A New '*Candidatus Liberibacter*' Species Associated with Diseases of Solanaceous Crops

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ABSTRACT

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A new disease of glasshouse-grown tomato and pepper in New Zealand has resulted in plant decline and yield loss. Affected plants are characterized by spiky, chlorotic apical growth, curling or cupping of the leaves, and overall stunting. Transmission electron microscopy revealed the presence of phloem-limited bacterium-like organisms in symptomatic plants. The strategy used to identify the bacterium involved using specific prokaryote polymerase chain reaction (PCR) primers in combination with universal 16S rRNA primers. Sequence analysis of the 16S rRNA gene, the 16S/23S rRNA spacer region, and the *rpLKAJL-rpoBC* operon revealed that the bacterium shared high identity with '*Candidatus Liberibacter*' species. Phylogenetic analysis showed that the bacterium is distinct from the three citrus liberibacter species previously described and has been named '*Candidatus Liberibacter solanacearum*'. This is the first report of a liberibacter naturally infecting a host outside the *Rutaceae* family. A specific PCR primer pair was developed for its detection.

In January 2008, a disease of glasshouse tomato (*Solanum lycopersicum*) crops was observed in Auckland, New Zealand. Symptoms included spiky, chlorotic apical growth, general mottling of the leaves, curling of the midveins, overall stunting of the plants, and in some cultivars fruit deformation. Cultivars varied in severity of symptoms and levels of disease incidence. This disease problem has been reported from three commercial glasshouse tomato growers in Auckland, and losses of up to NZ\$1 million have been reported.

Extensive testing was done to determine the etiology of this disease. No pathogenic fungi or culturable bacteria were isolated. Generic tests for viruses, including herba-

ceous indexing, transmission electron microscopy (leaf dip method), and dsRNA purification, were all negative. Polymerase chain reaction (PCR) tests for phytoplasmas, geminiviruses, tombusviruses, ilarviruses, potexviruses, *Beet pseudo yellows virus*, *Cucumber mosaic virus*, *Tomato spotted wilt virus*, *Tobacco mosaic virus*, *Tomato chlorosis virus*, *Tomato infectious chlorosis virus*, and pospiviridae were also negative.

The tomato/potato psyllid, *Bactericera cockerelli* (Homoptera: Psyllidae), was reported to occur in all tomato glasshouses where these disease symptoms were present. *B. cockerelli* was first discovered in an Auckland glasshouse tomato crop in May 2006, and is now established throughout the North Island and the top part of the South Island of New Zealand. Due to the similarity of symptoms with those reported as "psyllid yellows" and the absence of a positive result for other possible causal organisms, the symptoms were thought to be those of psyllid yellows, first reported by Richards (28), and thought to be caused by a toxin associated with feeding by the nymphal instars.

In April 2008, similar symptoms appeared in a glasshouse pepper (*Capsicum annuum*) crop on the same property as one of infected tomato crops. Peppers are not

known to be susceptible to psyllid yellows; therefore, further investigation was done. Transmission electron microscopy of thin sections of leaf tissue revealed the presence of bacterium-like organisms (BLOs) in the phloem of symptomatic tomato and pepper. There are no previous reports of BLOs in tomato or pepper, and only a small number of BLOs causing plant disease have been characterized at the molecular level. Of these, the three '*Candidatus Liberibacter*' species associated with huanglongbing of citrus are the best characterized (13,35). Other BLOs with 16S rRNA gene sequences available include '*Candidatus Phlomobacter fragariae*' of strawberry (38), yellow vine disease of cucurbits (2), and syndrome "basses richesses" of sugar beet (31). A difficulty in the characterization of BLOs is the inability to culture these organisms in vitro.

In this study, we report the initial discovery of a BLO in tomato and pepper by electron microscopy and the strategy used to determine its identity. Phylogenetic analysis of the 16S rRNA gene, the 16S/23S rRNA spacer region, and the *rpLKAJL-rpoBC* operon revealed that the BLO is a new species of the '*Candidatus Liberibacter*' genus. A specific PCR primer pair for its detection was developed.

