Re-classification of Clavibacter michiganensis subspecies on the basis of whole-genome and multi-locus sequence analyses

Xiang Li,1,* James Tambong,2 Kat (Xiaoli) Yuan,1 Wen Chen,2 Huimin Xu,1 C. André Lévesque2 and Solke H. De Boer1

Abstract

Although the genus Clavibacter was originally proposed to accommodate all phytopathogenic coryneform bacteria containing B2y dianaminobutyrate in the peptidoglycan, reclassification of all but one species into other genera has resulted in the current monospecific status of the genus. The single species in the genus, Clavibacter michiganensis, has multiple subspecies, which are all highly host-specific plant pathogens. Whole genome analysis based on average nucleotide identity and digital DNA–DNA hybridization as well as multi-locus sequence analysis (MLSA) of seven housekeeping genes support raising each of the C. michiganensis subspecies to species status. On the basis of whole genome and MLSA data, we propose the establishment of two new species and three new combinations: Clavibacter capsici sp. nov., comb. nov. and Clavibacter tessellarius sp. nov., comb. nov., and Clavibacter insidiosus comb. nov., Clavibacter nebraskensis comb. nov. and Clavibacter sepedonicus comb. nov.

TAXONOMIC DESCRIPTION

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The genus Clavibacter was originally proposed by Davis et al. [1] to accommodate all phytopathogenic coryneform bacteria containing B2y dianaminobutyrate in the peptidoglycan. This genus originally included six plant pathogenic species: Clavibacter michiganensis, Clavibacter iranicum, Clavibacter rathayi, Clavibacter toxicus, Clavibacter tritici and Clavibacter xyli. Subsequently, the grass-specific pathogens, C. iranicum, C. rathayi, C. toxicus and C. tritici, were reclassified into the genus Rathayibacter on the basis of DNA–DNA hybridization and their unique menaquinone structures [2]. The two subspecies of C. xyli were placed in the genus, Leifsonia [3, 4]. Currently, the genus Clavibacter consists of only one species, C. michiganensis, which is subdivided into seven subspecies of plant pathogenic bacteria with narrow host specificities and two subspecies with close association with tomato and pepper seeds. Five of the subspecies comprise well-known pathogens, namely, C. michiganensis subsp. michiganensis (Cmm; bacterial canker and wilt of tomato), C. michiganensis subsp. sepedonicus (Cms; bacterial ring rot of potato), C. michiganensis subsp. insidiosus (Cmi; wilting and stunting in alfalfa), C. michiganensis subsp. nebraskensis (Cmn; wilt and blight of maize), and C. michiganensis subsp. tessellarius (Cmt; leaf freckles and leaf spots in wheat). More importantly, the first three subspecies are quarantine or regulated pathogens of important agricultural crops in many countries. Recently, C. michiganensis subsp. phaseoli was described as the causal agent of bacterial leaf yellowing on bean [5] and C. michiganensis subsp. capsici (Cmc) as the causal agent of bacterial canker on pepper [6]. Another two subspecies, C. michiganensis subsp. californiensis and C. michiganensis subsp. chilensis were named to include bacterial isolates from tomato and pepper seeds produced in California and Chile, respectively [7]. Among these newly established subspecies, only C. michiganensis subsp. capsici with available genome sequence data (Table 1) was used in this study. The other three recently named subspecies were not included in this study.

To better define the taxonomic position of the subspecies of C. michiganensis, whole-genome sequences of two strains of Cms, six strains of Cmn, two strains of Cmt, and the type strains of Cmm, Cmi, and Cmt were decoded using PacBio single molecule real-time (SMRT) sequencing at Genome Quebec (McGill University and Genome Quebec Innovation Centre, Montreal, Quebec, Canada). The assembled sequences were compared with published sequences of C. michiganensis subsp. michiganensis and subsp. insidiosus, and other clavibacter sequences in GenBank (Table 1). Currently available genome sequences for most type strains of each subspecies of Clavibacter michiganensis were included in this study. The genome sequences generated in this study...
were deposited in Genbank with accession numbers of MZMQ00000000 (Cmt ATCC 33566), MZMM00000000 (Cms CFIA-Cs3N), MZMN00000000 (Cms CFIA-CsR14), MZMO00000000 (Cmi LMG 3663) and MZMP00000000 (Cmt ATCC 33566).

