et al., 2013; Zaluga et al., 2011). Subsequently, a loop-mediated amplification (LAMP) reaction was designed to specifically detect *Clavibacter michiganensis* subsp. *michiganensis* strains, and distinguish them from these seed-associated CMB (Yasuhara-Bell & Alvarez, 2012, 2015; Yasuhara-Bell et al., 2013). Additionally, PCR primers were designed to react only with *Clavibacter michiganensis* subsp. *michiganensis* and not with the seed-associated CMB (Yasuhara-Bell et al., 2014).

PCR primers designed for specific pathogenicity-associated genes of *Clavibacter michiganensis* subsp. *michiganensis* revealed that the CMB lacked the *ppal*, *pat-1*, *chpC*, *tomA*, *ppa* and *ppxC* genes (Jacques et al., 2012; Yasuhara-Bell et al., 2013). In previous studies utilizing replication initiation factor (RIF) marker sequence analysis of *dnaA* (Schneider et al., 2011), these non-pathogenic CMB strains formed a clade separate from all other associated subspecies of the genus *Clavibacter* (Yasuhara-Bell et al., 2013; Zaluga et al., 2013b). Similar findings were reported in studies using *gyrB* analysis (Zaluga et al., 2011, 2013b), while another used multilocus sequence analysis of six housekeeping genes (*atpD*, *dnaK*, *gyrB*, *ppK*, *recA* and *rpoB*) (Jacques et al., 2012). Together, these data suggest that the seed-associated members of the genus *Clavibacter* potentially warrant at least a novel subspecies designation.

Reports concerning these CMB are lacking and biological information regarding these strains is limited. A recent draft genome of CMB strain LMG 26808 (Zaluga et al., 2014)
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 CMB strains.

A virulent strain of *Clavibacter michiganensis* subsp. *michiganensis* (K73=H-160) previously determined to contain all genes necessary for pathogenicity (Yasuhara-Bell et al., 2013), *Clavibacter michiganensis* subsp. *insidiosus* (K0091=T=ATCC 10253), *Clavibacter michiganensis* subsp. *sepedonicus* (A2041=R8) and the seed-associated CMB strains C55T, C59-A, C63, C6-A, C74-A and C91-A were from the Pacific Bacterial Collection at the University of Hawai‘i at Mānoa, HI, USA. *Clavibacter michiganensis* subsp. *negraskensis* strains NCPPB 2579 (=LMG 3698), 20037 and 20080460 were from Anne Vidaver at the University of Nebraska, Lincoln, NE, USA. A strain of *Clavibacter michiganensis* subsp. *tessellarius* (LMG 7294=T=ATCC 33566) and the seed-associated CMB strains ZUM3064, ZUM3065, ZUM3936, ZUM4206, ZUM4207, ZUM4209, ZUM4210 and ZUM4211 were provided by Bert Woudt at Syngenta Seeds (Enkhuizen, the Netherlands). *Clavibacter* pepper strains PS003, PS005, PS006, PF007 and PF008 were provided by Kyu-Ock Yim and Hyok-In Lee at the National Plant Quarantine Service of Korea, and Jae-Soo Cha at Chungbuk National University, Korea. Strains were removed from −80 °C, plated onto TZC-S medium [17 g agar l⁻¹, 10 g peptone l⁻¹, 5 g sucrose l⁻¹ and 0.001 % 2,3,5-triphenyl-tetrazolium chloride (TZO)] and then incubated at 26 °C (±2 °C). Isolated colonies were streaked onto YSC medium (17 g agar l⁻¹, 10 g yeast extract l⁻¹, 20 g sucrose l⁻¹ and 20 g CaCO₃ l⁻¹) and incubated at 26 °C (±2 °C).

Medium 6 (15 g agar l⁻¹, 5 g peptone l⁻¹, 5 g yeast extract l⁻¹, 10 g glucose l⁻¹), CNS medium [Gross & Vidaver, 1979; 15 g agar l⁻¹, 8 g dehydrated nutrient broth (Difco) l⁻¹, 2 g yeast extract l⁻¹, 5 g glucose l⁻¹, 2 g K₂HPO₄ l⁻¹, 0.5 g KH₂PO₄ l⁻¹, 247 mg MgSO₄·7H₂O l⁻¹, 25 mg naldixic acid l⁻¹, 32 mg polymyxin B sulfate l⁻¹ and 200 mg cycloheximide l⁻¹] CMM1 medium (Alvarez & Kaneshiro, 1999; Alvarez et al., 2005; Kaneshiro et al., 2006; Koenraadt et al., 2009; 15 g agar l⁻¹, 2 g yeast extract l⁻¹, 10 g sucrose l⁻¹, 1.2 g Tris base l⁻¹, 250 mg MgSO₄·7H₂O l⁻¹, 5 g lithium chloride l⁻¹, 1 g NH₄Cl l⁻¹, 4 g casein acid hydrolysate l⁻¹, 28 mg naldixic acid l⁻¹, 10 mg polymyxin B sulfate l⁻¹ and 200 mg cycloheximide l⁻¹] and selective BCT medium [Fukay et al., 2011; 15 g agar l⁻¹, 2.5 g mannositol l⁻¹, 2 g yeast extract l⁻¹, 2 g K₂HPO₄ l⁻¹, 0.5 mg KH₂PO₄ l⁻¹, 0.5 g NaCl l⁻¹, 0.1 g MgSO₄·7H₂O l⁻¹, 0.015 mg MnSO₄·H₂O l⁻¹, 0.015 g FeSO₄·7H₂O l⁻¹, 0.6 g H₃BO₃ l⁻¹, 20 mg naldixic acid l⁻¹, 100 mg trimethoprim l⁻¹, 20 mg polymyxin B sulfate l⁻¹ and 1 ml 5 % Opus Top (BASF l⁻¹)] were used for strain characterization based on the ability of the strains to grow on these media. Growth on media was assessed by spot plating ~10⁴ cells, in triplicate, of each strain on each medium, with incubation at 26 °C (±2 °C) for 7 days.