MATERIALS AND METHODS

Plant and insect material. Symptomatic tomato and pepper plants were collected from commercial glasshouses in the Auckland region between January and May 2008. Healthy tomato and pepper were grown from seed in an insect-proof glasshouse to use as negative controls. *B. cockerelli* psyllids were collected from glasshouses with symptomatic plants. Total DNA from citrus infected with '*Candidatus Liberibacter asiaticus*' and '*Candidatus Liberibacter americanus*' was kindly provided by Diva Teixeira (Fundecitrus, Araquara, Brazil) and Helvécio Coletta-Filho (Centro APTA Citros Sylvio Moreira – Instituto Agronômico de Campinas, Brazil); and total DNA from citrus infected with '*Ca. L. africanus*' was provided by Gerhard Pietersen (Citrus Research Inter-

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national, University of Pretoria, South Africa).

Transmission electron microscopy. Secondary leaf veins of symptomatic and healthy plants were cut into 1×5 mm pieces and fixed in 2% freshly prepared paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 under vacuum for 1 h. The samples were then washed three times in buffer, post-fixed in 1% osmium tetroxide for 1 h, dehydrated in an ethanol series, infiltrated, and embedded in Spurr's resin. Sections 120 nm thick were cut and placed on Formvar coated grids and stained in 1% vol/vol aqueous uranyl acetate and lead citrate (29). Sections were viewed on a JEOL JEM-1200EX II transmission electron microscope (Tokyo) operating at 80 kV.

DNA isolation. Plant DNA was extracted from leaf midribs and petioles of symptomatic and healthy plants using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Psyllid nymphs in batches of approximately 20 were placed in 1.5-ml microcentrifuge tubes with 180 μ l of 0.01 M PBS (0.138 M NaCl, 0.0027 M KCl, pH 7.4; Sigma-Aldrich, St Louis, MO) and homogenized using a disposable microtube pestle. DNA was extracted from the homogenized psyllids using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions.

PCR amplification. PCR primers used in attempts to amplify a fragment of the BLO were as follows: Fra 4/Fra 5 (38), LSg2f/LSg2r (4), and 16S-23SF/16S-23SR (24). The primer pairs Fra 4/Fra 5 and LSg2f/LSg2r were designed to detect '*Ca. Phlomobacter fragariae*' and '*Ca. L. americanus*', respectively (4,38). The primer pair 16S-23SF/16S-23SR was designed by Martinati et al. (24) to amplify the 16S/23S rRNA spacer region of *Xylella fastidiosa*. These primers were selected because BLASTn analysis determined that they also match the 16S rRNA or 23S rRNA gene of a wide range of bacteria. The universal prokaryote 16S rRNA primer pair fD2/rP1 (36) was used to obtain PCR products for cloning. These primers amplify the 16S rRNA gene from bacteria and plants. The fD2/rP1 primers were also used in combination with each of the more specific primers described above. Amplification was performed in 20- μ l reactions containing 1 $\times$ PCR buffer (Invitrogen, Carlsbad, CA), 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.25 μ M of each primer, and 1 U of Platinum *Taq* polymerase (Invitrogen). The PCR conditions were: an initial cycle at 94°C for 5 min, then 40 cycles of 94°C for 30 s, 48°C for 30 s, and 72°C for 1 min, plus an additional cycle of 10 min at 72°C. All amplifications were performed in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The amplified DNA was

analyzed by agarose gel electrophoresis, and bands were visualized with SYBR Safe (Invitrogen).

DNA cloning and sequencing. PCR products were either gel purified using the Quantum Prep Freeze 'N Squeeze DNA Gel Extraction Spin Columns (Bio-Rad, Hercules, CA) or using the QIAquick PCR Purification Kit (Qiagen). The purified PCR products were either sequenced directly or cloned into the pCR 4-TOPO vector (Invitrogen) followed by transformation into One Shot TOP10 chemically competent *Escherichia coli* according to the manufacturer's instructions (Invitrogen). Plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen). The DNA fragments were sequenced in both directions using the M13 forward and reverse primers for cloned inserts or the relevant PCR primer for direct sequencing of PCR products. DNA was sequenced on an ABI Avant 3100 Genetic analyzer using BigDye 3.2 chemistry.

