Genome Analysis and Development of a Multiplex TaqMan Real-Time PCR for Specific Identification and Detection of *Clavibacter michiganensis* subsp. *nebraskensis*

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ABSTRACT

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The reemergence of the Goss's bacterial wilt and blight disease in corn in the United States and Canada has prompted investigative research to better understand the genome organization. In this study, we generated a draft genome sequence of *Clavibacter michiganensis* subsp. *nebraskensis* strain DOAB 395 and performed genome and proteome analysis of *C. michiganensis* subsp. *nebraskensis* strains isolated in 2014 (DOAB 397 and DOAB 395) compared with the type strain, NCPPB 2581 (isolated over 40 years ago). The proteomes of strains DOAB 395 and DOAB 397 exhibited a 99.2% homology but had 92.1 and 91.8% homology, respectively, with strain NCPPB 2581. The

majority (99.9%) of the protein sequences had a 99.6 to 100% homology between *C. michiganensis* subsp. *nebraskensis* strains DOAB 395 and DOAB 397, with only four protein sequences (0.1%) exhibiting a similarity <70%. In contrast, 3.0% of the protein sequences of strain DOAB 395 or DOAB 397 showed low homologies (<70%) with the type strain NCPPB 2581. The genome data were exploited for the development of a multiplex TaqMan real-time polymerase chain reaction (PCR) tool for rapid detection of *C. michiganensis* subsp. *nebraskensis*. The specificity of the assay was validated using 122 strains of *Clavibacter* and non-*Clavibacter* spp. A blind test and naturally infected leaf samples were used to confirm specificity. The sensitivity (0.1 to 1.0 pg) compared favorably with previously reported real-time PCR assays. This tool should fill the current gap for a reliable diagnostic technique.

Additional keywords: pseudogenes, stacking energy.

Clavibacter michiganensis is a gram-positive bacterium (family Microbacteriaceae Park et al. 1995 emend. Zhi et al. 2009) comprising five core subspecies, all of which are phytopathogens on specific important agricultural crops worldwide. The cells are rods of coryneform morphology. The cell wall contains B2γ-type peptidoglycan with the diamino butyric acid MK-9 as the predominant menaquinone, and phosphatidylglycerol and diphosphatidylglycerol as the basic polar lipids (Agarkova et al. 2011; Davis et al. 1984). The genomes have a high GC content of 72 to 74 mol% (Agarkova et al. 2011; Davis et al. 1984; Tambong et al. 2015). Four of these subspecies are classified as quarantine organisms due to the high economic threat they pose (Jacques et al. 2012). *C. michiganensis* subsp. *michiganensis* causes bacterial wilt and canker of tomato (*Solanum lycopersicum*) (Anonymous 2005), whereas *C. michiganensis* subsp. *sepedonicus* causes diseases on potato. *C. michiganensis* subsp. *insidiosus* is a pathogen of alfalfa, *C. michiganensis* subsp. *tessellarius* is pathogenic to wheat, and *C. michiganensis* subsp. *nebraskensis* infects corn. All subspecies seem to be able to invade seed, show only poor survival capabilities in soil (Davis et al. 1984), and may have an epiphytic lifestyle (Ahmad et al. 2015).

The reemergence and rapid spread of the Goss's bacterial wilt and blight disease in the United States and Canada has prompted investigative research to better understand the pathogen. *C. michiganensis* subsp. *nebraskensis* is the causal agent of the Goss's bacterial wilt and blight of corn or maize (*Zea mays* L.). The disease was first reported in 1969 in south-central Nebraska and neighboring states (Vidaver et al. 1981) but, in recent years, it has been confirmed in Minnesota, Wisconsin, Kansas, Colorado, Wyoming, South Dakota, Iowa, Illinois, Wisconsin, Texas, Indiana, and Canada (Agarkova et al. 2011; Dawson 2010; Heppner 2010; Jackson et al. 2007; Malvick et al. 2010; Ruhl et al. 2009; Tambong et al. 2015). Yield losses can be as high as 50% from systemic infections of the xylem (Eichenlaub et al. 2006; Vidaver et al. 1981). Such infections may be latent, without visible symptoms (Davis et al. 1984; Tambong et al. 2015). In addition, leaf symptoms may be confused with the disease of Stewart's wilt on corn caused by *Pantoea stewartii* subsp. *stewartii*. No characteristic symptoms of Goss's wilt are visible on seed. Based on these factors, Paul and Smith (1989) proposed that *C. michiganensis* subsp. *nebraskensis* is a candidate A1 quarantine organism. This attribute indicates potential regulatory challenges because export countries will be required to certify shipments destined to regions with no recent reports of the disease.

Unlike *C. michiganensis* subsp. *michiganensis* and *sepedonicus*, the genome organization of *C. michiganensis* subsp. *nebraskensis* is not well understood and the molecular mechanisms of its pathogenicity are unknown (Agarkova et al. 2011). Rapid expansion of nucleotide sequencing capacity with next-generation sequencing technology provides an opportunity to study *C. michiganensis* subsp. *nebraskensis* genomes and identify changes in chromosomal virulence

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*The e-Xtra logo stands for "electronic extra" and indicates that one supplementary table and five supplementary figures are published online.

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factors. Recently, the complete genome of the type strain (NCPBP 2581) of *C. michiganensis* subsp. *nebraskensis* was deposited in GenBank (HE614873) while Tambong et al. (2015) published the draft genome of strain DOAB 397 that was isolated in 2014 in the province of Manitoba, Canada. Preliminary analysis of these two genomes suggested that potential differences exist between *C. michiganensis* subsp. *nebraskensis* strain DOAB 397 and the type strain (NCPBP 2581). In addition, strain DOAB 397 showed significantly higher pathogenicity than the strain NCPBP 2581 on two Manitoban corn cultivars (F. Daayf, unpublished). In-depth analysis may show differences in the genome organization.

There are no standardized traditional techniques for the detection and identification of the Goss's wilt pathogen and certification of corn or maize seed. An enzyme-linked *C. michiganensis* subsp. *michiganensis* ImmunoStrip assay developed by Agdia Inc. is recommended with caution for detection of *C. michiganensis* subsp. *nebraskensis* due to strong positive reactions with four isolates of *Ochrobactrum* and *Microbacterium* spp. (<https://d163axztg8am2h.cloudfront.net/static/doc/81/62/5500cccb797e0661761c277f968e.pdf>). This creates some degree of uncertainty in *C. michiganensis* subsp. *nebraskensis* identification or detection with potential economic impact. Nevertheless, it is inexpensive and easy to use in the field. Polymerase chain reaction (PCR)-based methods would allow for a rapid and more reliable detection of the pathogen on corn plant tissues and identification of the pure cultures; however, very few PCR-based techniques are publicly available. Waleron et al. (2011) reported the use of specific random fragment length polymorphism patterns to differentiate *C. michiganensis* subspecies. Also, R. R. McNally, C. A. Ishimaru, and D. K. Malvick (unpublished data) reported a PCR-mediated assay using primers derived from a predicted gene while Feng et al. (2014) developed a nested-PCR using the 16S-23S intergenic transcribed spacer (ITS). Conventional PCR-based techniques used to date require post-PCR handling to visualize the results and, as such, are prone to errors.

