SHORT COMMUNICATION

MOLECULAR CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF 16S rRNA FROM A NEW "*CANDIDATUS* LIBERIBACTER" STRAIN ASSOCIATED WITH ZEBRA CHIP DISEASE OF POTATO (*SOLANUM TUBEROSUM* L.) AND THE POTATO PSYLLID (*BACTERICERA COCKERELLI* SULC)

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SUMMARY

The full-length 16S rRNA gene region of a new "*Candidatus* Liberibacter" strain was PCR- amplified from tubers of potato plants showing zebra chip (ZC) disease symptoms and also from the potato psyllid [*Bactericera* (= *Paratrioza*) *cockerelli* Sulc], the presumptive vector of the ZC disease causal agent. Sequences of the amplicons from ZC diseased potato and potato psyllids were 100% identical but differed from "*Ca.* L. asiaticus" (Las) and "*Ca.* L. africanus" (Laf) by 4% and from "*Ca.* L. americanus" (Lam) by 6.0-6.3%. Neighbor-joining analysis placed the ZC disease-associated sequences in a monophyletic clade consisting of all known "*Ca.* Liberibacter" spp., positioned in the tree basal to a node shared exclusively by Las and Laf but proximal to a node shared by Lam and all other "*Ca.* Liberibacter" spp. Here we present evidence for the association between a new "*Candidatus* Liberibacter" strain and ZC disease in field samples. The relationship of this Liberibacter to the other Liberibacter bacteria detected in psyllids, potato and other solanaceous crops as well as in citrus remains to be determined.

Key words: "*Candidatus* Liberibacter", zebra chip, potato psyllid, 16S rRNA.

Zebra chip (ZC) has emerged in recent years as a widespread and damaging disease of potato (*Solanum tuberosum* L.) in Mexico, Central America, and the southwestern United States (Secor and Rivera-Varas, 2004; Goolsby *et al*., 2007; Munyaneza *et al*., 2007a, 2007b). Disease symptoms on aboveground plant parts include stunting, chlorosis, leaf scorching and early decline (Munyaneza *et al*., 2007b). Tubers of ZC-affected plants have swollen lenticels and vascular tissue browning, and show poor to no germination as the result of dead eyes. Economic damage caused by the disease is due primarily to brown or dark (zebra) stripes in processed potato chips, rendering them unmarketable (Munyaneza *et al*., 2007b).

Munyaneza and co-workers (Munyaneza *et al*., 2007a, 2007b) provided evidence for a strong association between presence of potato psyllid (*Bactericera cockerelli* Sulc) and development of ZC disease. However, etiology of ZC disease in the U.S. has yet to be established. Earlier studies suggested an association of phytoplasmas, or *Xylella fastidiosa* with ZC disease (Lee *et al*., 2006; Secor *et al*., 2006; Goolsby *et al*., 2008). Although aboveground symptoms of ZC diseased plants are similar to those of potato purple top disease caused by phytoplasmas (Lee *et al*., 2004; Crosslin *et al*., 2005, 2006; Munyaneza *et al*., 2006), extensive testing of ZC symptomatic potato plants and tubers have not established an association of phytoplasma infection with ZC disease (Munyaneza *et al*., 2007a, 2007b).

Recently, MAF Biosecurity New Zealand (MAFBNZ) reported detection of a bacterium that appears to be new species belonging to the genus "*Candidatus* Liberibacter" in glasshouse-grown tomato and capsicum, and in potatoes grown in commercial fields (MAF Biosecurity web report, 2008, http://www.maf.govt.nz/mafnet/press/2008/083006-potatoes.htm). Diagnostic tests showed that the ZC disease-associated organism was different from three known "*Ca*. Liberibacter" spp. ["*Ca*. L. asiaticus" (Las), "*Ca*. L africanus" (Laf), and "*Ca*. L. americanus" (Lam)] associated with citrus Huanglongbing (HLB) disease and the Asian and African citrus pysllids. MAFBNZ further suggested that this newly discovered bacterium may be transmitted to plants by the tomato and potato psyllids (http://www.biosecurity.govt.nz/pests-diseases/plants/potato-tomato-psyllid.htm). The MAFBNZ report was followed by a USDA-APHIS announcement confirming identification of a "*Ca.* Liberibacter" spp. in tubers from two field-grown potato plants collected in Texas, USA and showing typical ZC symptoms

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(https://www.wpdn.org/common/news_events/candidatus_liberibacter/APHIS%20SPRO%202008-32_7-14-08.pdf). As a new strain of "*Ca*. Liberibacter" has been associated with ZC disease in the USA (Lin *et al*., unpublished data), there is an urgent need to determine its taxonomic position to facilitate detection and identification.

