

Aspects of Pathogen Genomics, Diversity, Epidemiology, Vector Dynamics, and Disease Management for a Newly Emerged Disease of Potato: Zebra Chip

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ABSTRACT

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An overview is provided for the aspects of history, biology, genomics, genetics, and epidemiology of zebra chip (ZC), a destructive disease of potato (*Solanum tuberosum*) that represents a major threat to the potato industries in the United States as well as other potato-production regions in the world. The disease is associated with a gram-negative, phloem-limited, insect-vectored, unculturable prokaryote, ‘*Candidatus* Liberibacter solanacearum’, that belongs to the *Rhizobiaceae* family of α -Proteobacteria. The closest cultivated relatives of ‘*Ca. L. solanacearum*’ are members of the group of bacteria known as the α -2 subgroup. In spite of the fact that Koch’s postulates *sensu stricto* have not been fulfilled, a great deal of progress has been made in understanding the ZC disease complex since discovery of the disease. Nevertheless, more research is needed to better understand vector biology, disease mechanisms, host response, and epidemiology in the context of vector–pathogen–plant interactions. Current ZC management strategies focus primarily on psyllid control. The ultimate control of ZC likely relies on host resistance. Unfortunately, all commercial potato cultivars are susceptible to ZC. Elucidation of the ‘*Ca. L. solanacearum*’ genome sequence has provided insights into the genetic basis of virulence and physiological and metabolic capability of this organism. Finally, the most effective, sustainable management of ZC is likely to be based on integrated strategies, including removal or reduction of vectors or inocula, improvement of host resistance to the presumptive pathogen and psyllid vectors, and novel gene-based therapeutic treatment.

Additional keywords: genetic diversity, potato psyllid, psyllid *Bactericera cockerelli*.

Zebra chip (ZC) is a destructive disease of potato (*Solanum tuberosum*) that has emerged in North America as well as other potato-production regions in the world. The disease has caused significant economic losses to the U.S. potato industry. ZC was first discovered around Saltillo, Mexico in the 1990s (24,37,98). In the early 2000s, it was reported in commercial potato fields in the Pearsall and lower Rio Grande valley areas of Texas. By 2006, the disease had spread to most potato-production areas in Texas as well as other potato-production states, including Arizona, Kansas, Colorado, California, Nevada, and New Mexico (74). ZC was reported in New Zealand in 2008 (52) and, more recently, from Idaho, the Columbia Basin of Washington, and Oregon (18,22).

ZC-affected potato plants exhibit a range of foliar symptoms that resemble those of the purple top wilt symptoms caused by the

clover proliferation phytoplasma (37). Foliar symptoms include stunting, chlorosis, swollen nodes, proliferated axillary buds, aerial tubers, browning of the vascular system in stems, and leaf scorching (Fig. 1A and B). Belowground plant symptoms include enlarged lenticels of the underground stem, collapsed stolons, brown discoloration of the vascular ring, necrotic flecking on internal tuber tissues, and, occasionally, streaking of the medullary ray tissues (Fig. 1C) (37). At the time the disease was identified, the causal agent was unknown. To investigate the possible causal agents, insect transmission and graft experiments were conducted to determine whether infectious agents were transmissible to healthy plants or due to nonbiological factors such as heat stress (heat necrosis) or toxicity (psyllid toxin). In addition, research also was conducted to determine whether the symptoms were caused by nutritional deficiency, a toxin or other abiotic factor, or some combination of these factors. Although researchers were not able to consistently associate the disease with any specific causes, results from graft- and insect-transmission experiments suggested that there was likely a transmissible factor, probably a vascular plant pathogen, virus, or prokaryote or bacterial-like organism (BLO), that was associated with ZC (24,71).

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These results were further supported by transmission electron microscopy studies, where BLOs were observed in the vascular tissue of infected plant parts that were similar to the phloem-restricted proteobacteria which are associated with the “basses riches” syndrome of sugar beet (24). Because the symptoms of ZC resembled those of phytoplasma-associated diseases, studies were subsequently carried out to determine whether leafhoppers or potato-tomato psyllid *Bactericera cockerelli* (Šulc) was a primary vector responsible for transmission of a ZC-causing agent. Results from cage trial studies demonstrated that plants infested by potato psyllids developed ZC foliar symptoms and characteristic ZC discoloration in tubers (71,74). However, at that time, the causal agent of ZC was still unknown. To identify the causal pathogen or pathogens, polymerase chain reaction (PCR) primers detecting pathogens associated with vascular-restricted diseases, including cucurbit yellow vine disease, marginal chlorosis of strawberry, Pierce’s disease of grapevine, and “stolbur-like” phytoplasma, were used to screen DNAs extracted from ZC-affected and healthy potato. However, attempts to amplify specific known disease-prokaryote products from a suspect ZC-associated prokaryote failed to obtain consistent results from ZC-diseased potato or psyllids or other insect vectors (37).