Colony colour and morphology was observed, as well as cell morphology following Gram staining. 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, Braunschweig, Germany). DNA–DNA hybridization and DNA G+C content analyses were performed by the Identification Service of the BCCM/LMG (Gent, Belgium). A variety of standard biochemical tests were performed to determine starch, casein and ascelin hydrolysis, H₂S production from peptone, and acid production from carbon sources. Methyl red and oxidase tests were performed to determine the ability to perform mixed-acid fermentation and the presence of oxidase, respectively. The GEN III MicroPlate (Biolog) was used, according to the manufacturer’s instructions, to determine carbon source utilization and chemical sensitivity. API Coryne and API ZYM test strips (bioMérieux) were used to determine the fermentation of carbohydrates and enzymatic activities, respectively.

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) in 1 × TE buffer (10 mM Tris/HCl and 1 mM EDTA at pH 8) with 10 % Triton X-100 (Sigma-Aldrich) was added to each tube. Samples were mixed with vigorous pipetting 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.

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) 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 *Clavibacter michiganensis* subsp. *michiganensis* strain K73 DNA was used as the positive control.

PCR amplifications were carried out using primer pairs designed to amplify dnaA (Schneider et al., 2011), partial 16S rRNA (Weisburg et al., 1991), atpD, dnaK, gyrB, ppK, recA (Jacques et al., 2012), rpoB, clVA, clVF and clVG (Yasuhara-Bell et al., 2014) gene sequences (Table 1). The rpoB primers used in this study were the reverse complements to those previously published by Jacques et al. (2012). PCRs for all primers were performed in a 10 μl reaction volume containing 1 μl bacterial DNA and 9 μl PCR master mix [0.5 μl of each primer (10 μM), 5.0 μl JumpStart REDTaq ReadyMix (cat. no. P1107; Sigma-Aldrich) and 3 μl double-distilled H₂O (ddH₂O)]. PCR conditions for all primers used are listed in Table 1. PCR products were resolved using 1.5 % agarose gel electrophoresis. Gels were analysed using the Foto/Analyst Express System (Fotodyne).

PCR products were cleaned for sequencing using ExoSAP-IT (cat. no. 78200; Affymetrix), 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.

Sequence alignment and phylogenetic analyses were performed using MEGA5 version 5.05 (Tamura et al., 2011). The dnaA sequence data for Clavibacter michiganensis subsp. michiganensis strains NCPPB 382, K0074, K0079, K84, K87, K0428, K0448, K0449, K0465 and K0469, Clavibacter michiganensis subsp. insidiosus strain K0091 (=ATCC 10253), Clavibacter michiganensis subsp. sepedonicus strain K0909 (=ATCC 9850), CMB strains C55T, C59-A, C63, C6-A, C74A, C91-A, X01 (=ZUM3064), X02 (=ZUM3065), X05T (=ZUM3936T), X06 (=ZUM4209), X07 (=ZUM4210), X09 (=ZUM4211), X11 (=ZUM4206) and X12 (=ZUM4207), CMB strains AFLP 50, AFLP 121, AFLP 173, NCB 4040, NCB 4041, NCB 4042, NCB 4043, NCB 4228 and NCB 4229, and out-group Aquifex aeolicus VF5 were obtained from NCBI GenBank. Sequences were trimmed to 661 nt and multiple alignments were made using CLUSTAL W, taking into account GenBank. The model of evolution for maximum-likelihood analysis was determined using Modeltest 3.7 in PAUP* (Swofford, 2000). The model of evolution for maximum-likelihood neighbour interchange at search level 3 (Nei & Kumar, 2000) was reconstructed using the Jukes–Cantor method (Jukes & Kumar, 1969) and NJ trees (Saitou & Nei, 1987) were concatenated and NJ trees (Saitou & Nei, 1987) were reconstructed using the Jukes–Cantor method (Jukes & Kumar, 1969) to compute evolutionary distances. Maximum-parsimony (MP) analysis was used to reconstruct trees with the closest neighbour 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 Clavibacter michiganensis subsp. michiganensis, Clavibacter michiganensis subsp. insidiosus, Clavibacter michiganensis subsp. nebraskensis, Clavibacter michiganensis subsp. sepedonicus, Clavibacter michiganensis subsp. tesselarius and CMB strains, and out-group Rathayibacter iranicus were obtained from NCBI GenBank (Table S1, available in the online Supplementary Material). 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 CLUSTAL W, taking into account the corresponding amino acid alignments for protein-coding genes. Neighbour-joining (NJ) trees (Saitou & Nei, 1987) were reconstructed using the Jukes–Cantor method (Jukes & Cantor, 1969) to compute evolutionary distances. Maximum-parsimony (MP) analysis was used to reconstruct trees with the closest neighbour interchange at search level 3 (Nei & Kumar, 2000). Confidence intervals were assessed using the bootstrap method with 1000 replications (Felsenstein, 1985).