Real-time PCR provides a better alternative for accurate detection and identification of specific plant pathogens (Tambong et al. 2008; Xu and Tambong 2011). TaqMan real-time PCR relies on the combination of a primer set and an additional dual-labeled fluorogenic probe to allow continuous monitoring of amplicon synthesis during thermocycling (Orlando et al. 1998). Because post-PCR processing steps are not required, the assay can be easily automated, leading to high sample throughput (Weller et al. 2000). Yasuhara-Bell et al. (2016) reported a loop-mediated PCR amplification system for detection of *C. michiganensis* subsp. *nebraskensis* in maize targeting the tripartite ATP-independent periplasmic-type C4-dicarboxylate transport system large permease component, while Bach et al. (2003) reported a TaqMan-PCR protocol for quantification and differentiation of *C. michiganensis* subspecies targeting 16S-23S ITS. Single-gene-probe real-time PCR have an inherent disadvantage of not having a confirmatory second probe.

The objectives of this study were to (i) generate and analyze the structural DNA atlas of the complete genome of type strain *C. michiganensis* subsp. *nebraskensis* NCPBP 2581, (ii) sequence and assemble a second-draft genome of a recent *C. michiganensis* subsp. *nebraskensis* strain DOAB 395, (iii) perform genome or proteome analysis of *C. michiganensis* subsp. *nebraskensis* strains isolated in 2014 (DOAB 397 and DOAB 395) compared with the type strain (NCPBP 2581) isolated over 40 years ago to provide an insight into any differences in their organization, and (iv) develop and validate a multiplex TaqMan real-time PCR assay targeting a secreted cellulase (CMN_02390) and 50S ribosomal protein L36 (syn. rpsJ) for reliable detection and identification of *C. michiganensis* subsp. *nebraskensis* in pure cultures and naturally infected corn leaves. The secreted cellulase gene is a functional endoglucanase with an ortholog in *C. michiganensis* subsp. *michiganensis* (CMM_2443) and *C. michiganensis* subsp. *sepedonicus* (CMS_0045). The *C. michiganensis* subsp. *michiganensis* ortholog is the *celB* gene, a putative secreted cellulase containing cellulose-binding domain

(Gartemann et al. 2008). In addition, the assay uses a λ DNA-derived internal control probe (Tambong et al. 2008) used to test for the presence of PCR inhibitors across DNA samples. Finally, the multiplex TaqMan assay was evaluated across frequently used real-time PCR platforms.

MATERIALS AND METHODS

Bacterial strains and DNA extraction. In total, 122 bacterial strains from various sources were used in this study (Table 1). Isolation of *C. michiganensis* subsp. *nebraskensis* strains was done using the *Corynebacterium nebraskense* semiselective medium (Gross and Vidaver 1978). Strains of *Pectobacterium* spp. were cultured in tryptone yeast extract liquid media while *C. michiganensis* subspecies were grown in nutrient broth supplemented with yeast extract (Smid et al. 1995). All other bacterial strains were grown using Luria-Bertani (tryptone at 10 g liter⁻¹, yeast extract at 5 g liter⁻¹, and NaCl at 10 g liter⁻¹) or nutrient broth media. Stock bacterial cultures were maintained on the same medium supplemented with 25% (wt/vol) glycerol at -80°C. Genomic DNA was purified using the Wizard SV Genomic DNA purification system (Promega Corp.). The concentration of DNA was determined using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). Purified DNA was stored at -20°C.

16S ribosomal RNA gene amplification and sequencing. The primer pair 16S27 (5'-AGAGTTTGATCMTGGCTCAG-3') and 1482R (5'-TACGGYTACCTTGTTACGACTT-3') (Lane 1991), was used. Primers were synthesized by Invitrogen Inc., and PCR amplifications were performed in a 10- μ l volume reaction using a thermal cycler (Biometa) with 10 ng of bacterial DNA (1 μ l), 1 μ l of 10 $\times$ PCR buffer, 0.75 μ l of 2 mM deoxynucleoside triphosphates (dNTP), 0.08 μ l of each primer (20 μ M), 0.1 μ l of Titanium Taq DNA polymerase (5 U/ μ l; BD/Biosciences/Clontech), and 6.99 μ l of MilliQ water. Optimal PCR conditions included an initial denaturation of 95°C for 5 min; followed by 40 cycles of 95°C for 45 s, 62°C for 45 s, and 72°C for 90 s; and a final extension at 72°C for 10 min. Sequencing was done using ABI BigDye Terminator chemistry v3.1 (Applied Biosystems) and run on an ABI 3130xl automated sequencer (Applied Biosystems/Hitachi).

Also, the identity of strains exhibiting a positive TaqMan real-time PCR reaction were validated by sequencing three ribosomal protein gene (*rpsB*, *rpsG*, and *rpmF*) fragments followed by Basic Local Alignment Tool (BLAST) analysis. Primer sequences of the ribosomal gene fragments are given in Table 2. PCR amplification was performed as described above at an annealing temperature of 70°C and sequencing was done as described above.

Genome downloads. Whole- and draft-genome data from eight strains—*C. michiganensis* subsp. *sepedonicus* ATCC 33113 (AM849034), *C. michiganensis* subsp. *michiganensis* NCPBP 382 (AM711867), *C. michiganensis* subsp. *nebraskensis* NCPBP 2581 (HE614873), *C. michiganensis* subsp. *insidiosus* R1-1 (CP011043), *C. michiganensis* subsp. *nebraskensis* DOAB 397 (LAKL000000000), *C. michiganensis* subsp. *tessellarius* DOAB 609 (LQXA000000000), *C. michiganensis* CF11 (JROD000000000), and *C. michiganensis* LMG 26808 (AZQZ000000000)—representing the *C. michiganensis* subspecies were downloaded from the GenBank database (Benson et al. 2012) at the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/genome/>). NCBI GenBank International Nucleotide Sequence Database Collaboration or whole-genome-sequence (WGS) numbers were used to download each genome in the NCBI GenBank format, using the getgbk.pl script as implemented in CMG-Biotools (Vesth et al. 2013). DNA sequences were extracted from GenBank files and saved in FASTA format using the sacco_convert script (Jensen and Knudsen 2000).

de novo sequencing of *C. michiganensis* subsp. *nebraskensis* strain DOAB 395. Preliminary analysis of the genome of a recent (2014) *C. michiganensis* subsp. *nebraskensis* strain and the type strain isolated over 40 years ago indicated potential differences (Tambong

TABLE 1. Bacteria used to evaluate the specificity of the real-time polymerase chain reaction (PCR) assay, TaqMan detection data, and BLAST results of ribosomal proteins of positive strains