Sequence analysis. The sequences were assembled using the SeqMan software of the LaserGene package (DNASTAR, Madison, WI). Searches of the GenBank database for homologous sequences were performed using the BLASTn network service available at the National Center for Biotechnology Information (Bethesda, MD, USA). The software tRNAscan-SE (23) was used to find transfer RNA genes.

Phylogenetic analysis. Multiple sequence alignments were performed using Clustal X 2.0 (17). Modeltest 3.7 (27) was used to determine the optimal analysis method. The general time reversible model with the proportion of invariant sites and the gamma shape parameter (GTR+I+ Γ) was selected. Neighbor-joining (NJ) trees were constructed using HKY85 parameters, maximum likelihood (ML) trees were constructed using the Modeltest parameters, with 10 replicates, and both the NJ and ML trees were analyzed in PAUP* 4.0b10 (32). Bayesian inference trees were constructed with MrBayes 3.12 (12,30) with a flat Dirichlet distribution, and run for seven million generations with the burnin set past the point of log probability leveling off.

Primer design and PCR protocol for specific detection of the BLO in tomato and pepper. The forward primer OA2 (5'-GCGCTTATTTTAAATAGGAGCGGCA-3') was designed from the 16S rRNA sequence of the tomato/pepper BLO. This primer is located in the same region as OI1 and OA1, and when used in combination with OI2c, a 1,160-bp product is expected (14). The forward primers OI1 and OA1 were designed by Jagoueix et al. (14) to be specific to '*Ca. L. asiaticus*' and '*Ca. L. africanus*', respectively, while the reverse primer OI2c matches both liberibacter species. The sequences of OI1 and OA1 are identical except for a trinucleotide located 12 bp from the 3' end. PCR condi-

tions were the same as above except that the annealing step was carried out at 65°C.

RESULTS

Symptomatology. Symptoms associated with the BLO in tomato vary in severity and prevalence between cultivars. New leaves develop interveinal chlorosis (Fig. 1A), and the growing tips have a spiky appearance and may show purpling of the midveins depending on the cultivar (Fig. 1B). Severely affected leaflets can sometimes have a curled midvein forming a cupped structure, with extended petioles (Fig. 1C). The plants exhibit overall stunting. Fruit may be misshapen with a strawberry-like appearance and uneven development of fruit locules (data not shown). In some cases, there is no fruit set at all.

Pepper plants infected with the BLO have chlorotic or pale green leaves (Fig. 1D), shortened internodes, and overall stunting. Other symptoms include leaf cupping (Fig. 1E) and sharp tapering of the leaf apex, resulting in a spiky appearance. Apical meristem necrosis and flower abortion may also occur.

Transmission electron microscopy. Transmission electron microscopy revealed the presence of numerous BLOs in the phloem sieve tubes of symptomatic tomato plants (Fig. 2) but not in healthy tomato plants grown from seed (data not shown). Fewer BLOs were observed in symptomatic pepper plants, which may reflect uneven distribution in the plant rather than a lower titer (data not shown). A total of 50 cross-sections and 20 longitudinal-sections were measured, and the BLOs were determined to be approximately 0.2 μ m in width and 4 μ m in length.

Identification of the BLO. Two strategies were used to obtain sequence from the 16S rRNA gene of the BLO from symptomatic tomato plants. The first was that used to isolate the 16S rRNA gene of '*Ca. L. africanus*' (13), '*Ca. L. asiaticus*' (13), '*Ca. L. americanus*' (35), and '*Ca. Phlomobacter fragariae*' (38). This method involved amplifying the 16S rRNA gene from symptomatic tomato plants with the fD2 and rP1 primers (36), which are universal primers for amplification of the 16S rRNA gene of prokaryotes as well as plant organelles. The resulting PCR product was cloned, and 56 transformants were analyzed by digestion with *Bcl*I and *Eco*RI. The clones exhibited two different types of restriction profiles (data not shown), and database searches of the sequences of these clones indicated that they were of plant mitochondrial and chloroplast origin.