"*Ca.* Liberibacter" species are Gram-negative, non-culturable α-proteobacteria. Amplification of the "*Ca*. Liberibacter" species 16S rRNA gene has been accomplished using primers f-D1 and rP1 that universally amplify prokaryotic 16S rRNA gene sequences (Weisburg *et al*., 1991). Analysis of 16S rRNA gene sequences led to identification of three species, Las, Laf (Jagoueix *et al*., 1996), and Lam (Teixeira *et al*., 2005), associated with citrus HLB disease. Each species was named based on presumptive origins in Asia, Africa, and the Americas, respectively (Bové, 2006). However, etiology of HLB has not been established as Koch's postulates have not been completed. Therefore, genomic analysis of these presumptive pathogens is challenging, as pure genomic DNA is not available.

The objectives of this research were to: (i) obtain full-length 16S rRNA gene sequences of the "*Ca.* Liberibacter" spp. from potato psyllids and ZC-diseased potato plants by PCR using conserved primers based on citrus HLB-associated "*Ca*. Liberibacter" spp.; and (ii) determine phylogenetic relationships of ZC-associated "*Ca.* Liberibacter" strains with citrus HLB-associated "*Ca*. Liberibacter" spp.

Potato psyllid adults and nymphs were collected from a severely ZC-affected potato field in Dalhart, Texas. Additional psyllid nymphs and adults were obtained from different laboratory-reared colonies maintained at the USDA-ARS in Wapato (WA, USA). ZC-symptomatic potato (vars. FL1867 and Atlantic) plants and tubers were collected from Garden City (KS, USA), and Dalhart (TX, USA). Healthy potato plants were used as negative controls.

Total genomic DNA was extracted from ZC-symptomatic potato tubers and psyllids as previously described (Lin and Walker, 1997). Total genomic DNA also was extracted from HLB-affected citrus plants infected with Las (Guangxi, China; Florida, USA; São Paulo, Brazil), Laf (Nelspruit, Mpumalanga, South Africa), or Lam (São Paulo, Brazil) and sent as microbe-free, non-infectious nucleic acid samples to the USDA-ARS, San Joaquin Valley Agricultural Sciences Center in Parlier (CA, USA) for analysis. DNA concentration was determined spectrophotometrically and adjusted to 50 ng/µl.

PCR was performed in a 20 µl reaction volume as previously described (Lin *et al*., 2008). For potato psyllid and ZC-diseased tuber DNA samples, forward primer, "CL-16S-F" (5'TTAACACATGCAAGTCGAGCG3') and reverse primer "CL-16S-R", (5'CTACCGACCTCACCCTTATCAG3') were used. Using genomic walking protocol developed previously (Lin *et al*., 2008), 16S rRNA gene amplicons for multiple strains of the three citrus HLB-associated "*Ca* Liberibacter" spp. also were obtained. Amplicons of the expected size were recovered from gels and purified using a PCR purification kit (Invitrogen Inc. USA). Purified PCR products were used directly for sequencing reactions with BigBye Terminator v3.1 Cycle Sequencing Kits (ABI, Foster City, CA). DNA sequences were obtained using an ABI 3130x Genetic Analyzer.

Gene Runner (version 3.05, Hastings Software, Inc.