Average nucleotide identity (ANI) values of whole genomes represent the degree of identity/similarity between homologous regions shared by two genomes and has emerged as a powerful genome-based criterion for establishing species identity amongst genetically related micro-organisms [8, 9]. The approach evaluates a large number of genes, including both slow and fast evolving genes, in the calculation and thus minimizes the effect of variable evolutionary rates or horizontal gene transfer events [9]. In this study, ANI was calculated using the JSpecies software [10] with the Nucleotide MUMmer algorithm (NUCmer) and default parameter settings. The degree of pairwise genome-based relatedness was calculated as an ANI value following the ANI calculation method described by Goris et al. [10]. ANI was calculated based on comparisons between all strains sequenced in this study and those sequenced previously [12], were well below the 96 % cutoff for species delineation. The digital pairwise estimator for the relatedness of genomes serves as an in silico replacement for the wet-lab based DNA–DNA hybridization. In this study dDDH values were calculated using GGDC 2.0 server (http://ggdc.dsmz.de/distcalc2.php) by means of genome-to-genome sequence comparison and pairwise dDDH values were estimated using the GGDC calculator [14]. Consistency with ANI data and dDDH values clearly differentiated the Clavibacter subspecies into distinct clades with high degree of congruency with genomospecies allocation (Table 2). The dDDH values between different subspecies were within the range of 37–60 % (Table 2), below the suggested 70 % cut-off for species delineation [14]. Significantly, but not unexpectedly, evaluations between strains of the same subspecies showed dDDH values of more than 93 % (Table 2).

The ANI values among the subspecies of Clavibacter were generally below the 96 % cutoff value for species delineation suggested by Richter and Rosselló-Móra [10]. ANI values between subspecies were 89.18–95.01 %, whereas ANI values between strains of the same subspecies were >99 % (99.17–99.98 %) (Table 2). Comparative ANI scores of ~90 % for the two strains, CF 11 and LMG 26808, tentatively identified as non-pathogenic isolates of Clavibacter michiganensis [12], were well below the 96 % cutoff for species delineation. The taxonomic status of these strains requires further study.

While ANI represents core genome homology, genome-genome distance calculation (GGDC) or digital DNA–DNA hybridization (dDDH) [13, 14] measures the genome-to-genome distances between pairs of entirely or partially sequenced genomes. The digital pairwise estimator for the relatedness of genomes serves as an in silico replacement for the wet-lab based DNA–DNA hybridization. In this study dDDH values were calculated using GGDC 2.0 server (http://ggdc.dsmz.de/distcalc2.php) by means of genome-to-genome sequence comparison and pairwise dDDH values were estimated using the GGDC calculator [14]. Consistency with ANI data and dDDH values clearly differentiated the Clavibacter subspecies into distinct clades with high degree of congruency with genomospecies allocation (Table 2). The dDDH values between different subspecies were within the range of 37–60 % (Table 2), below the suggested 70 % cut-off for species delineation [14]. Significantly, but not unexpectedly, evaluations between strains of the same subspecies showed dDDH values of more than 93 % (Table 2).