A breakthrough came in April 2008, when researchers at the Plant Health and Environment Laboratory, MAF Biosecurity, New Zealand reported that a new disease of glasshouse-grown tomato (*S. lycopersicum*) and pepper (*Capsicum annuum*) plants was identified using universal prokaryote 16S ribosomal DNA primer pairs that amplified a PCR product specifically from symptomatic plants (52,54). This result, along with transmission electron microscopy evidence (24,99), confirmed the presence of a phloem-inhabiting BLO in diseased plants, but not in healthy plants. The same New Zealand group subsequently confirmed that the same pathogen was associated with ZC of potato (52,54). Sequence analysis of the amplicon indicated that it shared high similarity with the bacterial species associated with citrus huang-

longbing (HLB) (also known as citrus greening). Subsequently, it was given the name ‘*Candidatus Liberibacter solanacearum*’ (55). In August 2008, a research group at the University of California–Riverside also reported a new citrus “HLB species” bacterium named ‘*Ca. L. psyllauros*’ responsible for tomato yellow disease caused by a bacterium transmitted by the tomato/potato psyllid (39). Because the sequences of ‘*Ca. L. solanacearum*’ and ‘*Ca. L. psyllauros*’ share >99% similarity in the 16S rRNA gene, it is likely these two bacterial names are synonymous (99,110). ‘*Ca. L. solanacearum*’ is recommended as the reference to this bacterium because this name and a description of the bacterium were published in the International Journal of Systematic and Evolutionary Microbiology (55), a preferred journal for descriptions of uncharacterized bacterial species.

Since the discovery that ‘*Ca. L. solanacearum*’ was responsible for ZC disease, various studies have been carried out (1,53,56, 76,99,110). All results were consistent with the conclusion that ‘*Ca. L. solanacearum*’ is the putative causal agent of ZC of potato. Due to its fastidious nature, the ‘*Ca. L. solanacearum*’ bacterium is, as of yet, unculturable and, thus, Koch’s postulates have not been fulfilled. Consequently, conclusive detailed information regarding the general biology, physiology, and pathogenicity of ‘*Ca. L. solanacearum*’ is lacking. In spite of these limitations, using DNA enrichment techniques and next-generation sequencing technology, the whole genome sequence of ‘*Ca. L. solanacearum*’ has been obtained (58). Annotation of genome information provides insights into gene inventory account for biological functions, mode of pathogenicity, metabolic pathways, and nutritional requirements for this obligate intracellular pathogenic bacterium.

Evolution, speciation, and genetic diversity. The genus of ‘*Candidatus Liberibacter*’ belongs to the *Rhizobiaceae* family of α -Proteobacteria. There are four plant-pathogenic species of bacteria in this genus (8,46,55). Among the four pathogenic *Liberibacter* spp., three are associated with citrus HLB worldwide