Bacterial species, strain	Year ^c	AFLP ^d	16S rRNA ^e	BLAST results ^a			TaqMan real-time PCR reaction ^b	
				<i>rpsB</i>	<i>rpsG</i>	<i>rpmF</i>	cell_cmn38pb	cmn_rpsJ27pb
<i>Clavibacter michiganensis</i> subsp. <i>nebraskensis</i>								
CN18-1	1969	A1	<i>Clavibacter</i>	+	+	+	+	+
CN18-5, CN18-6, CN38-1	1970	A1	<i>Clavibacter</i>	+	+	+	+	+
CN298, NCPPB 2581	1971	A1	<i>Clavibacter</i>	+	+	+	+	+
CN30-1	1972	A1	<i>Clavibacter</i>	+	+	+	+	+
CN37-1, CN37-2	1972	A1	<i>Clavibacter</i>	+	+	+	+	+
CN28-1, CN4-2	1973	A1	<i>Clavibacter</i>	+	+	+	+	+
CN15-1	1974	A1	<i>Clavibacter</i>	+	+	+	+	+
CN49-2, CN62-1	1975	A1	<i>Clavibacter</i>	+	+	+	+	+
1994 NC+	1994	A1	<i>Clavibacter</i>	+	+	+	+	+
1996 CIBA, 1996 NC+, 1996 Pioneer	1996	A1	<i>Clavibacter</i>	+	+	+	+	+
9918-1, 9918-3, 9910	1999	A1	<i>Clavibacter</i>	+	+	+	+	+
20032, 20037	2003	A1	<i>Clavibacter</i>	+	+	+	+	+
31341, 32719	2004	A1	<i>Clavibacter</i>	+	+	+	+	+
2006 Box Butte 1, 2006 Scotts Bluff 3	2006	A1	<i>Clavibacter</i>	+	+	+	+	+
2007 15-17 (103), 200710-C, 20071-2	2007	A1	<i>Clavibacter</i>	+	+	+	+	+
200800460, 200800417 Indiana	2008	A1	<i>Clavibacter</i>	+	+	+	+	+
Fuss/Debus (F16), 200900406	2009	A1	<i>Clavibacter</i>	+	+	+	+	+
CN18-4, CNN-1, H.D.363	1970	A2	<i>Clavibacter</i>	+	+	+	+	+
K293E, 313.2 PDG101	1971	A2	<i>Clavibacter</i>	+	+	+	+	+
CNK-2	1972	A2	<i>Clavibacter</i>	+	+	+	+	+
CN4-1, CN81-2	1973	A2	<i>Clavibacter</i>	+	+	+	+	+
CN72-1	1974	A2	<i>Clavibacter</i>	+	+	+	+	+
CN68-1, CN76-2	1975	A2	<i>Clavibacter</i>	+	+	+	+	+
CN72-10, CN72-11A, CN72-23A, CN72-25A, CN72-28, CN72-29	1982	A2	<i>Clavibacter</i>	+	+	+	+	+
200717-C	2007	A2	<i>Clavibacter</i>	+	+	+	+	+
Butcher (G2)	2008	A2	<i>Clavibacter</i>	+	+	+	+	+
200900798 Texas	2009	A2	<i>Clavibacter</i>	+	+	+	+	+
DOAB 391, DOAB392, DOAB393, DOAB 395, DOAB 396, DOAB 397, DOAB 398, DOAB 399, DOAB 400, DOAB 401, DOAB 402	2014	nd	<i>Clavibacter</i>	+	+	+	+	+
DOAB 799, DOAB 800, DOAB 802, DOAB 803, DOAB 804, DOAB 805, DOAB 806, DOAB 806, DOAB 807, DOAB 808, DOAB 810, DOAB 811, DOAB 812, DOAB 813, DOAB 814, DOAB 815, DOAB 876	2015	nd	<i>Clavibacter</i>	+	+	+	+	+
Other <i>C. michiganensis</i> subspecies								
subsp. <i>michiganensis</i> : LMG 7333 ^T , JD 83-1, DC 93-1, DC 94-1, DC 96-1, DC 96-2, DC97-1	1910, 1983, 1993, 1994, 1996, 1997	n/a	<i>Clavibacter</i>	–	–	–	–	–
subsp. <i>tessellarius</i> : ATCC 33566 ^T , CMT 7621, CMT 80001	UNK	n/a	<i>Clavibacter</i>	–	–	–	–	–
subsp. <i>sepedonicus</i> : CR10	UNK		<i>Clavibacter</i>	–	–	–	–	–
subsp. <i>insidiosus</i> : GSPB 2225, LMG 3663 ^T	UNK	n/a	<i>Clavibacter</i>	–	–	–	–	–
Other bacterial species								
<i>Pantoea stewartii</i> : LMG 2632 ^T , LMG 2715 ^T , DOAB 21, DOAB 22	1970, 1970, 2006, 2006	n/a	<i>Pantoea</i>	–	–	–	–	–
<i>P. ananatis</i> : LMG 2665 ^T , LMG 2667	1966, 1958		<i>Pantoea</i>	–	–	–	–	–
<i>P. allii</i> : LMG 24248	2005	n/a	<i>Pantoea</i>	–	–	–	–	–
<i>P. eucalypti</i> : LMG 24197 ^T , LMG 24198	2004, 2004	n/a	<i>Pantoea</i>	–	–	–	–	–
<i>Dickeya dadantii</i> : EC46, GSPB 413	UNK	n/a	<i>Pantoea</i>	–	–	–	–	–
<i>P. agglomerans</i> : LMG 1286 ^T , LMG 2102	1920, UNK		<i>Pantoea</i>	–	–	–	–	–
<i>Pseudomonas syringae</i> : Psgly-2, HBA2, T1	UNK	n/a	<i>Pseudomonas</i>	–	–	–	–	–
<i>Xanthomonas campestris</i> var. <i>phaseoli</i> : ATCC 10199, ATCC 1019315, XCC007	UNK	n/a	<i>Xanthomonas</i>	–	–	–	–	–
<i>Pectobacterium carotovorum</i> : ECC71, Eca9204t	UNK	n/a	<i>Pectobacterium</i>	–	–	–	–	–
<i>Pseudomonas fluorescens</i> : LMG 1794, pf-5	1951, 1979	n/a	<i>Pseudomonas</i>	–	–	–	–	–
<i>Bacillus subtilis</i> : CH10, Ch15	2007		<i>Bacillus</i>	–	–	–	–	–
<i>Streptomyces scabies</i> : CG1	UNK	n/a	<i>Streptomyces</i>	–	–	–	–	–
<i>S. acidiscabies</i> : ATCC49003	UNK	n/a	<i>Streptomyces</i>	–	–	–	–	–
<i>S. europaescabies</i> : CFPB 4497	1986	n/a	<i>Streptomyces</i>	–	–	–	–	–

^a *rpsB*, *rpsG*, and *rpmF* were sequenced and BLAST performed for strains showing positive TaqMan detection. Positive (+) BLAST results indicates a hit to *C. michiganensis* subsp. *nebraskensis* NCPPB 2581 genome (HE614873) with 100% homology while a negative sign (–) indicates no hits or PCR amplification using the respective *rps* primers. n/a, not applicable; nd, not determined.

^b Symbols: + indicates positive reaction based on detectable fluorescence (with mean threshold values between 19 and 22) and – indicates no detectable fluorescence. *C. michiganensis* subsp. *nebraskensis* strains from 1969 to 2009 were kindly provided by Dr. Anne Vidaver (University of Nebraska-Lincoln). Other strains were obtained from culture collections: LMG, Belgian Coordinated Collections of Microorganisms/Laboratory of Microbiology; ATCC, American Type Culture Collection; CFPB, French Culture Collection for Plant Associated bacteria; GSPB: Göttingen Collection of Phytopathogenic Bacteria. *Streptomyces scabies* strain CG1 was kindly provided by Carol Beaulieu (Université de Sherbrooke, Quebec, Canada). Strains with prefix DOAB were isolated from infected corn leaves in Manitoba, Canada.

^c Year of isolation; UNK = unknown.