Multi-locus sequence analysis (MLSA) of housekeeping genes was performed according to previously published methods (Jacques et al., 2012). Briefly, sequences were concatenated and NJ trees (Saitou & Nei, 1987) were reconstructed using the Jukes–Cantor method (Jukes & Cantor, 1969) to compute evolutionary distances. MP analysis was used to reconstruct trees with the closest neighbour 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 et al., 2013a). 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/phylml/. 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 1000 replications (Felsenstein, 1985).

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 (approx. 1 mm) on the stem of juvenile tomato (S. lycopersicum ‘Kewalo’), chilli pepper (Capsicum annuum ‘Jalapeños’ and ‘Pasilla Bajo’) and hybrid cabbage [Brassica oleracea (capitata group) ‘Early Green farao’ f1) plants that were approximately 3–4 weeks old. Bacteria (~10^9 c.f.u. ml^-1) were applied to the wound, using the scalpel tip. Negative controls were made by applying approximately 500 μl ddH₂O to the wound. Inoculated plants and controls were then placed carefully 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 weeks in a growth room (30 °C) under Philips F40/ AGRO Agri-Lite fluorescent lamps (cat. no. 392287). Plants were checked periodically for symptoms and pathogenicity was determined at 3–4 weeks post-inoculation, relative to controls. Two plants were used per test for each strain, with the entire set of tests being repeated three times.

Conventional bacteriological tests were performed on two different CMB bacterial strains (C55T and ZUM3936T) that were isolated from tomato seed (California and Chile, respectively). A summary of these results can be found in Tables 2 and 3. Both of these strains have mucoid colony morphology similar to Clavibacter michiganensis subsp. michiganensis. Colonies of C55T appeared to be yellow–orange in colour, and produced larger amounts of exopolysaccharide, making them thick and gummy. Colonies of ZUM3936T were light to dark yellow and consistency was similar to that of Clavibacter michiganensis subsp. michiganensis. Both strains were Gram-stain-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 the genus Clavibacter. Acid production from various carbohydrates, utilization of nitrate/nitrite, hydrolysis of casein and aesculin, and activity of certain enzymes have been used to differentiate between genera of the family Microbacteriaceae (Table 2). 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 members of the genus Clavibacter are rhamnose, galactose and mannose. Strain C55T had the characteristic rhamnose, galactose and...
mannose, but also possessed fucose. In contrast, strain ZUM3936\textsuperscript{T} had galactose, mannose and ribose, but no rhamnose. These data together show that these two strains not only differ from known species of the genus Clavibacter, 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 & Kerr (2012), the major fatty acids possessed by members of the genus Clavibacter are straight-chain fatty acids dodecanoic (C\textsubscript{12} : 0) and hexadecanoic (anteiso-C\textsubscript{15} : 0) and 14-methylpentadecanoic (iso-C\textsubscript{16} : 0) acids. Significant amounts of octadecenoic acid (C\textsubscript{18} : 1\textsubscript{n}) are also found (Saddler & Kerr, 2012). Both C55\textsuperscript{T} and ZUM3936\textsuperscript{T} possessed the same major fatty acids, suggesting inclusion in the genus Clavibacter; however, they did not possess C\textsubscript{18} : 1. Additionally, Clavibacter michiganensis subsp. michiganensis possesses the saturated, straight-chain fatty acids dodecanoic (C\textsubscript{12} : 0) and hexadecanoic (C\textsubscript{16} : 0) acids in significant amounts (Saddler & Kerr, 2012). Both C55\textsuperscript{T} and ZUM3936\textsuperscript{T} possessed C\textsubscript{16} : 0; however, they did not possess C\textsubscript{12} : 0. Saddler & Kerr (2012) reported that the presence of the unsaturated, branched-chain acid 12-methyltetradecenoic acid (anteiso-C\textsubscript{15} : 1\textsubscript{n}) was considered diagnostic for Clavibacter michiganensis subsp. michiganensis. Interestingly, strain ZUM3936\textsuperscript{T} contained anteiso-C\textsubscript{15} : 1 in significant amounts, while strain C55\textsuperscript{T} did not. This would suggest that strain ZUM3936\textsuperscript{T} is more closely related to Clavibacter michiganensis subsp. michiganensis than is C55\textsuperscript{T}, which is also reinforced by ZUM3936\textsuperscript{T}’s more similar colony colour and morphology. However, as stated previously, primary cell-wall sugar analyses showed that strain C55\textsuperscript{T} has all the cell-wall sugars found in members of the genus Clavibacter, though it does contain an extra sugar not found in any species of the genus Clavibacter, while strain ZUM3936\textsuperscript{T} lacked one of the Clavibacter cell-wall sugars and contained one sugar not found in members of the genus Clavibacter (ribose). Additionally, phylogenetic analysis of the genus Clavibacter based on dnaA sequence analysis showed that C55\textsuperscript{T} grouped closer to Clavibacter michiganensis subsp. michiganensis strains (Yasuhara-Bell et al., 2013), again contradicting an identification based on the presence of anteiso-C\textsubscript{15} : 1 in strain ZUM3936\textsuperscript{T}. Nevertheless, all results presented to this point justify inclusion of strains C55\textsuperscript{T} and ZUM3936\textsuperscript{T} within the genus Clavibacter, and establish that these strains are different from Clavibacter michiganensis subsp. michiganensis, as well as each other.