Multi-locus sequence analysis (MLSA) based on concatenated segments of housekeeping genes is used in phylogenetic studies to resolve taxonomic relationships among closely related species [15–17]. MLSA was employed on seven housekeeping genes, acnA, gapA, lcaA, mdh, mtlD, pgi and proA (Fig. 1). Strains within each of the five C. michiganensis subspecies clearly formed five distinct phylogenetic clusters, well-supported by high bootstrap values (Fig. 1). The grouping coincided perfectly with the five apparent genomospecies based on ANI and dDDH values (Table 2). Of the two non-pathogenic strains, LMG 26808 clustered most closely to C. m. subsp. michiganensis but separate from CF11, which formed a unique cluster. In addition, single

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Table 1. Bacterial strains and their genome sequences analysed in this study

| Bacterial strains | Strain no | GenBank accession no | Isolated from | Reference |
|------------------|-----------|----------------------|---------------|-----------|
| Clavibacter sp.   | CF 11     | JROD01000001         | Soil          | [22]      |
| Clavibacter sp.   | LMG 26808 | AZQZ01000000         | unknown       | [12]      |
| C. michiganensis subsp. insidiosus | LMG 3663^T | MZMO00000000 | Alfalfa | This work |
|                   | R-1       | NZ_CP011043          | Alfalfa       | [23]      |
| C. m. subsp. michiganensis | LMG 7333^T | MZMP00000000 | Tomato | This work |
|                   | NCMP 382  | NC_009480            | Tomato        | [24]      |
| C. m. subsp. nebraskensis | NCPPB 2581~LMG 3700^T | NC_020891 | Maize | Gartemann unpublished |
|                   | DOAB 397  | LAKL01000001         | Corn          | [25]      |
|                   | DOAB 395  | LSOE01000000         | Corn          | [21]      |
| C. m. subsp. sepedonicus | ATCC 3313^T | NC_010407 | Potato | [26]      |
|                   | CFIA-Cs3N | MZMM00000000         | Potato        | This work |
|                   | CFIA-CsR14 | MZMN00000000 | Potato | This work |
| C. m. subsp. tessellarius | ATCC 33566^T | MZMQ00000000 | Wheat | This work |
| C. m. subsp. capsici | PF 008^T | NZ_CP012573          | Pepper        | [6]       |
| Liefsonia xyli subsp. xyli | 356_LXYL | NZ_JVOK00000000 | Sugarcane | [1] |
| Liefsonia xyli subsp. cyanodontis | DSM 46306 | NC_022438 | Bermuda Grass | [1] |

T. Type strain for the subspecies.
Table 2. Average nucleotide identity (ANI; lower diagonal) and digital DNA–DNA hybridization (dDDH; upper diagonal) values among *Clavibacter michiganensis* and related species and subspecies. Cut-off values for species delineation are 96.0 and 70.0 % for ANI and dDDH, respectively.