^d Composite amplified fragment length polymorphism (AFLP)-BOX grouping of *C. michiganensis* subsp. *nebraskensis* strains according to Agarkova et al. (2011); nd = not done and n/a = not applicable.

^e 16S rRNA identification.

et al. 2015). To confirm these differences, a second strain isolated in 2014 was sequenced. Genomic DNA shearing, library preparation, and the whole-genome shotgun sequence of *C. michiganensis* subsp. *nebraskensis* DOAB 397 were performed by the Gnme-Qubec Innovation Centre. The draft genome was determined by paired-end sequencing using Illumina MiSeq technology (Gnme-Qubec). In total, 1,347,336 paired-end reads, each 250 bp in length, totaling 336,834,000 bp, were obtained. The quality of the reads was checked by using FastQC version 0.11.3 (Andrews 2015). De novo assembly was performed using ABySS version 1.5.2 (Simpson et al. 2009) at different k-mer values (75 to 113). Scaffolds with length <300 bp were discarded. A k-mer value of 89 gave the best assembly quality (no misassembled scaffolds) based on QUAST version 2.3 (Gurevich et al. 2013). The 25 scaffolds of *C. michiganensis* subsp. *nebraskensis* DOAB 397 were ordered and oriented using the CAR software (Lu et al. 2014) based on the complete genome sequence of the type strain, *C. michiganensis* subsp. *nebraskensis* NCPPB 2581 (NC_020891). Contigs were annotated using the NCBI Prokaryotic Genome Automatic Annotation Pipeline (PGAAP) (Angiuoli et al. 2008) with the best-placed reference protein set. The assembled contigs were deposited into the GenBank WGS database with accession number LSOE00000000.

independently through a pipeline and outputted in a circular plot (Vesth et al. 2013). The gene annotations are obtained from GenBank files containing the coding regions. Atlases were not generated from the draft genomes of *C. michiganensis* subsp. *nebraskensis* strains DOAB 395 and DOAB 397 because a genome atlas can only be visualized from one single replicon but not from a multifasta file such as draft genomes.

Also, the pan- and core-genome plot was also used to compare proteomes (using the 50/50 cut-off, as described above). The program (pancoreplot) produces a plot and a table which can be used to look up the underlying values of the plot (Vesth et al. 2013). The pan-genome is the entire set of protein families from all genomes in the comparison while the core-genome consists of protein families with representatives found in all investigated genomes. Using the `pancoreplot_tree` command (Vesth et al. 2013), a pan-genome phylogenetic tree was created based on the shared gene families as defined in the pan- and core-genome analysis.

TABLE 2. Oligonucleotide primers, probes, approximate amplicon length, and their locations on *Clavibacter michiganensis* subsp. *nebraskensis* genome sequence (HE614873)

^a Oligonucleotide code.

(AMR) (McArthur et al. 2013). The RGI uses a BLASTp search against the set of curated resistance genes in the Comprehensive Antibiotic Resistance Database (<https://card.mcmaster.ca/>) with precise filtering criteria based on different types of resistance classes. The analysis of pathogen-associated genes (PAG) was performed as reported by Ho Sui et al. (2009) and implemented in IslandViewer 3 (Dhillon et al. 2015). GI identification on the draft genome of *C. michiganensis* subsp. *nebraskensis* DOAB 395 was performed using the complete genome of *C. michiganensis* subsp. *nebraskensis* strain NCPPB 2581 as a reference, as recommended by Dhillon et al. (2015).

Primers and TaqMan probe design and real-time PCR conditions. Using comparative genomics data, the genes *celB* and *rpsJ* were identified as candidates for TaqMan probes based on nucleotide variation across the *C. michiganensis* subspecies. The *celB* and *rpsJ* sequences of all the subspecies were downloaded or extracted from GenBank and aligned in MegAlign (version 7.0; DNASTAR). Specific primers and dual-labeled probes were designed as previously reported (Tambong et al. 2008; Xu and Tambong 2011). A similarity search was performed using the Blastn (nucleotide versus nucleotide comparison) with the primers as queries against all publicly available GenBank database, as previously described (Tambong et al. 2006). The location of the primers and probes on the *C. michiganensis* subsp. *nebraskensis* genome (HE614873), nucleotide sequences, melting temperatures, and other characteristics are shown in Table 2. The primers and probe were designed and analyzed for dimer or hairpin loop structures by using oligo 6.71 software (Molecular Biology Insights). Primers and probes were synthesized by Sigma-Aldrich and labeled with the corresponding fluorescent reporter dyes covalently coupled to the 5' end and with quencher at the 3' end (Table 2).

Initial TaqMan real-time PCR was conducted in a Chromo4 real-time PCR System (MJ Research Bio-Rad Laboratories). PCR was performed by using 5 µl of 2× SsoAdvanced Universal Probes Supermix (Bio-Rad Laboratories), 0.2 µl of each primer at 20 µmol liter⁻¹ and probe at 10 µmol liter⁻¹, 1 µl of template or standard

DNA, and 3.4 µl of sterile ultrapure water to a total reaction volume of 10 µl. The thermal cycling optimized conditions were an initial denaturation at 95°C for 15 min followed by 50 cycles at 95°C for 1 s and 60°C for 1 min. Detection and quantification of the fluorescence was read after every cycle and data were assembled using Opticon analysis software (version 3.1; MJ Research/Bio-Rad Laboratories). Triplicates of standards and samples were subjected simultaneously to real-time PCR analysis, with each run including a negative control (no DNA template).

Sensitivity and specificity. The detection limit (sensitivity) of the assay was determined using purified genomic DNA of *C. michiganensis* subsp. *nebraskensis* strain NCPPB 2581 (type strain). The DNA was serially diluted 10-fold from 10 ng to 1 fg in PCR water (Thermo Scientific) and subjected to TaqMan PCR amplification using the corresponding probes and primers. All reactions were performed in triplicate.

Specificity of the assay was evaluated on genomic DNA of 122 strains, including *C. michiganensis* subsp. *nebraskensis* ($n = 82$) and closely ($n = 12$) and distantly ($n = 28$) related bacterial species (Table 1). The *C. michiganensis* subsp. *nebraskensis* strains used were isolated between 1972 and 2015. The DNA for all the strains was diluted to 1 ng µl⁻¹ and TaqMan real-time PCR was performed using the same conditions indicated above. Each strain was run in duplicate and repeated once. DNA samples that did not show detectable fluorescent signals were evaluated for PCR inhibition using the IC-65for/IC-65rev primer pair with the hex-labeled probe (IC-88pb), as previously described (Tambong et al. 2008).

Comparing real-time PCR platforms. The compatibility of the multiplex assay across three widely used real-time PCR platforms was evaluated using the Chromo4 System (MJ Research Bio-Rad Laboratories), LightCycler 480 instrument (Roche), and CFX96 Detection System (Bio-Rad Laboratories). The assay used three probes (*cell_cmn38pb*, *cmn_rps27pb*, and *IC-88pb*) labeled with Cy5, 6FAM, and hex reporter dyes, respectively, in a multiplex run in a 25-µl total volume. The sensitivity using dilutions (1 ng to 1 fg) of the DNA of NCPPB 2581 and the

Fig. 1. Dot-plot graph showing a perfect reordering of the scaffolds of *Clavibacter michiganensis* subsp. *nebraskensis* strain DOAB 395 relative to strain NCPPB 2581. Graph was created by the program CAR (Lu et al. 2014).

specificity of the different instruments were evaluated using 15 strains, consisting of *C. michiganensis* subsp. *nebraskensis* ($n = 6$), other *C. michiganensis* subspecies ($n = 4$), and distantly related bacterial species ($n = 2$) in triplicate. The PCR conditions were as described above.