The second strategy was to use primers specific for prokaryote 16S rRNA sequences in combination with the universal fD2 and rP1 primers (36). Séméty et al. (31) used this strategy to amplify the 16S rRNA gene from syndrome "basses richesses" BLO of sugar beet. The primer

pair LSg2f/rP1 amplified a 1.4-kb fragment from DNA of both healthy (Fig. 3A, lanes 1 and 2) and symptomatic (Fig. 3A, lanes 3 and 4) tomato plants. This primer pair amplified an additional 1-kb fragment from symptomatic plants only, indicating that this fragment is specific to the BLO (Fig. 3A, lanes 3 and 4). The primer combinations fD2/LSg2r and LSg2f/LSg2r produced the same size fragment from both healthy and symptomatic plants, indicating that these fragments are of plant origin (Fig. 3B and C).

The 1-kb band from symptomatic tomato plants obtained using the primer pair LSg2f/rP1 was gel-purified, cloned, and sequenced. A search of the GenBank database revealed that this initial sequence shared 97% identity with the 16S rRNA gene of both '*Ca. L. asiaticus*' and '*Ca. L. africanus*'. However, PCR with primer pairs OI1/OI2c and OA1/OI2c (14) (designed for detection of '*Ca. L. asiaticus*'

and '*Ca. L. africanus*', respectively) using DNA extracted from symptomatic tomato plants failed to amplify the expected product (data not shown).

Sequence analysis of the 16S rRNA gene. The primer OI2c was identical to the initial sequence from the tomato BLO, and therefore the remainder of the 16S rRNA sequence was obtained using the primer pair fD2/OI2c. This 16S rRNA gene sequence has been deposited in the GenBank database with accession number EU834130. Analysis of this sequence revealed that the OI1 and OA1 primers, designed from '*Ca. L. asiaticus*' and '*Ca. L. africanus*', respectively, differed significantly from the sequence of the tomato BLO. The unique signature sequence regions from the three previously identified liberibacter species were not present in the tomato BLO. The 16S rRNA sequences of the BLO from tomato and pepper were identical. The sequence shared 96% iden-

tity with the 16S rRNA gene of both '*Ca. L. asiaticus*' and '*Ca. L. africanus*' and 95% identity with '*Ca. L. americanus*'.

Sequence analysis of the 16S/23S rRNA spacer region and ribosomal protein genes of the β -operon. To further compare the tomato BLO to the known liberibacter species, two additional fragments of the genome were amplified and sequenced. The 16S/23S rRNA spacer region was amplified with the primer pair OI2/23S1 (15) and the sequence assembled with the 16S rRNA gene sequence (GenBank accession number EU834130). This region was found to contain two tRNA genes, those for isoleucine and alanine. The 16S/23S rRNA spacer regions of '*Ca. L. asiaticus*' (15) and '*Ca. L. americanus*' (35) also contained tRNA^{Ile} and tRNA^{Ala}, while that of '*Ca. L. africanus*' contained only tRNA^{Ala} (15). The 16S/23S rRNA spacer region of the tomato BLO showed the highest identity with '*Ca. L. asiaticus*'

Fig. 1. Symptoms associated with bacterium-like organism (BLO) infection in tomato and pepper. **A**, Chlorotic tomato plant in the foreground surrounded by asymptomatic plants; **B**, spiky growing tip of tomato with purpling of midveins; **C**, chlorotic, curled midveins of tomato leaflets compared with normal leaves at bottom right of photo; **D**, chlorotic pepper plant in the foreground surrounded by asymptomatic plants; and **E**, cupped leaves of infected pepper.

(80%) followed by '*Ca. L. americanus*' (79%) then '*Ca. L. africanus*' (78%).