Strains C55\textsuperscript{T} and ZUM3936\textsuperscript{T} are representatives of two separate populations representing the genus 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 3. These tests, along with all remaining tests in this study, also include a proposed Clavibacter michiganensis subsp. michiganensis strain (PS005) isolated from pepper (Capsicum annum) and characterized as a phenotypic variant of Clavibacter michiganensis subsp. michiganensis by Yim et al. (2012). Repetitive element (rep)-PCR and phylogenetic analyses based on 16S rRNA gene and internal transcribed spacer (ITS) sequences all showed that the pepper Clavibacter michiganensis subsp. michiganensis formed a clade separate from all known subspecies of Clavibacter michiganensis. Since their work clearly demonstrated that the pepper strains were not typical Clavibacter michiganensis subsp. michiganensis, but perhaps another unknown subspecies, the pepper strain PS005 was included in the current study.

Growth of all strains tested was assessed on two complex media and three selective media (data not shown), under the conditions of this study. All strains grew on both YSC and LMG medium-6 (Table 3). On YSC, strains C55\textsuperscript{T} and ZUM3936\textsuperscript{T} and Clavibacter michiganensis subsp. michiganensis K73 produced a yellow pigment, whereas strains PS005, Clavibacter michiganensis subsp. nebraskensis NCPPB 2579 and

<table>
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<th>Gene</th>
<th>Primers</th>
<th>Cycle conditions (35 cycles of)</th>
<th>Size (bp)</th>
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<td>16S rRNA</td>
<td>fD1/rP2</td>
<td>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</td>
<td>1500</td>
<td>Weisburg et al. (1991)</td>
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<tr>
<td>dnaA</td>
<td>dnaAF/R</td>
<td>94 °C for 5 min, (94 °C for 30 s, 61 °C for 1 min, 72 °C for 10 min)</td>
<td>933</td>
<td>Schneider et al. (2011)</td>
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<td>atpD</td>
<td>atpDF/R</td>
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<td>697</td>
<td>Jacques et al. (2012)</td>
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<td>gyrB</td>
<td>gyrBF/R</td>
<td></td>
<td>909</td>
<td></td>
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<tr>
<td>ppK</td>
<td>ppKF/R</td>
<td></td>
<td>604</td>
<td></td>
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<td>recA</td>
<td>recAF/R</td>
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<td>724</td>
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<td>rpoB*</td>
<td>rpoBF/R</td>
<td></td>
<td>662</td>
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<td>clvA</td>
<td>clvA-F/R</td>
<td>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</td>
<td>338</td>
<td>Yasuhara-Bell et al. (2013)</td>
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<td>clvF</td>
<td>clvF-F/R</td>
<td>72 °C for 1 min, 72 °C for 30 s</td>
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<td></td>
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<td>clvG</td>
<td>clvG-F/R</td>
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*Primers were made in this study from the reverse complement sequences published by Jacques et al. (2012).
Table 2. Characteristics differentiating plant-associated genera of the family *Microbacteriaceae*


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<tr>
<th>Characteristic</th>
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<td>Colony colour</td>
<td>OY</td>
<td>Y</td>
<td>O/Y</td>
<td>O/W/Y</td>
<td>I/OY</td>
<td>Y</td>
<td>I/R/Y/W</td>
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<td>Y</td>
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<td>Motility</td>
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<td>-</td>
<td>+</td>
<td>V</td>
<td>+</td>
<td>V</td>
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<td>DL-DAB</td>
<td>DL-DAB</td>
<td>l-DAB; D-Orn; Hyg</td>
<td>DL-DAB</td>
<td>D-Orn</td>
<td>l-Lys</td>
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<td>14-Methylhexadecanoic; 12-methyltetradecanoic; 14-methylpentadecanoic</td>
<td>14-Methylhexadecanoic; 12-methyltetradecanoic; 14-methylpentadecanoic</td>
<td>14-Methylhexadecanoic; 12-methyltetradecanoic; 14-methylpentadecanoic</td>
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<td>14-Methylhexadecanoic; 12-methyltetradecanoic; 14-methylpentadecanoic</td>
<td>14-Methylhexadecanoic; 12-methyltetradecanoic; 14-methylpentadecanoic</td>
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<td>PG; DPG; GL</td>
<td>PG; DPG; GL</td>
<td>PG; DPG; GL</td>
<td>PG; DPG; GL</td>
<td>PG; DPG; GL</td>
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<td>ND</td>
<td>SPM</td>
<td>SPD; SPM; (PUT; CAD)</td>
<td>SPD; SPM</td>
<td>SPD; SPM</td>
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<td>SPD; SPM; (PUT; CAD)</td>
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<td>MK-9</td>
<td>MK-10</td>
<td>MK-9</td>
<td>MK-9</td>
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<td>Primary cell-wall sugars</td>
<td>Rhamnose; galactose; mannosse; fucose</td>
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<td><em>myo</em>-Inositol</td>
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<td>-</td>
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<td>-/v</td>
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Table 2. cont.