Detection of *C. michiganensis* subsp. *nebraskensis* in corn leaf samples by TaqMan real-time PCR. Validation of the assay was done using growth-chamber-inoculated plants in a blind test and naturally infected corn plants collected in 2014 and 2015. The growth-chamber inoculations of eight plants were performed by the Canadian Food Inspection Agency (CFIA). After inoculations, leaves were harvested, dried, coded for blind testing, and sent to the Bacteriology Laboratory of Agriculture and Agri-Food Canada for processing and detection. Also, 25 field-infected leaves were collected in Manitoba and shipped to the Bacteriology Lab for processing. Total DNA was extracted from leaf samples (200 mg) with Ultraclean soil DNA isolation kits (Mo Bio Laboratories) following the manufacturer's protocol. The DNA quality was verified by electrophoresis on a 1.0% agarose gel and the concentration was determined using the Nano-Drop ND-1000 Spectrophotometer (NanoDrop Technologies). The presence of PCR inhibition was evaluated using the IC-65for/IC-65rev primer pair with the hex-labeled probe (IC-88pb), as previously described

(Tambong et al. 2008). Real-time PCR detection was performed on the DNA as indicated above. To confirm the presence of *C. michiganensis* subsp. *nebraskensis* in field samples, plate isolation was performed using a semiselective medium, as previously described (Gross and Vidaver 1978). Five peach-colored bacterial colonies were removed from agar and suspended in 20 μ l of autoclaved milliQ water and colony PCR was performed for 16S ribosomal RNA (rRNA). The generated amplicons were sequenced as indicated above. A BLAST search was performed with the nucleotide sequences for genus-level identification.

RESULTS

Summary statistics of *C. michiganensis* subsp. *nebraskensis* strain DOAB 395 draft genome. The draft genome comprised 25 scaffolds (minimum, 2,814 bp; maximum, 373,300 bp; N_{50} , 197,574 bp; total size, 3,077,355 bp). The quality of the draft genome of DOAB 395 was good because no misassembled fragments were detected using Quast software (Gurevich et al. 2013), and the dot-plot graph of scaffolds reordering against the complete strain NCPPB 2581 showed an almost perfect alignment (Fig. 1). The G+C content of the draft genome is 72.6%, with an

Fig. 2. DNA structural features of the complete genome of *Clavibacter michiganensis* subsp. *nebraskensis* based on genomic atlases. Data of DNA, RNA, and gene annotation are from the published GenBank entry HE614873. Each lane of the circular representation of the chromosome shows a different DNA feature. From innermost circle: size of genome (axis), percent AT (red = high AT), GC skew (blue = most G), inverted and direct repeats (color = repeats), position preference, stacking energy, and intrinsic curvature. Dark-brown arrows highlight areas of the genome with significantly different DNA structures than the remaining of the genome. Black arrows show regions of low DNA curvature and stacking energy. Changes in the skew of G and C are depicted by orange arrows. Blue arrows shows the locations of rRNA operons as annotated in the GenBank file. Genome atlas was generated using CMG-Biotools (Vesth et al. 2013), which calculates a numerical value for each nucleotide and is saved in a file that is read by GeneWiz software. See “Materials and Methods” for details.

overall estimated coverage of 110x. PGAAP annotation identified 2,792 putative protein-coding sequences. Four *rrn* operons, 45 transfer RNA (tRNA), 3 noncoding RNA, and 67 pseudogenes are present in the draft genome.

Structural features of a DNA atlas of the NCPPB 2581 genome. A structural DNA atlas was created for the complete genome of *C. michiganensis* subsp. *nebraskensis* NCPPB 2581 (Fig. 2). The genome atlas shows three categories of data: (i) base composition (AT content, GC skew), (ii) global repeats (direct and inverted), and (iii) DNA structural properties (position preference, DNA stacking energy, and curvature). Genes (blue for leading and red for lagging strand), tRNA, and rRNA are shown as found in the genome annotations. The DNA is used for simple base count information as well as AT content and GC skew. The DNA atlas showed a low AT content consistent with actinomycetales. The GC skew lane (Fig. 2) consists of changes from most G (blue) to more C (pink) or vice versa (Fig. 2; orange arrows). Note how the GC skew changes with genes of the leading and lagging strands. There is a C bias toward the leading strand and G on the lagging. The genome atlas of *C. michiganensis* subsp. *nebraskensis* NCPPB 2581 showed no global inverted repeats. Very few direct repeats were observed on the DNA atlas. These corresponded to the location of the three *rrn* operons (blue arrows). The *rrn* operons are also located in regions of high intrinsic curvature and stacking energy and low position preference (Fig. 2; brown arrows). Black arrows (Fig. 2) depict

regions with low intrinsic curvature and stacking energy and high position preference.

The predicted GI on the complete genome of *C. michiganensis* subsp. *nebraskensis* NCPPB 2581 is shown in Supplementary Figure S1A. The positions of the GI aligned perfectly with regions of high intrinsic curvature and stacking energy and low position preference (Supplementary Fig. S2, superimposed image). GI predictions are represented as blocks (integrated results in red, IslandPath-DIMOB in blue, and SIGI-HMM in orange) (Supplementary Fig. S1). Circular glyphs within the radial panel indicate putative antimicrobial resistance (green arrows) and virulence (purple arrows) genes. Similar GI were identified for *C. michiganensis* subsp. *nebraskensis* DOAB 395 but PAG and AMR were not detected, probably because it is a draft genome of several scaffolds or contigs (Supplementary Fig. S1B). A summary of the GI identified, length, and genes involved is presented in Supplementary Table S1. Genes identified within the GI of *C. michiganensis* subsp. *nebraskensis* strain NCPPB 2581 (92 genes) and DOAB395 (79 genes) include hypothetical protein or putative excisionase, LD-carboxypeptidase, serine peptidase family S66, and the putative toxin component of a toxin/antitoxin system. The largest GI found on the genome of *C. michiganensis* subsp. *nebraskensis* strain NCPPB 2581 is 22.62 kb while strain DOAB 395 draft genome has a 25.13-kb fragment. The genome of NCPPB 2581 exhibited 6 PAG and 5 AMR, including MFS permease, putative efflux MFS permease, putative multidrug

Fig. 3. BLAST matrix of proteomes of nine strains of *Clavibacter michiganensis* subspecies based on all against-protein comparison with define homologs. A hit is considered significant if a 50 and 50% (identity and length coverage) requirement between proteomes is met. Paralogs (internal homology) are proteins within a genome matching the same 50-50 rule. Self-matches here are ignored. An XML input file was generated by the program makebmdest and BLAST comparison implemented in CMG-Biotools (Vesth et al. 2013).

ABC transporter, fused permease and ATP-binding protein, and ABC transporter consisting of fused permease and ATPase domains.