A 1.7-kb fragment of the *rplKAL-rpoBC* operon from the tomato BLO was amplified with the primer pair fp 1898/rp 1897 (26) and sequenced (GenBank accession number EU834131). The sequence shared 78 to 79%, 76 to 79%, and 70% identity to '*Ca. L. asiaticus*', '*Ca. L. africanus*', and '*Ca. L. americanus*' sequences, respectively. The primer pair, A2/J5, also designed from this operon (11), amplified the same sized product from healthy and symptomatic tomato and pepper plants, so this primer pair was not useful for characterization (data not shown). There were 5 mismatches at the 3' end of the A2 primer

when compared to the sequence of the *rplKAL-rpoBC* operon from the tomato BLO, suggesting that this primer will not successfully amplify the target sequence from this organism.

Phylogenetic analysis. Phylogenetic trees using 16S rRNA sequences were constructed using neighbor-joining, maximum likelihood, and Bayesian inference methods. All trees were congruent. The Bayesian inference analysis is presented here as representative (Fig. 4). The phylogeny is robust with good posterior probability support for most clades. The tree-island profile of the maximum likelihood tree indicated 14 very similar trees that differed only in the rearrangement of the

'*Ca. L. asiaticus*' sequences (data not shown).

These data show that the organism is clearly a member of the genus '*Ca. Liberibacter*' and is distinct from the currently described species and strains (Fig. 4), and is hereafter referred to as the tomato/pepper liberibacter. Phylogenetic analysis of the 16S/23S rRNA spacer region and the *rplKAL-rpoBC* operon confirmed this conclusion (data not shown). The phylogenetic relationship with respect to the other liberibacter species is uncertain. In all analyses, the tomato/pepper liberibacter does not appear in the '*Ca. L. americanus*' and '*Ca. L. asiaticus*'/'*Ca. L. africanus*' clades, but terminates on its own

Fig. 2. Transmission electron micrographs of tomato sieve tube elements (st) infected with bacterium-like organisms (BLOs). Small arrows indicate the BLOs in cross-section and large arrows indicate the BLOs in longitudinal section. **A**, Low magnification of several sieve tubes; **B**, higher magnification of BLOs in a cross-section of a sieve tube element; and **C**, higher magnification of a longitudinal section of a sieve tube element.

branch. The percent nucleotide identity shows the tomato/pepper liberibacter marginally more closely related to '*Ca. L. asiaticus*' and '*Ca. L. africanus*' than to '*Ca. L. americanus*'.

Specific PCR detection of the tomato/pepper liberibacter. The primer OA2 designed from the 16S rRNA sequence of the tomato/pepper liberibacter amplified the expected 1,160-bp fragment from DNA extracted from symptomatic tomato and pepper plants when used in

combination with the OI2c primer (Fig. 5A, lanes 3 and 4, respectively). No fragment was amplified from DNA from healthy tomato or pepper (Fig. 5A, lanes 1 and 2, respectively) or from DNA extracts from citrus infected with '*Ca. L. asiaticus*', '*Ca. L. africanus*', or '*Ca. L. americanus*' (Fig. 5A, lanes 5, 6, and 7, respectively). The OI1/OI2c and OA1/OI2c primer pairs (14) amplified a fragment from both '*Ca. L. asiaticus*' and '*Ca. L. africanus*' as expected (Fig. 5B and C, lanes 5 and 6, re-

spectively), whereas the primer pair GB1/GB3 (34) amplified a product of the expected size only with DNA from citrus infected with '*Ca. L. americanus*' (Fig. 5D, lane 7) as previously reported (18).

The OA2/OI2c primer pair detected the liberibacter in all symptomatic tomato and pepper plants tested to date (approximately 42 samples) (data not shown). A fragment of the correct size was also amplified from DNA extracted from a batch of 20 *B. cockerelli* psyllids collected from symptomatic plants, and the origin of this fragment was confirmed to be from the tomato/pepper liberibacter by sequence analysis (data not shown).