| Data on the upper diagonal | Clavibacter sp. | Cmm | Cmm | Cmm | ATCC | Cms | Cms | Cmi | Cmt | Cmt | Cmc |
|----------------------------|----------------|-----|-----|-----|------|-----|-----|-----|-----|-----|-----|
| Cell | LMG 26808 | LMG 7333 | NCPPB 382 | NCPPB 2581 | NCPPB 397 | DOAB 395 | DOAB 395 | ATCC 33113 | CFIA 3NM | CFIA R14 | LMG 3663 | R1-1 | ATCC 33566 | PF 008 |
| CF 11 | 100 | 39.5 | 40 | 40 | 40.7 | 40.6 | 40.7 | 39.1 | 39 | 39 | 42.2 | 40.2 | 38.5 | 58.5 |
| LMG 26808 | 90.04 | 100 | 58.5 | 58.7 | 47.1 | 47.1 | 47.2 | 45.8 | 45.7 | 45.8 | 47.8 | 47.9 | 37 | 39.6 |
| *C. michiganensis* subsp. *michiganensis* | 90.14 | 94.5 | 100 | 93.3 | 48 | 47.9 | 48.1 | 45.8 | 45.7 | 45.7 | 48.6 | 48.7 | 37.2 | 40.2 |
| LMG 7333<sup>3</sup> | 90.05 | 94.46 | 99.17 | 100 | 48 | 47.9 | 48.1 | 46.3 | 46.2 | 46.2 | 48.6 | 48.7 | 37.2 | 40.2 |
| NCPPB 382 | 90.28 | 92.08 | 92.32 | 92.23 | 100 | 99.9 | 99.5 | 45.2 | 45.2 | 45.2 | 59.9 | 60 | 37.2 | 40.7 |
| *C. m. subsp. nebraskensis* (Cmm) | 90.26 | 92.09 | 92.24 | 92.26 | 99.97 | 100 | 98.3 | 45.2 | 45.2 | 45.2 | 59.9 | 60 | 37.1 | 40.7 |
| DOAB 397 | 90.30 | 92.08 | 92.25 | 92.21 | 99.97 | 100 | 100 | 45.3 | 45.2 | 45.3 | 60.1 | 60.2 | 37.8 | 40.8 |
| DOAB 395 | 89.91 | 91.88 | 91.92 | 91.87 | 91.68 | 91.66 | 91.71 | 100 | 99.8 | 98.8 | 45.1 | 45.2 | 36.2 | 39.1 |
| ATCC 33113<sup>3</sup> | 89.91 | 91.86 | 91.88 | 91.86 | 91.67 | 91.69 | 91.76 | 99.97 | 100 | 99.8 | 44.9 | 45.1 | 36.2 | 39 |
| CFIA Cs3NM | 89.91 | 91.88 | 91.92 | 91.88 | 91.68 | 91.66 | 91.72 | 99.98 | 99.96 | 100 | 44.9 | 45.2 | 36.2 | 39.1 |
| CFIA Cs R14 | 89.91 | 91.88 | 91.92 | 91.88 | 91.68 | 91.66 | 91.72 | 99.98 | 99.96 | 100 | 44.9 | 45.2 | 36.2 | 39.1 |
| *C. m. subsp. insidiosus* (Cmi) | 90.17 | 92.3 | 92.45 | 92.52 | 94.98 | 94.96 | 94.98 | 91.75 | 91.77 | 91.78 | 900 | 94.3 | 37.4 | 40.5 |
| LMG 3663<sup>3</sup> | 90.17 | 92.27 | 92.51 | 92.43 | 94.94 | 95.01 | 94.95 | 91.76 | 91.76 | 91.77 | 900 | 94.3 | 37.4 | 40.5 |
| R1-1 | 89.85 | 89.45 | 89.45 | 89.57 | 89.72 | 89.77 | 89.78 | 89.18 | 89.20 | 89.19 | 89.71 | 89.72 | 100 | 38.4 |
| ATCC 33566<sup>4</sup> | 89.85 | 90.10 | 90.11 | 90.17 | 90.30 | 90.35 | 90.30 | 89.97 | 89.93 | 89.98 | 89.24 | 90.26 | 89.87 | 100 |
| *C. m. subsp. ccppsici* (Cmc) | 94.58 | 90.10 | 90.11 | 90.17 | 90.30 | 90.35 | 90.30 | 89.97 | 89.93 | 89.98 | 89.24 | 90.26 | 89.87 | 100 |

Data on the lower diagonal

| Cell | LMG 26808 | LMG 7333 | NCPPB 382 | NCPPB 2581 | NCPPB 397 | DOAB 395 | ATCC 33113 | CFIA 3NM | CFIA R14 | LMG 3663 | R1-1 | ATCC 33566 | PF 008 |
| CF 11 | 89 | ANI % | 100 | | | | | | | | | | | |
gene phylogenies confirmed the distinct clustering of the five subspecies studied (Fig. S1, available in the online version of this article).

Re-classifying *C. michiganensis* subspecies does not undermine classification based on phenotypic characterization of this group of plant pathogenic bacteria but rather supports their classification as individual species which are easily differentiated by classical bacteriological methods as previously reported [5, 18, 19]. As already noted, each of the *C. michiganensis* subspecies is highly host-specific and in culture can also be readily differentiated by colony pigmentation on many commonly used growth media and substrate utilization (Table 3). Biochemical and physiological test reactions also differentiate each of the *Clavibacter* groups (Table 3).