Proteome comparisons. The proteomes were constructed with nine genomes. The BLAST matrix illustrates that the conservation between genomes is generally higher within than between subspecies (Fig. 3). The proteomes of *C. michiganensis* subsp. *nebraskensis* strains DOAB 395 and DOAB 397 exhibited a 99.2% homology but had 92.1 and 91.8% homologies, respectively, with type strain NCPPB 2581 (Fig. 3). The majority (99.9%) of the protein sequences have a 99.6 to 100% homology between *C. michiganensis* subsp. *nebraskensis* strains DOAB 395 and DOAB 397 while only four protein sequences (0.1%) exhibited a similarity of <70%. In contrast, 3.0% ($n = 75$) of the protein sequences of strains DOAB 395 and DOAB 397 showed low homologies (<70%) with type strain NCPPB 2581. Proteomes of the three *C. michiganensis* subsp. *nebraskensis* strains showed only 67.3 to 75.5% similarity with the other *C. michiganensis* subspecies (Fig. 3). The genome of *C. michiganensis* subsp. *michiganensis* NCPPB 382 showed a relatively high similarity (75.3%) with the genome of *C. michiganensis* subsp. *nebraskensis* DOAB 397 (Fig. 3). The lowest proteome homology of 61.0% was between *C. michiganensis* subsp. *sepedonicus* and *tessellarius* genomes (Fig. 3). The homology within proteomes (Fig. 3, red squares) ranged from 2.3 to 3.0%. *C. michiganensis* subsp. *michiganensis* showed the highest paralogous homology within the proteomes. The pan-genomic dendrogram (Fig. 4) confirms the similarities and differences indicated above. The analysis clustered all the *C. michiganensis* subsp. *nebraskensis* strains in a unique subcluster (Fig. 4). However, strains DOAB 395 and DOAB 397 clustered distinctly from type strain NCPPB 2581 and were well supported by a high bootstrap value (Fig. 4). All the other *C. michiganensis* subspecies clustered distinctly (Fig. 4).

Validation of TaqMan primers and probes. The primers and probes were analyzed by BLAST to assess their specificity against GenBank entries. The results of a comprehensive BLAST search of sequences for the primers and probe confirmed their specificities. All the primers and probes exhibited a perfect (100%) match with the genome of *C. michiganensis* subsp. *nebraskensis* (HE614873) whereas the other *C. michiganensis* subspecies showed low to no homology with the query nucleotide sequences. For example, the nucleotide sequence of the TaqMan probe cell_cmn38pb had coverage and identity of 100% (e-value = 0.069) with only the genome of *C. michiganensis* subsp. *nebraskensis*. The closest sequence hit for the nucleotide sequence of this probe is *Exophiala aquamarina* CBS 119918 hypothetical protein (XM_013407968), with an 85% identity (e-value = 4.2). Also, the forward primer cell_cmn3F exhibited 100% coverage and identity (e-value = 0.04) with only the target subspecies (*C. michiganensis* subsp. *nebraskensis*). No significant hits were recorded with any of the other *C. michiganensis* subspecies.

Sensitivity and specificity of TaqMan real-time PCR assay. TaqMan real-time PCR performed on serial dilutions of genomic DNA of *C. michiganensis* subsp. *nebraskensis* NCPPB 2581 exhibited consistent amplification of the respective target genes at the 0.1-pg level (Supplementary Fig. S3) for the primer/probe combination targeting the secreted cellulase gene (*celB*) at a fluorescence cut-off value = 0.1. Primer/probe combinations derived from 50S ribosomal protein L36 (*rpsJ*) showed a similar amplification but at the 1.0-pg level of sensitivity. The cycle threshold (C_T) values increased at each dilution, demonstrating the validity of the assay as the target DNA concentration decreased. An increment in C_T value of 2.1 to 4.3 or 1.3 to 3.8 cycles for *celB* or *rpsJ* probes, respectively, was recorded as the genomic DNA concentration was decreased by 10-fold. Linear negative correlations between the C_T values and purified DNA concentration can be established with high r^2 values of 0.99 and 0.98 for the respective primers/probe combinations.

Eighty-two strains of *C. michiganensis* subsp. *nebraskensis* isolated between 1969 and 2015 were subjected to TaqMan real-time PCR using the different primer/probe combinations. The base

pairing of the probes to the targeted respective gene amplicons confirmed the specificity of detection (Table 1). The increase in fluorescence crossing the threshold values at a specific cycle ($C_T = 19$ to 24) indicated the positive amplification of the targeted gene (Table 1). All *C. michiganensis* subsp. *nebraskensis* strains exhibited positive amplification of the expected 107- and 94-bp amplicons for the *celB* and *rpsJ* genes, respectively. Agarose gel electrophoresis (data not shown) confirmed the presence of a band of the expected amplicon size. No increase in fluorescence (Table 1) was recorded for 40 bacterial strains consisting of other *C. michiganensis* subspecies and non-*Clavibacter* spp. using the TaqMan real-time PCR assay (Table 1).

Detection of *C. michiganensis* subsp. *nebraskensis* in blind-test and naturally infested corn leaf samples. The developed TaqMan real-time PCR was validated in a blind test of growth-chamber-inoculated plants (Supplementary Fig. S4) and naturally infested field leaf samples (Table 3). The assay accurately and reliably detected the Goss's wilt pathogen in all three plants that were inoculated in the growth chamber in a blind test of eight plants. The *celB* probe assay had a positive detection at C_T values of 25 to 26 and the probe targeting *rpsJ* gene exhibited C_T values of 28 to 30 on the same blindly coded samples. Both probes did not show any detectable fluorescence with five samples. The blind samples were decoded by CFIA staff, showing a perfect match to the results obtained using the developed TaqMan assay.

Also, validation of the assay was done using naturally infected corn plants collected in 2014 and 2015. All leaf samples with typical Goss's wilt disease symptoms ($n = 17$) showed detectable fluorescence at C_T values of 19.6 to 30.4 and 20.7 to 27.8 for *celB* and *rpsJ* probes, respectively (Table 3). The TaqMan detections were validated by colony isolation followed by 16S rRNA gene sequencing. Eight leaf samples with noncharacteristic symptoms did not exhibit detectable fluorescence (Table 3). *C. michiganensis* subsp. *nebraskensis* strains were not recovered from these samples. Also, no PCR inhibition was detected based on the internal control TaqMan real-time PCR assay (Tambong et al. 2008).

Assay reliability across different real-time PCR platforms. The compatibility of the assay across three widely used real-time PCR platforms was evaluated using the Chromo4 System (MJ Research Bio-Rad Laboratories), LightCycler 480 instrument (Roche), and CFX96 Detection System (Bio-Rad Laboratories). In a multiplex assay, all three platforms showed positive fluorescence detection with the six *C. michiganensis* subsp. *nebraskensis* strains within the acceptable C_T values (Fig. 5). The fluorescence kinetics graphs are shown in Supplementary Figure S4. Strain NCPPB 2581 showed the lowest C_T values on all the platforms and the means of repeated experiments were comparable (Fig. 5). As expected, the *celB* probes showed lower C_T values compared with the probe targeting the *rpsJ* gene. None of the other strains of *C. michiganensis* subspecies or *P. stewartii* subsp. *stewartii* exhibited detectable fluorescence.