DISCUSSION

In this study we have identified a new '*Candidatus Liberibacter*' species that is an important disease in glasshouse tomato and pepper crops. Previous to this finding, three liberibacter species had been recognized, '*Ca. L. asiaticus*', '*Ca. L. africanus*', and '*Ca. L. americanus*', collectively associated with the citrus disease huanglongbing (formerly known as citrus greening). Typical symptoms of huanglongbing disease include blotchy leaf

Fig. 3. Agarose gel electrophoresis of polymerase chain reaction (PCR) products amplified from DNA extracts of two healthy (lanes 1 and 2) and two symptomatic (lanes 3 and 4) tomato plants with primer pairs **A**, LSg2f/rP1; **B**, fD2/LSg2r; and **C**, LSg2f/LSg2r. Lane 5 depicts the product of a no template control reaction. Lanes marked (M) depict the 100-bp size marker ladder (Invitrogen).

Fig. 4. A 50% majority rule consensus Bayesian inference phylogenetic tree based on 16S rRNA sequences showing the relationships of the four '*Candidatus Liberibacter*' species and representatives of the alpha subdivision of the *Proteobacteria*. *Escherichia coli* was used as an outgroup. GenBank accession numbers are given in parentheses. Posterior probabilities are indicated above nodes. Scale bar shows inferred number of changes per site.

mottle, the development of yellow shoots, tree decline and stunting, and the production of small, lopsided fruit that may fail to color properly (3). The symptoms of huanglongbing were first observed in southern China in the late nineteenth century, but it wasn't until 1994 that the causal agent was characterized by its 16S rRNA gene and 16S/23S intergenic region sequences (13). The bacterium was found to be a new lineage of the alpha subdivision of the *Proteobacteria* and was given the trivial name liberobacter (13), later replaced by liberibacter (9). The liberibacters are phloem-limited, Gram-negative, unculturable bacteria that are spread between plants by grafting and psyllid insect vectors (reviewed by Bové [3]).

Defining a new species of bacterium is typically achieved by a combination of sequence data and biochemical tests. However, since the liberibacters are not culturable, identification must rely primarily on DNA sequence data and phylogenetic analysis. To ensure a robust and accurate result, we sequenced three different genome regions (16S rRNA, 16S/23S rRNA spacer region, and the *rp1KAJL-rpoBC* operon) and analyzed them with three different phylogenetic analysis methods, neighbor-joining, maximum likelihood, and Bayesian inference. In every analysis, the tomato/pepper liberibacter was unambiguously assigned as a member of the liberibacter clade, but was distinct from the currently described species. Based on the sequence and the novel host range, we consider the tomato/pepper liberibacter to be a new species of the genus '*Candidatus Liberibacter*'. We have named this new liberibacter species '*Candidatus Liberibacter solanacearum*' (21), the specific epithet referring to the family of plant hosts from which the bacterium has been isolated.

The solanaceae liberibacter described in this study is the first recognized natural infection of a host outside the *Rutaceae*. '*Candidatus Liberibacter*' species were originally reported to naturally occur in citrus, with all *Citrus* species and cultivars being sensitive to infection by this destructive disease (3). The ornamental rutaceous trees, *Calodendrum capense* (Cape chestnut) (9) and *Murraya paniculata* (orange jasmine) (37), are also natural hosts for the

citrus-infecting liberibacters. Experimental transmission of '*Ca. L. asiaticus*' by dodder has been demonstrated for tomato, where resulting symptoms included vein clearing, leaf mottle, thick and leathery older leaves, with development of some lopsided fruits (6), as well as for periwinkle (*Catharanthus roseus*) (7) and tobacco (*Nicotiana tabacum* 'Xanthi') (8).

'*Ca. L. solanacearum*' likely has a different insect vector from the citrus liberibacters. The two known psyllid vectors of citrus-infecting liberibacters, *Diaphorina citri* and *Trioza erytreae*, do not occur in New Zealand, and therefore another insect is responsible for disease spread. We suspect that the *B. cockerelli* psyllid is the vector of '*Ca. L. solanacearum*'. This psyllid was present in all three glasshouses where the liberibacter has been detected. The liberibacter was detected in *B. cockerelli* by PCR, indicating that it can acquire the bacteria. The bacteria must pass through the gut barrier and multiply within the insect until it reaches the salivary glands before it can be transmitted to another plant. Transmission studies with infected psyllids are needed to confirm its vector status.