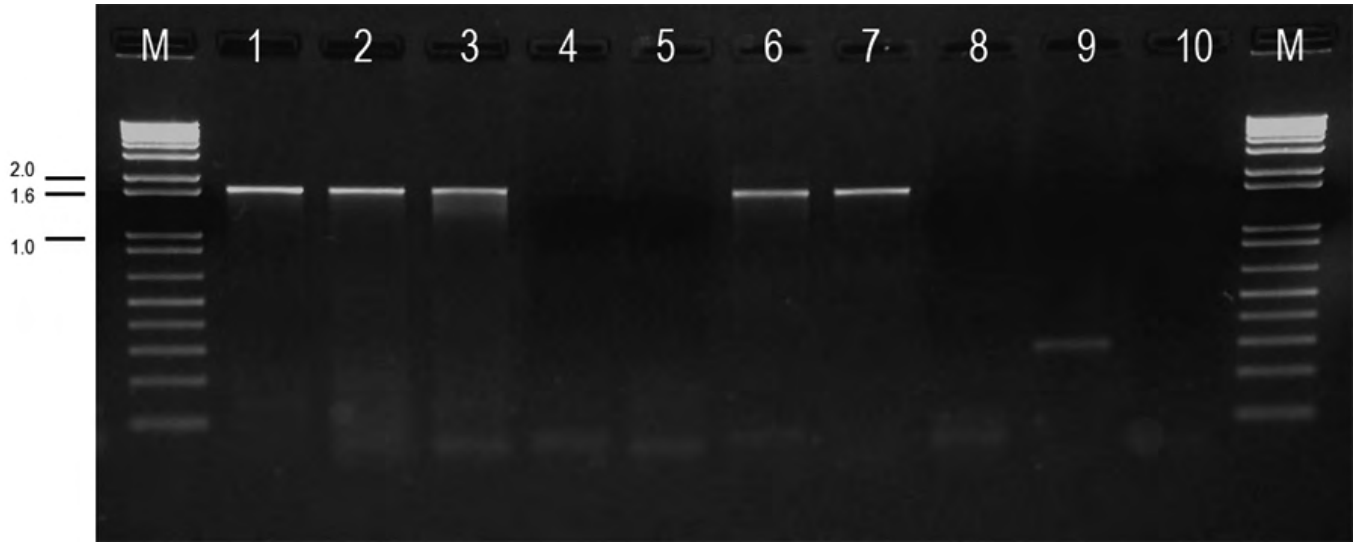

Fig. 1. Electrophoretic image of DNA (1,695 bp) run on a 1.2% agarose gel after amplification from adult potato psyllids (lanes 1, 2, and 3) and zebra chip diseased potato tubers of cvs FL1867 (lane 6) and Atlantic (lane 7). No expected target product was amplified from samples of potato psyllid nymphs (lane 4), negative control psyllids (lane 5), healthy potato tuber (lane 8), healthy potato shoot (lane 9), or the no template negative control (Lane 10). The 1Kb plus DNA marker (Invitrogen Inc, USA) was loaded in the extreme left and right lanes as size standards.