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<td>Hydrolysis of casein</td>
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Clavibacter michiganensis subsp. tessellarius LMG 7294<sup>T</sup> were orange. Clavibacter michiganensis subsp. insidiosus A1149<sup>T</sup> was yellow–orange with indigoidine production that made it appear greenish and Clavibacter michiganensis subsp. sepedonicus 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 Clavibacter michiganensis subsp. insidiosus A1149<sup>T</sup> (greenish) when enough indigoidine was produced (5–7 days post-inoculation), as well as strain PS005 (orange) at ≥ 5 days post-inoculation. The fact that all strains were of similar colour when grown on medium-6 makes subspecies differentiation difficult based on appearance. Additionally, growth on this medium was not as robust as compared with YSC, with very weak growth observed for Clavibacter michiganensis subsp. sepedonicus. This confirms previous reports that subspecies of the genus Clavibacter grow better on sucrose than glucose (Chun, 1982; Kaneshiro, 2003; Kaneshiro et al., 2006). BCT inhibited growth of both Clavibacter michiganensis subsp. insidiosus and Clavibacter michiganensis subsp. sepedonicus, while strains of all other subspecies showed various levels of growth. Only growth of Clavibacter michiganensis subsp. sepedonicus was inhibited on CNS. Inhibition of Clavibacter michiganensis subsp. sepedonicus by CNS and BCT confirms data presented by Saddler & Kerr (2012) and Ftayeh et al. (2011), respectively. Growth of Clavibacter michiganensis subsp. insidiosus on CNS and inhibition of Clavibacter michiganensis subsp. insidiosus by BCT contradicts data reported by Saddler & Kerr (2012) and Ftayeh et al. (2011), respectively. Clavibacter michiganensis subsp. nebraskensis was pigmented orange on BCT, in contrast to data reported by Ftayeh et al. (2011). Strains C55<sup>T</sup> and ZUM3936<sup>T</sup> were yellow on BCT, while strain PS005 was orange. At an inoculum of 10<sup>4</sup> cells per spot, only Clavibacter michiganensis subsp. insidiosus, Clavibacter michiganensis subsp. tessellarius, C55<sup>T</sup> and ZUM3936<sup>T</sup> showed growth on CMM1 plates at 7 and 9 days post-inoculation, with Clavibacter michiganensis subsp. insidiosus and C55<sup>T</sup> showing very little growth, just enough to be seen. At a higher inoculum (~5 × 10<sup>5</sup> c.f.u. per spot), more robust growth was seen on all media (data not shown). Again, on CNS, Clavibacter michiganensis subsp. insidiosus growth was apparent, though very slow and far less than that observed for all other strains. Clavibacter michiganensis subsp. sepedonicus still showed no growth. BCT medium again inhibited growth of both Clavibacter michiganensis subsp. insidiosus and Clavibacter michiganensis subsp. sepedonicus when inoculated at the higher titre. All strains, except those of Clavibacter michiganensis subsp. sepedonicus, grew on CMM1 media when applied at higher titre and produced pigments with varying shades of yellow to yellow–orange. Strain ZUM3936<sup>T</sup> showed the most robust growth on CMM1, followed by Clavibacter michiganensis subsp. nebraskensis. All other strains tested grew more slowly.

To compare the results obtained from Clavibacter strains from tomato seed and from pepper to data presented by Saddler & Kerr (2012), strains were tested for their ability to produce acid aerobically from various carbohydrates,
hydrolyse gelatin and starch, produce mixed acid from glucose (methyl red test), and produce H₂S from peptone. As seen in Table 3, strains C55⁵, ZUM3936⁶ and PS005 were unable to produce acid aerobically from inulin, mannitol, mannose or sorbitol. Only strain PS005 hydrolysed gelatin. All three strains were negative for the methyl red test. In our study, Clavibacter michiganensis subsp. insidiosus was negative, and all other subspecies of Clavibacter michiganensis are variable in their ability to hydrolyse starch.