Traditional classification of plant pathogens faces critical challenges in the genome era as sequence data become routinely accessible through next-generation sequencing methods. The growing number of sequenced genomes of plant pathogens provides a rich source of information for new approaches to resolve complex taxonomic questions. In this study, the draft genomes of three type strains of *Clavibacter* species/subspecies, not previously available, were generated and compared with all publicly available GenBank entries so as to accurately define the taxonomic status of the five subspecies within *C. michiganensis*. On the basis of the genome data (ANI and dDDH values) and multi-locus phylogenetic analysis presented in this paper and previously reported phenotypic characteristics, we propose that the bacteria presently classified as *Clavibacter michiganensis* subsp. *capsici* Oh et al. 2016, *Clavibacter michiganensis* subsp. *nebraskensis* (Vidaver and Mandel 1974) Davis et al. 1984, *Clavibacter michiganensis* subsp. *insidiosus* (McCulloch 1925) Davis et al. 1984, *Clavibacter michiganensis* subsp. *sepedonicus* (Speckermann and Kotthoff 1914) Davis et al. 1984, and *Clavibacter michiganensis* subsp. *tessellarius* (Carlson and Vidaver 1982) Davis et al. 1984 be reclassified as *Clavibacter capsici* sp. nov., comb. nov., *Clavibacter nebraskensis* comb. nov., *Clavibacter insidiosus* comb. nov., *Clavibacter sepedonicus* comb. nov., and *Clavibacter tessellarius* sp. nov., comb. nov., respectively. The original type strains of the subspecies become type strains for each of the new species and species descriptions remain the same as for the former descriptions of corresponding subspecies [20].

**DESCRIPTION OF CLAVIBACTER CAPSICI SP. NOV., COMB. NOV.**

*Clavibacter capsici* (cap’si.ci. N.L. neut. gen. n. *capsici*, referring to *Capsicum*, the genus name of pepper).

Basonym: *Clavibacter michiganensis* subsp. *capsici* Oh et al. 2016.

The species description is unchanged from its description as *Clavibacter michiganensis* subsp. *capsici* given by Oh et al. [6].

The type strain is PF008T (=KACC 18448T=LMG 29047T).

**DESCRIPTION OF CLAVIBACTER INSIDIOSUS COMB. NOV.**

*Clavibacter insidiosus* (in.si.di.o’sus. L. masc. adj. *insidiosus*, deceitful, insidious).
### Table 3. Phenotypic characteristics of Clavibacter michiganensis subsp. [5, 6, 18, 19]

| Characteristic                | C. michiganensis subsp. michiganensis | C. m. subsp. insidiosus | C. m. subsp. nebraskensis | C. m. subsp. sepedonicus | C. m. subsp. tessellarius | C. m. subsp. capsici |
|------------------------------|---------------------------------------|-------------------------|---------------------------|--------------------------|---------------------------|-----------------------|
| Major host plant             | Tomato                                | Alfalfa                 | Maize                     | Potato                   | Wheat                     | Pepper                |
| Colony pigment               | Yellow*                               | Yellow/blue             | Orange/yellow             | White                    | Orange                    | Orange                |
| Colony type                  | Fluidal                               | Fluidal                 | Domed, mucoid             | Fluidal                  | Domed, mucoid             | Mucoid                |
| Growth on CNS                | +                                     | –                       | +                          | –                        | +                          | N/A                   |
| Growth on TTC                | +                                     | +                       | –                          | –                        | +                          | +                     |
| Gelatin liquefaction         | +                                     | –                       | –                          | –                        | –                          | +†                   |
| Levan production             | –                                     | –                       | +                          | –                        | +                          | +                     |
| Acid from sorbitol           | –                                     | –                       | +                          | +                        | +†                        | +                    |
| Acid from mannitol           | –                                     | –                       | –                          | –                        | +†                        | +                    |
| Utilization of melibiose     | +                                     | –                       | +                          | –                        | –                          | +                    |
| Utilization of trehalose     | w                                     | +                       | +                          | +                        | +                          | +                    |
| Utilization of fucose        | +                                     | –                       | –                          | –                        | –                          | –                    |
| Utilization of acetate       | +                                     | –                       | +                          | –                        | –                          | N/A                  |
| Utilization of glycerol      | +                                     | +                       | +                          | –                        | –                          | N/A                  |
| Utilization of succinate     | +                                     | –                       | +                          | +                        | –†                         | N/A                  |
| Hydrolysis of aesculin       | +                                     | +                       | +                          | +                        | +                          | N/A                  |
| Alkaline phosphatase activity| +                                     | –                       | +                          | ±                        | +                          | +                    |
| α-Mannosidase activity       | –                                     | –                       | –                          | –                        | –                          | – W                  |