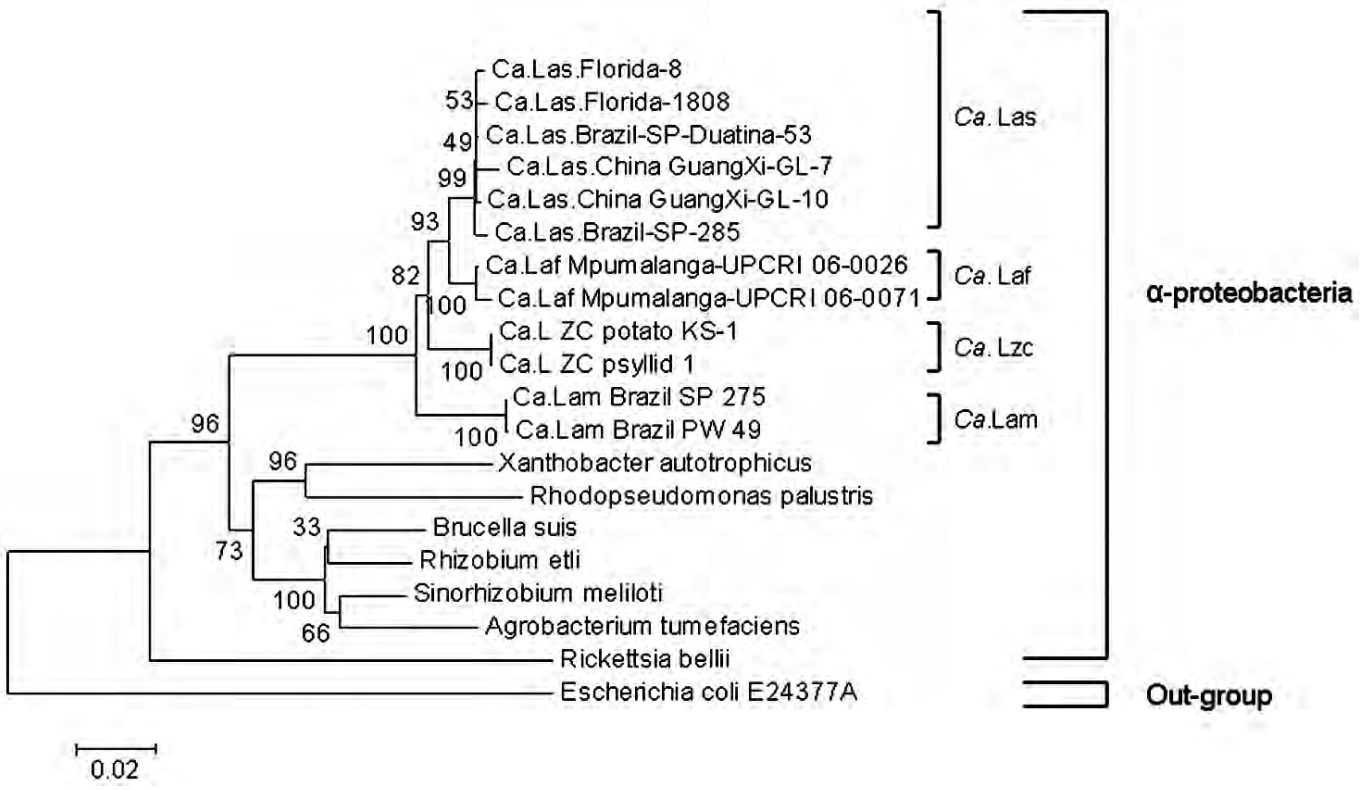

Fig. 2. Phylogenetic relationship of "*Ca*. Liberibacter" associated with zebra chip (ZC)-diseased tubers and ZC-exposed potato psyllids. The Neighbor-Joining tree of 16S rRNA gene sequences is based on 12 "*Ca.* Liberibacter" samples, seven related species of α-proteobacteria, and *Escherichia coli* designated as the outgroup. Evolutionary distances correspond to branch lengths computed using the Maximum Composite Likelihood method and are in units of the number of base substitutions per site. Bootstrap values are shown at the nodes.

Hastings, NY, USA) was used to analyze amplicon sequences. Initial sequence identification was done by BLAST searches of the GenBank 'nr' database. DNA sequences from different "*Ca.* Liberibacter" spp., seven distinct α-proteobacteria (*Agrobacterium tumefaciens, Brucella melitensis*, *Mesorhizobium loti, Rhodopseudomonas palustris*, *Rickettsia bellii*, *Rhizobium etli, Xanthobacter autotrophicus*), and *Escherichia coli* (used as the outgroup) were aligned using CLUSTALW. Neighbor-Joining distance trees were constructed using the Molecular Evolutionary Genetics Analysis software (MEGA software) (Kumar *et al*., 2004). Statistical confidence of tree topology was assessed by 1,000 bootstrap replications. All positions containing gaps or missing data were eliminated from the dataset. The final dataset contained a total of 1,398 nucleotide positions.

Using primers CL-16S-F and CL-16S-R, a 1,695 bp product including the 1,418 bp full-length 16S rRNA region was amplified from multiple samples of ZC-symptomatic tubers and from field-collected adult potato psyllids exposed to ZC-affected potato plants. No product of expected size was amplified using DNA template from psyllid nymphs, healthy potato, clean psyllids, or PCR negative controls (Fig. 1). In addition, 16S rRNA gene sequences were amplified from DNA samples of all three HLB-associated "*Ca.* Liberibacter" spp. New 16S rRNA gene sequences determined in this study for "*Ca*. Liberibacter" from ZC-diseased potato (EU921626), the potato psyllid (EU921627), and HLB-associated species Las (EU921613, EU921614, EU921615, EU921617, EU921618 and EU921622), Laf (EU921620 and EU921621), and Lam (EU921624 and EU921623) were submitted to GenBank.

"*Ca*. Liberibacter" 16S rRNA gene sequences amplified from ZC-diseased potato and potato psyllid samples were 100% identical. Among the 10 citrus HLB-associated "*Ca.* Liberibacter" sequences, Lam was unique with respect to a 20 bp deletion within the 16S rRNA gene region. The ZC-associated 16S rRNA gene sequences were 96% identical to sequences from Las and Laf, and 93.7 to 94% identical to sequences from Lam. Genetic divergence estimates revealed a divergence of 0.029±0.004 between the ZC-associated 16S rRNA gene sequence and Las and Laf sequences, and a divergence of 0.040±0.005 between ZC-associated and Lam 16S rRNA gene sequences. The citrus HLB-associated and ZC-associated "*Ca.* Liberibacter" spp. were genetically distant from three groupings of α-proteobacteria by 0.101±0.011 to 0.134±0.012 (Rhizobiaceae), 0.134±0.012 to 0.153±0.014 (Rhodobacteraceae), and

0.188±0.015 to 0.205±0.016 (Rickettsiales).