The Biolog system was used to determine the utilization of a vast array of carbon sources and chemical sensitivity (Table S2). The metabolic profiles obtained from the Biolog system are compared with those in a database (Table S3) in order to provide a bacterial identification. The Biolog program returned bacterial IDs of Clavibacter michiganensis subsp. insidiosus for the Clavibacter michiganensis subsp. michiganensis, Clavibacter michiganensis subsp. nebraskensis, Clavibacter michiganensis subsp. tessellarius, tomato-seed Clavibacter strains C55⁵ and ZUM3936⁶, and pepper Clavibacter strain PS005 used in this study. These bacterial IDs had an average similarity and probability index of 0.748 and 0.898, 0.564 and 0.668, 0.812 and 0.912, 0.605 and 0.698, 0.728 and 0.896, and 0.740 and 0.899 for Clavibacter michiganensis subsp. michiganensis, Clavibacter michiganensis subsp. nebraskensis,
Clavibacter michiganensis subsp. tessellarius, tomato seed Clavibacter strains C55T and ZUM3936T, and pepper Clavibacter strain PS005, respectively. These results are similar to those found previously (Harris-Baldwin & Gudmestad, 1996; Kaneshiro, 2003; Kaneshiro et al., 2006). Biolog gave an ID of Clavibacter michiganensis subsp. sepedonicus/Clavibacter michiganensis subsp. insidiosus for Clavibacter michiganensis subsp. sepedonicus and Clavibacter michiganensis subsp. sepedonicus for Clavibacter michiganensis subsp. sepedonicus. These bacterial IDs had an average similarity and probability index of 0.379 for Clavibacter michiganensis subsp. insidiosus (could not provide probability) and 0.559 and 0.697 for Clavibacter michiganensis subsp. sepedonicus, respectively. Only the Clavibacter michiganensis subsp. sepedonicus and Clavibacter michiganensis subsp. tessellarius strains were properly identified and Clavibacter michiganensis subsp. insidiosus was partially identified. The Biolog data for the known subspecies of Clavibacter michiganensis differed from data reported by Holt (2000) with respect to utilization of acetate, lactate, citrate, propionate and succinate. It is apparent that the Biolog database should be re-evaluated for plant-pathogenic bacteria, especially for the closely related subspecies of Clavibacter michiganensis. Regardless, the Biolog system did 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 S2. Tomato seed-associated Clavibacter strain C55T differed from Clavibacter michiganensis subsp. michiganensis 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, 2-ketoglutaric acid, D-malic acid, bromosuccinic acid, Tween 40 and propionic acid, and differed in chemical sensitivity to guanidine hydrochloride. These differences reflect strain C55T’s inability to utilize these carbon sources, whereas strain K73 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 ZUM3936T differed from Clavibacter michiganensis subsp. michiganensis strain K73 by its ability to utilize N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, L-rhamnose, methyl pyruvate, L-lactic acid, and α-ketobutyric acid, whereas strain K73 could not utilize these carbon sources. 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 ZUM3936T differed from Clavibacter michiganensis subsp. michiganensis 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, D-malic acid, propionic acid, or acetic acid. Strain ZUM3936T differed from strain K73 in chemical sensitivity to 8% NaCl, guanidine hydrochloride 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 differed from Clavibacter michiganensis subsp. michiganensis strain K73 in that it could not use D-fucose, L-fucose, glucuronamide, quinic acid, D-lactic acid methyl ester, D-malic acid, or propionic acid. Pepper strain PS005 differed from strain K73 in that it was able to use methyl pyruvate, gelatin and glycyrl-L-proline. Strain PS005 also differed from strain K73 in chemical sensitivity to 8% NaCl, guanidine hydrochloride and sodium butyrate. Strains C55T, ZUM3936T and PS005 all differed from Clavibacter michiganensis subsp. michiganensis as well as from each other. It should be noted that Clavibacter michiganensis subsp. michiganensis 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 pepper strain was most similar to Clavibacter michiganensis subsp. tessellarius on YSC medium. Pepper strain PS005 differed from Clavibacter michiganensis subsp. tessellarius strain LMG 7294T in that it could not use α-hydroxybutyric acid, β-hydroxy DL-butyr acid, α-ketobutyric acid or propionic acid, and that it utilized D-glucose 6-phosphate, D-fructose 6-phosphate, glycyrl-L-proline, L-aspartic acid, mucic acid, methyl pyruvate and 2-ketoglutaric acid. Strain PS005 did not differ from Clavibacter michiganensis subsp. tessellarius in its chemical sensitivity.

The API Coryne and API ZYM test strips were used to determine enzymic activities and fermentation of carbohydrates. All strains tested showed similar profiles by both the API Coryne (Table S4) and API ZYM tests (Table S5). All strains showed alkaline phosphatase, β-galactosidase, α-glucosidase, β-glucosidase and catalase activity on the API Coryne test strip (Table S4). Only strain PS005 hydrolysed gelatin (Table S4). Strain PS005’s ability to hydrolyse gelatin confirms the Biolog data (Table S2). Clavibacter michiganensis subsp. tessellarius showed weakly positive reactions with gelatin in the Biolog test (Table S2) but was negative for gelatin hydrolysis via API (Table S4), which is supported by data from Holt (2000). Both Biolog and API suggest that Clavibacter michiganensis subsp. michiganensis cannot hydrolyse gelatin, which contradicts data reported by Holt (2000). The API ZYM test also contained alkaline phosphatase, β-galactosidase, α-glucosidase, β-glucosidase and α-glucuronidase and results were consistent with API Coryne (Table S5). All strains had reactions for esterase (C4), esterase lipase (C8), leucine aryamidase and acid phosphatase. Clavibacter michiganensis subsp. insidiosus A1149T and Clavibacter michiganensis subsp. sepedonicus A2041 were the only strains negative for α-glucosidase while Clavibacter michiganensis subsp. insidiosus A1149T was the only strain positive for α-mannosidase. Strain ZUM3936T, Clavibacter michiganensis subsp. insidiosus A1149T and Clavibacter michiganensis subsp. michiganensis K73 were negative for naphthol-AS-BI-phosphohydrolyase, while all other strains had weakly positive reactions. These API tests have a reference database for strain identification; however, one does not exist for plant-associated
members of the genus *Clavibacter*. The results of the API ZYM tests in this study are highly similar to those presented previously by Palomo *et al.* (2006). These tests, like the Biolog tests, are aimed at identification of medically relevant bacteria. Results show that subspecies of *Clavibacter michiganensis* produce highly similar profiles, which 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.* (2006), could be used to create a reference database that could allow for identification of plant-associated members of the genus *Clavibacter*. As *Clavibacter* strains generally grow slowly, an alternative protocol would most likely be needed for identification of these bacteria using the API test.

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 *et al.*, 2010). The DNA G+C content for strains C55T and ZUM3936T were 72.6 % and 73.6 %, respectively. The DNA G+C content for these strains is similar to those of other subspecies of *Clavibacter michiganensis* (Table S6). The DNA G+C contents of strains C55T and ZUM3936T show less than 3 % difference, the generally accepted DNA G+C content range within a well-defined species (Vandamme *et al.*, 1996), from that of the type strain of *Clavibacter michiganensis* (73 %) reported by Sasaki *et al.* (1998).