DISCUSSION

The reemergence and rapid spread of the Goss's bacterial wilt and blight disease has prompted increased research to better understand the genome organization of this pathogen and develop reliable diagnostic tools. This study reports on the comparative genomics of *C. michiganensis* subsp. *nebraskensis* strains isolated in 2014 and the type strain NCPPB 2581 that was isolated over 40 years ago. The data generated expand our knowledge of the *C. michiganensis* subsp. *nebraskensis* genome organization, which is poorly understood. The results significantly add to the body of information that is required to understand the current reemergence of this phytopathogenic bacterium. Also, the data were exploited to develop a reliable diagnostic tool. The TaqMan assay reported here is robust and comprehensive, including a probe to detect PCR inhibitors. This multiplex assay can process a single sample within 3 h, with a high-throughput capability for rapid and accurate detection of

C. michiganensis subsp. *nebraskensis* in 96 samples in less than 16 h. This tool should fill the current gap of a comprehensive diagnostic technique for specific detection and identification of *C. michiganensis* subsp. *nebraskensis* in pure cultures and naturally infected corn leaves.

The draft genome size of *C. michiganensis* subsp. *nebraskensis* DOAB 395 generated in this study is within the range (3.06 to 3.42 Mb) of previously published draft and complete genomes of the

C. michiganensis subspecies (Bentley et al. 2008; Gartemann et al. 2008; Tambong et al. 2015). This indicates that the draft genome of strain DOAB 395 in this study is of good quality (no misassembled fragments), as determined by Quast (Gurevich et al. 2013) and dot-plot graph of scaffolds reordering against strain NCPPB 2581.

Both *C. michiganensis* subsp. *nebraskensis* strains isolated in 2014 show relatively higher pseudogenes. Comparable high levels of nonfunctional genes such as in DOAB 395 and DOAB 397 are

Fig. 4. Pan-genome phylogenetic tree based on shared gene families shows distinct clustering (shaded area) of the *Clavibacter michiganensis* subsp. *nebraskensis* strains DOAB 395 and DOAB 397 (isolated in 2014) relative to strain NCPPB 2581 (isolated over 40 years ago). The distinctiveness is supported by high bootstrap values. Bootstrap values <50% are not shown. The pan-genome tree was created using the pancoreplot_tree command (Vesth et al. 2013) based on shared gene families.

TABLE 3. Summary of the TaqMan real-time polymerase chain reaction detection of the Goss's wilt pathogen in corn field samples collected in Manitoba, Canada^a

Collection ^b	Field location	Date collected	Mean C _T value ± SD		Detection
			cmn_rpsJ27pb	cell_cmn38pb	
Field 6	West of Emerson	26 June 2015	23.32 ± 0.91	24.43 ± 0.78	+
Field 5	Alton	26 June 2015	20.57 ± 0.05	19.6 ± 0.33	+
Field 7	Manitoba	20 July 2015	n/d	n/d	–
Field 5	Manitoba	20 July 2015	23.47 ± 2.34	23.95 ± 2.27	+
Field 8	South of Roland	21 July 2015	25.27 ± 2.90	25.68 ± 1.82	+
Field 6a	West of Emerson	21 July 2015	24.84 ± 3.50	25.21 ± 2.06	+
Field 1	Manitoba	12 August 2015	n/d	n/d	–
Field 3	Manitoba	12 August 2015	26.50 ± 2.81	27.52 ± 1.42	+
Field 9	Lavenham, MB	17 August 2015	25.22 ± 2.32	24.99 ± 1.74	+
Field 9	Lavenham, MB	17 August 2015	25.77 ± 0.13	25.72 ± 0.92	+
Field 10	Haywood, MB	17 August 2015	n/d	n/d	–
Field 15	Elm Creek	12 September 2015	22.82 ± 0.60	21.6 ± 0.42	+
Field 16	Roland S	12 September 2015	24.87 ± 1.51	23.46 ± 0.38	+
Field 17	Steinbach, MB	3 September 2015	24.84 ± 0.43	22.60 ± 0.12	+
Field 18	Myrtle, MB	2 September 2015	n/d	n/d	–
Field 24	NE of Gretna, Rhineland	16 September 2015	27.79 ± 0.18	24.45 ± 0.26	+
Field 25	NE of Emerson, Rhineland	16 September 2015	25.59 ± 0.16	22.77 ± 0.03	+
Field 26	NE of Alton, Moncalm	16 September 2015	n/d	n/d	–
Field 27	NE of Gretna, Rhineland	16 September 2015	n/d	n/d	–
Field 32	New Bothwell	29 September 2015	27.15 ± 1.02	23.46 ± 0.54	+
Field 33	West of Steinbach	29 September 2015	25.25 ± 0.85	23.01 ± 0.72	+
Field 35	Whitemouth	29 September 2015	26.75 ± 0.78	30.40 ± 0.26	+
Field 36	Whitemouth	29 September 2015	n/d	n/d	–
DOAB232	Greenhouse inoculated	19 August 2015	25.34 ± 2.12	25.86 ± 1.74	+
DOAB232	Greenhouse inoculated	29 September 2015	26.86 ± 0.78	21.81 ± 0.41	+

^a Abbreviations: C_T = cycle threshold; SD = standard deviation; n/d = no detectable fluorescence; + and – indicate positive and negative, respectively; and NE = northeast.

^b Collection or field number. DOAB232 = DOAB232-inoculated; greenhouse-inoculated positive controls.

often linked with the degeneration of the genome (Bentley et al. 2008). This process is often associated with bacterial species that seem to have recently adapted to a new niche, thus rendering certain genes expendable and genetically selected for their functional removal (Blanc et al. 2007; Parkhill et al. 2001; Tyagi and Saini 2004). Niche adaptation has evolutionary shortcoming that may lead to an increased number of deleterious mutations and size of insertion sequence elements (Bentley et al. 2008). These could lead to reduced selective pressure (Blanc et al. 2007; Niernan et al. 2004; Parkhill et al. 2001).

The low AT observed on the DNA atlas of *C. michiganensis* subsp. *nebraskensis* strain NCPPB 2581 is characteristic of members of the actinomycetales. The GC skew data shown on the atlas of NCPPB 2581 does not distinctively depict the lagging and leading strands as in some other bacterial species. For example, gram-negative bacteria such as *Acidaminococcus fermentans* strain DSM 20731 (Vesth et al. 2013) and *P. ananatis* AJ13355 (unpublished data) have distinct GC skew for the lagging and leading strands. The juncture of separation is often considered the origin and terminus of replication (Vesth et al. 2013), with G biased toward the leading strand (Marín and Xia 2008). Contrary to Marín and Xia (2008), this study shows that the C are biased toward the leading strand. Similar results were observed on DNA atlases of complete genomes of other *C. michiganensis* subspecies (data not shown).

The atlas of *C. michiganensis* subsp. *nebraskensis* strain NCPPB 2581 also shows varying structural properties on the genome molecule. According to Vesth et al. (2013), the nature of these properties can have significant effects on gene expression and genome rearrangement. Due to differences in base composition, mobile elements can be indicated by regions of different position preference, stacking energy, or intrinsic curvature. For example, the position of the identified GI coincided perfectly with regions of high intrinsic curvature and stacking energy (can easily melt) and low position preference (flexible). Genomic islands (gene clusters potentially from horizontal transfer) are reported to encode a high number of genes involved in important ecological or medical adaptations, including virulence and antimicrobial resistance (Dhillon et al. 2015). In *Megasphaera elsdenii* and *Selenomonas*

sputigena, these regions are thought to correlate well with high gene expression and are associated with the location of *rrn* operons (Vesth et al. 2013). In our study, the rRNA genes on the genome of NCPPB 2581 are located within regions of high intrinsic curvature and stacking energy. DNA curvature plays a significant role in many biologically important processes such as transcription initiation (Angermayr et al. 2002; Kozobay-Avraham et al. 2006; Ohyama 2001; Schroth et al. 1992) termination (Hosid and Bolshoy 2004), recombination (Mazin et al. 1994), DNA replication (Ueguchi et al. 1994), and nucleosome positioning (Kiyama and Trifonov 2002).