The citrus liberibacters were first observed by transmission electron microscopy by Laffèche and Bové (16) and by scanning electron microscopy by Tanaka et al. (33). Transmission electron microscopy of '*Ca. L. solanacearum*' revealed that the organisms are 0.2 µm in width and 4 µm in length. The width of '*Ca. L. solanacearum*' is comparable to that of the citrus liberibacters with measurements between 0.1 and 0.3 µm (16,33). The length of '*Ca. L. solanacearum*' is longer than the 2 to 3 µm reported for the citrus liberibacters, possibly reflecting the inaccuracy of measuring this dimension, as they are often tangential to the plane of the longitudinal section.

The two strategies used in this study to identify the new liberibacter species have been used by other researchers. The use of specific 16S rRNA prokaryote primers in combination with the universal fd2 and rP1 primers enabled amplification of a fragment of the 16S rRNA gene. At this stage, we did not know the identity of the BLO, and it was a coincidence that LSg2f/rP1 was the successful primer pair.

The LSg2f primer was specifically designed for PCR detection of '*Ca. L. americanus*' (4), but we selected this primer to use in combination with the universal 16S rRNA primer because BLAST analysis suggested that it would also bind to the 16S rRNA gene of other bacteria. We do not understand why the strategy of amplifying the 16S rRNA gene of the liberibacter using the universal prokaryote 16S rRNA PCR primers failed. It could possibly have been due to preferential amplification of the host plant DNA over the bacterial DNA. Bové (3) recommends that the amount of plant mitochondrial 16S rRNA can be minimized by digestion of the DNA extract with the restriction enzyme *BclI* prior to amplification with the universal 16S rRNA primers. However, this would have been detrimental in this study, as '*Ca. L. solanacearum*' contains a *BclI* site in the universal forward primer (fd2) binding region, which would have precluded the amplification of its 16S rRNA gene.

The origin of '*Ca. L. solanacearum*' is uncertain. The emergence of this disease approximately 2 years after the incursion of *B. cockerelli* into New Zealand fits with the theory that the liberibacter arrived with the psyllid. If *B. cockerelli* is the vector of '*Ca. L. solanacearum*', the host range of the liberibacter could potentially include other plants in the family Solanaceae due to the vector's strong preference to feed on these hosts. We have already detected the new liberibacter species in three additional solanaceous plants, potato (*Solanum tuberosum*) (19), tamarillo (*Solanum beta-ceum*), and cape gooseberry (*Physalis peruviana*) (20). A new disease of potato called "zebra chip" associated with *B. cockerelli* (25) observed in Mexico, Guatemala, and the United States has been determined to be caused by a BLO (5). While this manuscript was under review, several researchers in the United States detected the solanaceous liberibacter species described in this study in potato showing zebra chip disease symptoms, as well as in tomato and *B. cockerelli* (1,10,22).

Work currently in progress to further characterize '*Ca. L. solanacearum*' includes (i) distribution of the bacterium in the host plant and fruit, (ii) seed, graft, and insect transmission studies, (iii) extent of

Fig. 5. Agarose gel electrophoresis of polymerase chain reaction (PCR) products amplified from DNA extracts of healthy tomato (lane 1), healthy pepper (lane 2), symptomatic tomato (lane 3), symptomatic pepper (lane 4), or citrus tissue infected with '*Candidatus Liberibacter asiaticus*' (lane 5), '*Ca. L. africanus*' (lane 6), or '*Ca. L. americanus*' (lane 7) with primer pairs **A**, OA2/OI2c; **B**, OI1/OI2c; **C**, OA1/OI2c; and **D**, GB1/GB3. Lane 8 depicts product from no template control reactions. Lanes marked (M) indicate the 100-bp size marker ladder (Invitrogen).

its distribution in New Zealand, and (iv) alternative host plants. This information will aid in the development of management strategies for this new disease of solanaceous crops.

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