DNA–DNA hybridization was performed on strains C55T and ZUM3936T 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 C55T and ZUM3936T were hybridized to each other, as well as to the *Clavibacter michiganensis* subsp. *michiganensis* type strain LMG 7333T. The hybridization values for C55T/ZUM3936T, C55T/LMG 7333T and ZUM3936T/LMG 7333T were 69 %, 73 % and 70 %, respectively. From these results, it can be concluded that strains C55T and ZUM3936T belong to the species *Clavibacter michiganensis*, as 70 % DNA–DNA relatedness 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 *Clavibacter michiganensis*, as high hybridization values are obtained amongst the different subspecies (unpublished data, personal communication from S. Van Trappen, BCCM/LMG, Belgium). Therefore, these strains were not tested against type strains of the other four known subspecies of *Clavibacter michiganensis*. Thus, while hybridization values suggest that these strains do not represent a novel species of the genus *Clavibacter*, it is still likely that these two strains belong to two novel subspecies.

PCR amplifications of the clvA, clvF and clvG genes produced bands of corresponding product size only for *Clavibacter michiganensis* subsp. *michiganensis* 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, 2014) that the clvA (formerly micA) gene, and associated clvF and clvG genes are highly specific for *Clavibacter michiganensis* subsp. *michiganensis*. A LAMP assay was performed using previously reported primers (Yasuhara-Bell *et al.*, 2013) and a master mix containing a novel polymerase. Again, only *Clavibacter michiganensis* subsp. *michiganensis* reacted with the LAMP reaction. This also further reinforces the utility of the clvA LAMP (Yasuhara-Bell & Alvarez, 2012, 2015; Yasuhara-Bell *et al.*, 2013) to discriminate all non-*Clavibacter michiganensis* subsp. *michiganensis* strains, including the most recently described *Clavibacter michiganensis* subsp. *michiganensis* variant from pepper that was isolated by Yim *et al.* (2012).

Analyses of the dnaA gene combined data from previously published results by Yasuhara-Bell *et al.* (2013) and Zaluga *et al.* (2013b) with new data acquired for strains tested in this study. The resulting MP tree (Fig. 1) shows congruent topology to trees presented by Yasuhara-Bell *et al.* (2013) and Zaluga *et al.* (2013b). CMB strain AFLP 50 grouped with tomato seed-associated *Clavibacter* strains from California. These strains formed a separate clade that was closest to *Clavibacter michiganensis* subsp. *michiganensis*. Note that strain LMG 26808 grouped more closely with the tomato seed-associated *Clavibacter* strains from California than with those from Chile/India. *Clavibacter* strains from pepper formed a separate clade and CMB strain AFLP 121 was grouped closely with these. The pepper strain clade was close to another clade formed by tomato seed-associated *Clavibacter* strains from Chile/India and the majority of the CMB strains presented by Zaluga *et al.* (2013b). Nj analysis produced consensus trees that were topologically congruent with that produced by MP analysis (Fig. S1).

MLSA analyses of concatenated housekeeping genes atpD, dnaK, gyrB, ppK, recA and rpoB was performed using data previously reported by Jacques *et al.* (2012) combined with data acquired in this study. MP analysis produced a tree with congruent topology to that produced previously (Jacques *et al.*, 2012) (Fig. 2). Nj and ML analysis of concatenated sequences (Fig. S2) produced consensus trees with congruent topology to that produced by MP analysis, as well as to each other and to ML trees produced previously (Jacques *et al.*, 2012). Again, tomato seed-associated *Clavibacter* strains from California and Chile/India, as well as...
Fig. 1. Phylogenetic analysis of dnaA sequences. MP trees were based on partial dnaA sequences of 59 Clavibacter strains and rooted using Aquifex aeolicus (accession AE000657.1) as an outgroup. Sequences were aligned and trimmed to 661 nt. Bootstrap values were generated from 1000 replications and bootstrap values of 50% or more are shown to the left of corresponding nodes.
Fig. 2. MLSA of concatenated atpD, dnaK, gyrB, ppk, recA and rpoB gene sequences. MP trees were based on sequences of 61 Clavibacter strains and rooted using sequences obtained from Rathayibacter iranicus CFBP 807 (Table S1) as an outgroup. Bootstrap values were generated from 1000 replications and bootstrap values of 50% or more are shown to the left of corresponding nodes.
Clavibacter strains from pepper, formed clades separate from all known subspecies of the genus Clavibacter and each other. The clade formed by tomato seed-associated Clavibacter strains from California was closest to Clavibacter michiganensis subsp. michiganensis. Tomato seed-associated Clavibacter strains from Chile/India grouped with the majority of the CMB strains presented by Jacques et al. (2012) to form its clade. Clavibacter strains from pepper formed a separate clade and CMB strain CFBP 7576 was grouped closely. Results of phylogenetic analyses show that strains C55T, ZUM3936T and PS005 are not Clavibacter michiganensis subsp. michiganensis and represent individually unique subspecies.

The positive control strain of Clavibacter michiganensis subsp. michiganensis (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 chilli pepper cultivars. These results may simply reflect the mode of inoculation used in this study, as different pathogens have varying 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 CMB strains appear to be non-pathogens on the hosts tested, especially on tomato with which they are found associated 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.