The BLAST matrix showed a pairwise proteome comparison which identified whether two proteins are shared between genomes (Binnewies et al. 2005). The BLAST matrix indicated that conservation between genomes is generally higher within than among subspecies. The two draft genomes of strains DOAB 395 and DOAB 397 isolated in 2014 exhibited a proteome homology of 99.2% but had approximately 92% similarity with that of *C. michiganensis* subsp. *nebraskensis* strain NCPPB 2581, the type strain isolated over 40 years ago. This indicates potential differences in genome arrangements between *C. michiganensis* subsp. *nebraskensis* strains. In Enterobacteriaceae genomes, a 76 to 98.8% similarity was found between seven genomes of *Escherichia coli*, with an internal homology of approximately 0.3 to 3% (Marín and Xia 2008). Also, a study performed on genomes from the Vibrionaceae family showed that different strains of *Vibrio cholera* share between 70 and 80% proteins and internal homology ranges from 1.3 to 5.3% (Vesth et al. 2010). Genome analysis of these two recently isolated *C. michiganensis* subsp. *nebraskensis* strains (DOAB 395 and DOAB 397) and the type strain (NCPPB 2581) has indicated potential differences given the relatively low homology between the proteomes. Although work is being done to identify these specific differences and determine their significance, we exploited the genome data for the development of a TaqMan real-time PCR tool for rapid detection of *C. michiganensis* subsp. *nebraskensis*.

Diagnosis of Goss's wilt disease using field inspection for symptoms is insufficient to meet potential plant quarantine requirements. Also, there is no reliable and acceptable enzyme-linked immunosorbent assay-based test for detection or

Fig. 5. Multiplex real-time polymerase chain reaction (PCR) assay is compatible across three frequently used real-time PCR platforms (Chromo4, LightCycler 480, and CFX96). The assay was repeated once, with three replicates per bacterial strain used.

certification of corn grain shipments. In this study, we have developed the first multiplex TaqMan real-time PCR assay for detection of *C. michiganensis* subsp. *nebraskensis* in pure cultures and plant leaf samples. TaqMan real-time PCR assays have several advantages over conventional PCR and culture-dependent methods. Because post-PCR processing steps are not required, the assay can be easily automated, leading to high sample throughput (Weller et al. 2000). This, together with the high specificity and sensitivity of the assay, offers significant advantages. Real-time PCR assays have been developed to detect a wide range of bacterial plant pathogens, including *P. stewartii* subsp. *stewartii* on corn (Tambong et al. 2008), *Pseudomonas syringae* on tomato (Xu and Tambong 2011), and *C. michiganensis* subsp. *sepedonicus* (Schaad et al. 1999) and *Ralstonia solanacearum* (Weller et al. 2000) in potato tubers. The only publicly available TaqMan real-time PCR assay for detection of *C. michiganensis* subsp. *nebraskensis* is that of Bach et al. (2003) that was designed to target the 16S-23S ITS regions. This region is known to display intragenomic heterogeneity in some bacteria (Acinas et al. 2004; Gürtler 1999; Liguori et al. 2011; Tambong et al. 2009), partly due to frequent homologous recombination (Gürtler 1999). Regions of DNA molecules that are prone to high recombination are generally not suitable diagnostic markers. The TaqMan real-time PCR assay reported here uses two genes known to exhibit low recombination events. Also, the use of dual TaqMan probes (*celB* and *rpsJ*) provides an added advantage and high degree of certainty on the specificity and reliability of the assay. A sample (pure culture or infected corn leaf) is reported to be positive for the presence of *C. michiganensis* subsp. *nebraskensis* only when detectable fluorescence thresholds are achieved with both probes.

Using the optimum TaqMan real-time PCR temperature cycling parameters, the sensitivities (0.1 pg for *celB* and 1.0 pg for *rpsJ*) of the assay compared favorably with previously reported real-time PCR assays (Panicker and Bej 2005; Tambong et al. 2008; Xu and Tambong 2011). The *celB* probe (0.1 pg) exhibited a higher sensitivity compared with the probe *rpsJ* (1 pg). The quantum yield of fluorescent dyes attached to nucleotide probes depends on many factors. One of these factors is the fluorescence quenching by adjacent nucleotides (Behlke et al. 2005; Kelley and Barton 1999). This slight difference in sensitivity levels could be attributed to a low quenching effect of cytosine nucleotide on the fluorescence reporter dye (6FAM) at the 5' end of the *rpsJ* probe sequence.

The assay was used successfully to detect the presence of the *C. michiganensis* subsp. *nebraskensis* in 17 infected leaf samples. Positive reactions were consistent, suggesting that the process from DNA extraction to detection is reliable. This indicates that the soil DNA kit used to extract total DNA from infected corn leaves produced high-quality DNA with minimal potential PCR inhibitors. This was confirmed using a TaqMan real-time assay derived from λ DNA (Tambong et al. 2008) used to demonstrate the presence of PCR inhibitions. Seven leaf samples, however, did not exhibit detectable fluorescence, suggesting that *C. michiganensis* subsp. *nebraskensis* might not be present. The sample did not indicate the presence of PCR inhibition using the assay mentioned previously. Examination of the leaf samples did not show typical symptoms (characteristic dark "freckles") of the Goss's wilt disease pathogen.

In this study, we sequenced and assembled the draft genome of *C. michiganensis* subsp. *nebraskensis* DOAB 395 and performed comparative genomics with previously reported draft genomes of *C. michiganensis* subsp. *nebraskensis* strain DOAB 397 (Tambong et al. 2015) and the type strain, NCPPB 2581. Strains DOAB 395 and DOAB 397, isolated in 2014, and strain NCPPB 258, isolated over 40 years ago, seem to show significant genome or proteome differences. Both *C. michiganensis* subsp. *nebraskensis* strains isolated in 2014 have a relatively high number of pseudogenes, attributes of potential adaptive ecological significance. Certainly, the genome sequence data of DOAB 395 obtained in this study should provide a better understanding of *C. michiganensis* subsp.

nebraskensis and, ultimately, allow for new approaches for rapid identification of strains and taxonomic description of novel subspecies. The genome data were exploited to develop a multiplex TaqMan real-time PCR tool for rapid detection. This tool could bridge the current gap of diagnostic techniques for accurate detection or identification of the Goss's wilt disease pathogen. This procedure is robust and rapid and can be automated with high sample throughput potential, permitting analysis of up to 96 samples within 24 h of receiving samples. Avoidance of post-PCR gel electrophoresis greatly reduces the opportunity for contamination of the reaction mixtures with target DNA, further increasing the suitability of this assay for routine diagnostic testing. In future work, the assay will be adapted, optimized, and validated for detection of *C. michiganensis* subsp. *nebraskensis* in corn seed or grain destined for export. The assay would be useful domestically as well to monitor disease and variability of the pathogen with time and location.

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