A phylogenetic tree constructed using the Neighbor-Joining method clustered all "*Ca*. Liberibacter" taxa into a monophyletic group (Fig. 2). All three previously known citrus HLB-associated "*Ca*. Liberibacter" spp. grouped as expected based on previous analyses (Bové, 2006). Interestingly, the newly discovered "*Ca*. Liberibacter" 16S rRNA gene sequences associated with potato psyllids and ZC-diseased potato were positioned at a node proximal to the basal node shared by all "*Ca*. Liberibacter" spp., inferring early divergence of Lam from other HLB-associated "*Ca*. Liberibacter" spp. and the ZC-associated "*Ca*. Liberibacter".

Previously, all known species of "*Ca*. Liberibacter" (Las, Laf, and Lam) were associated only with citrus HLB and transmitted by two psyllid vector species, *Diaphorina citri* and *Trioza erytreae* (Bové, 2006). In this study, we amplified and characterized full-length 16S rRNA gene sequences from "*Ca*. Liberibacter" associated with ZC disease of potato. Sequences from adult potato psyllid (*Bactericera cockerelli* Sulc) and ZC-symptomatic potatoes collected in the field were identical, suggesting that the same bacterium is present in both potato tubers and potato psyllids. Our study therefore supports the conclusion of Munyaneza *et al*. (2007a, 2007b) of a strong association between the potato psyllid and ZC disease. In addition, these results provide molecular evidence for a bacterial pathogen as the presumptive causal agent of ZC disease.

Recently, two new "*Ca.* Liberibacter"' 16S rRNA gene sequences from New Zealand amplified from tomato (*Solanum lycopersicum* L.) and potato (*Solanum tuberosum* L.) became available in NCBI (EU834130 and EU849020, respectively). Sequence alignment showed that both sequences have 99.8% identity with our potato psyllid and potato ZC-diseased tuber sequences. Two single nucleotide polymorphisms (SNPs) differentiate the North American ZC-associated "*Ca*. Liberibacter" sequences from the New Zealand sequences.

DNA sequence divergence data, particularly divergence in 16S rRNA, are widely used for defining species (Cohan, 2002). Bacteria with >3% divergence in 16S rRNA gene sequence are nearly always members of a different species. Therefore, a cut-off of 3% divergence was recommended as a criterion for demarcating species (Stackebrandt and Goebel, 1994). This is particularly useful for classification of unculturable bacterial taxa, such as "*Ca*. Liberibacter", for which phenotypic and ecological characters are limited or not available. DNA sequence comparisons of the ZC-associated 16S rRNA gene sequence revealed ~94-96% identity with known "*Ca*. Liberibacter" spp. and indicated that the ZC-associated "*Ca*. Liberibacter" was more closely related to Las and Laf than to Lam. As the divergence value exceeded 3% for all two-way comparisons among the ZC-associated "*Ca* Liberibacter" and other taxa, our results suggest that the ZC-associated "*Ca*. Liberibacter" may warrant evaluation as a new species. That the monophyletic clade comprising all known "*Ca*. Liberibacter" taxa shares a common node with all other clades of the α-proteobacteria, except the order Rickettsiales, suggests early divergence of the "*Ca*. Liberibacter" lineage from most other α-proteobacteria.

In this study, we describe molecular characterization and phylogenetic analysis of the 16S rRNA gene of a new strain of "*Ca.* Liberibacter" associated with ZC disease in the USA. While this manuscript was being prepared, we became aware of a report (Hansen *et al*., 2008) referring to a new 'HLB "*Ca.* Liberibacter" species', based on 16S rRNA gene information. This newly described bacterium, designated as "*Ca*. Liberibacter psyllaurous" by Hansen *et al*. (2008), was detected in tomato and potato and vectored by the potato psyllid. Further work is needed to define relationships among the "*Ca*. Liberibacter" bacteria detected in solanaceous crops and associated psyllids in North America (Hansen *et al*., 2008; this report) and New Zealand (Liefting *et al*., 2008).

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