CNS, Corynebacterium nebraskense semi-selective medium [28]; TTC, 2,3,5 triphenyl tetrazolium chloride medium [29].

*Also various other pigments (e.g. pink, red, orange, white or colourless).
†This work; w, less than 50 % positive results; N/A, not available.

Basonym: Corynebacterium insidiosum (McCulloch 1925) Jensen 1934, Corynebacterium michiganense subsp. insidiosum (McCulloch 1925) Carlson and Vidaver 1982, Clavibacter michiganensis subsp. insidiosus (McCulloch 1925) Davis et al. 1984.

Gram-stain-positive, non-spore forming, aerobic bacterium without flagella. Produces yellow to orange colonies on common laboratory growth media. It grows on CNS but does not grow on TTC medium. It does not liquefy gelatin but it does produce levan. It produces acid from sorbitol but it does not produce acid from mannitol. It utilizes acetate, glycerol and succinate. It hydrolyses aesculin, it has alkaline phosphatase activity, but it does not have α-mannosidase activity. It causes leaf freckles and a wilt disease of maize (Zea mays L.) DNA G+C content of the type strain is 73.0 %. The type strain is NCPPB 2581T (=ATCC 27794T =LMG 3700T).

### DESCRIPTION OF CLAVIBACTER NEBRASKENSIS COMB. NOV.

Clavibacter nebraskensis (ne.bras.ken’sis. N.L. masc. adj. nebraskensis, pertaining to the state of Nebraska, USA).

Basonym: Corynebacterium nebraskense Vidaver and Mandel 1974, Corynebacterium michiganense subsp. nebraskense (Vidaver and Mandel 1974) Carlson and Vidaver 1982, Clavibacter michiganensis subsp. nebraskensis (Vidaver and Mandel 1974) Davis et al. 1984.

Gram-stain-positive, non-spore forming, aerobic bacterium without flagella. Produces white mucoid colonies at an optimum growth temperature of 20–23 °C. It does not grow on
The type strain is ATCC 33566 with quarantine and regulated plant pathogens.

subsp. The species description is unchanged from its description as subsp. established genus, problem of having only a single species within the well-

This new taxonomy not only resolves the long-standing problem of having only a single species within the well-established genus, Clavibacter, but it also provides a practical solution for plant pathologists and policy makers dealing with quarantine and regulated plant pathogens. *C. michiganensis*, *C. sepedonicus* and *C. insidiosus* are quarantine or regulated pathogens of important agricultural crops in many countries, while *C. capsici* is a newly described plant pathogen for which the range of distribution and risk to agriculture need to be assessed. The revised classification, and accordingly a simpler nomenclature, uncomplicates regulatory documents and more accurately reflects biological reality.

While this manuscript was under review, one of the co-authors [21] of this manuscript carried out an independent investigation titled 'Comparative genomics of *Clavibacter michiganensis* subspecies, pathogens of important agricultural crops'. It is quoted here 'the study also assessed the taxonomic position of the subspecies based on 16S rRNA and genome-based DNA homology and concludes that there is ample evidence to elevate some of the subspecies to species-level'.

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