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; Zaluaga et al., 2013b), make it apparent that the Clavibacter strains from tomato seed (C55T and ZUM3936T) and pepper represent 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 C55T were all isolated from seed produced in California; therefore, we propose Clavibacter michiganensis subsp. californiensis nov. for strains represented by strain C55T. Strain ZUM3936T was isolated from seed produced in Chile, and strains represented by ZUM3936T 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 nov. for strains represented by ZUM3936T. Strains of Clavibacter isolated from pepper, and represented by strain PS005, should also be given a separate subspecies designation, as evidence suggests that they represent a novel pathogenic subspecies. Further studies on these strains are currently under way in Korea (personal communication from Kyu-Ock Yim, National Plant Quarantine Service of Korea, and Jae-Soon Cha, Chungbuk National University, Korea). Strains C55T and ZUM3936T were isolated from tomato seed and may have arisen from Clavibacter michiganensis subsp. michiganensis (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 Clavibacter michiganensis subsp. tessellarius. Further pathogenicity testing on a broader range of hosts, as well as whole-genome comparisons, will elucidate the lineage of these strains. Additionally, due to the fact that the tomato seed-associated strains look like Clavibacter michiganensis subsp. michiganensis 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 under way.

**Description of Clavibacter michiganensis subsp. californiensis subsp. nov.**

Clavibacter michiganensis subsp. californiensis (ca.li.for.mi.en’ sis. N.L. masc. adj. californiensis pertaining to California, referring to the location of isolation of the type strain).

Cells are Gram-stain-positive, aerobic, coryneform and non-motile. Colonies are yellow–orange, round and mucoid, with a thick and gummy consistency. Differential characteristics include presence of fucose as a primary cell-wall sugar and the absence of major fatty acids C18:1, C12:0 and anteiso-C15:1. Growth can occur on YSC, YDC, medium-6, CNS, CMM1 and BCT media and sucrose appears to be the preferred carbon source. The bacterium is oxidative on Ayers’ mineral medium supplemented with glucose and glycerol, positive for catalase and asacilin and starch hydrolysis, but negative for oxidase and casein hydrolysis. Can utilize acetate, dextrin, maltose, trehalose, cellobiose, gentiobiase, sucrose, turanose, stachyose, raffinose, α-lactose, melibiose, methyl β-D-glucoside, D-salicyl, α-D-glucoside, D-mannose, D-fructose, D-galactose, D-fucose, inosine, D-sorbitol, D-mannitol, myo-inositol, glycerol, L-alanine, L-aspartic acid, L-glutamic acid, pectin, D-glucuronamide, quinic acid, L-malic acid, acetoacetic acid and acetic acid; shows chemical sensitivity to 8% NaCl, fusidic acid, D-serine, treoleandomycin, rifamycin SV, minocycline, lincomycin, guanidine hydrochloride, niapropofor 4, vancomycin, tetrathion violet, tetrathion blue and sodium butyrate; has alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase and β-glucosidase activities.

The type strain is C55T (=ATCC BAA-2691T=CFBP 8216T).

**Description of Clavibacter michiganensis subsp. chilensis subsp. nov.**

Clavibacter michiganensis subsp. chilensis (chil.en’sis. N.L. masc. adj. chilensis pertaining to Chile, referring to the location of isolation of the type strain).

Cells are Gram-stain-positive, aerobic, coryneform and non-motile. Colonies are light to dark yellow, round and mucoid, with a consistency similar to that of Clavibacter
michiganensis subsp. michiganensis. Differential characteristics include the presence of ribose and lack of rhamnose as a primary cell-wall sugar, and the absence of major fatty acids C<sub>18:1</sub>, C<sub>12:0</sub> and anteiso-C<sub>15:1</sub>. Growth can occur on YSC, YDC, medium-6, CNS, CMM1 and BCT media and sucrose appears to be the preferred carbon source. The bacterium is oxidative on Ayers’ mineral medium supplemented with glucose and glycerol, positive for catalase and starch hydrolysis, but negative for oxidase and casein hydrolysis. Can utilize citrate, lactate, succinate, dextrin, maltose, trehalose, cellobiose, gentiobiose, sucrose, turanose, stachyose, raffinose, α-lactose, melibiose, methyl β-D-glucoside, D-salicin, N-acetyl-D-glucosamine, α-D-glucose, D-mannose, D-fructose, D-galactose, L-rhamnose, inosine, D-sorbitol, D-mannitol, myo-inositol, glycerol, L-alanine, L-aspartic acid, L-glutamic acid, L-serine, pectin, D-gluconic acid, methyl pyruvate, L-lactic acid, L-citrlic acid, α-ketoglutaric acid, L-malic acid, bomosuccinic acid, Tween 40, α-ketobytyric acid and acetoacetic acid; shows chemical sensitivity to fusidic acid, D-serine, troleandomycin, rifamycin SV, minocycline, lincomycin, guanidine hydrochloride, Niaproat 4, vancomycin, tetrazolium violet, hydrochloride, Niaproof 4, vancomycin, tetrazolium violet; has alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, α-galactosidase, β-galactosidase, and β-glucosidase activities.

The type strain is ZUM3936<sup>T</sup> (=ATCC BAA-2690<sup>T</sup> = CFBP 8217<sup>T</sup>).

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