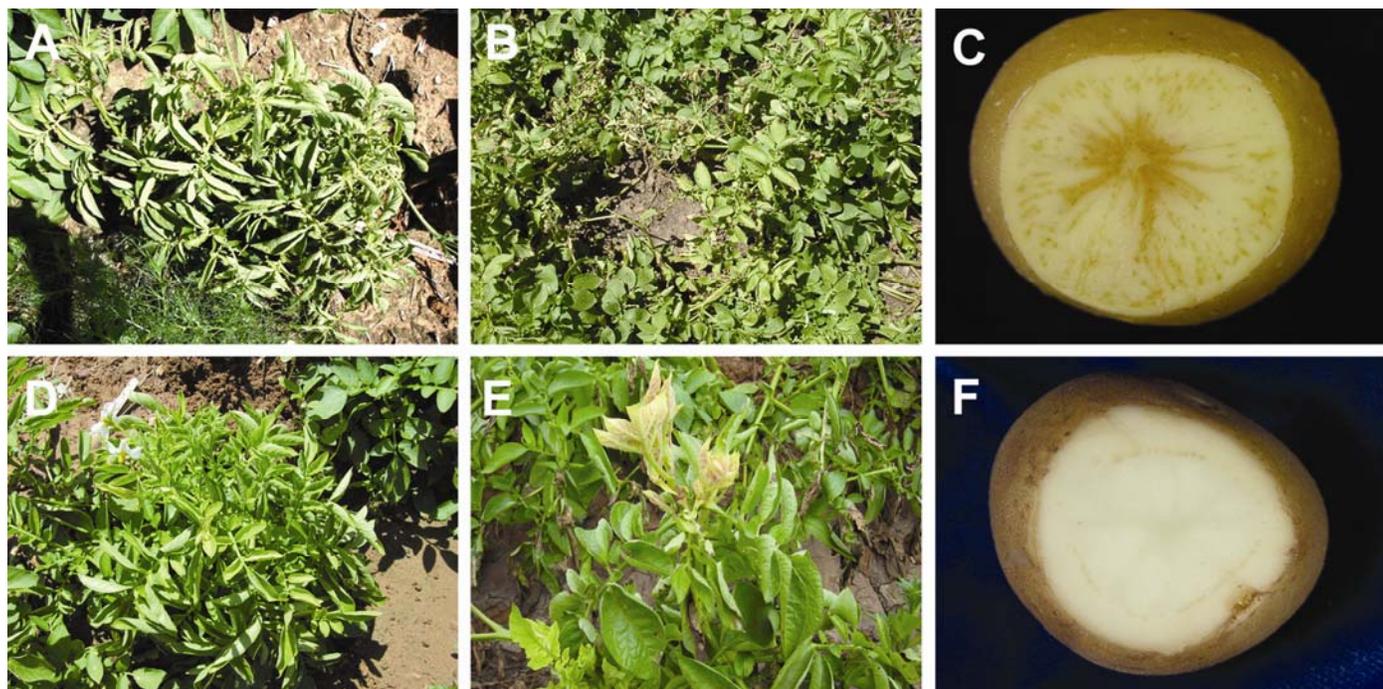


FIGURE 1

Disease symptomatology associated with **A to C**, zebra chip (ZC) and **D to F**, psyllid yellows (PY) of potato. **A**, Early symptom development of ‘*Candidatus Liberibacter solanacearum*’ infections in potato foliage; **B**, advanced foliar symptoms of ZC; and **C**, medullary streaking and flecking in tuber flesh. **D**, Early symptom development of PY in potato foliage; **E**, advanced symptom development of PY; and **F**, mild vascular discoloration of potato tubers due to PY.

(96). Not surprisingly, two major lineages were also identified in U.S. '*Ca. L. solanacearum*' populations (Table 1). Although genetic analyses detected two lineages in agricultural population systems, the biological implication is yet to be determined. Interestingly, genetic analyses indicated that the occurrence of identical haplotypes was not restricted to any particular geographical area or host cultivar. These overlapping patterns were also observed by SNP-based typing analyses (82). In contrast to natural populations, it is expected that frequent exchange of plant materials in agricultural productions could facilitate the movement of ZC-affected materials, which could largely account for such non-pattern geographical distribution among identical haplotypes. Additionally, SSR DNA markers associated with hypervariable sequence regions can provide sufficient resolution for differentiating closely related isolates and for tracking genotypes of interest. Thus, these markers could help identify the source of invasive strains.

Pathogens with higher diversity and evolutionary potential will likely pose a higher risk of overcoming host resistance or evolving to reduce the efficacy of disease control measures (65). Understanding genetic diversity and evolutionary potential of '*Ca. L. solanacearum*' populations is critical for development of disease management strategies and tactics. Currently, information regarding the population structure and genetic diversity, as well as evolutionary and epidemiological relationships, among '*Ca. L. solanacearum*' isolates in agricultural populations is limited. Pathogen genetic analyses, in combination with advances in the understanding of the spatial and temporal dynamics of the epidemiology of the disease, will facilitate development of effective integrated pest and disease management for the ZC problem.

General features of '*Ca. L. solanacearum*' genome. The genome of '*Ca. L. solanacearum*' is composed of 1.26 Mbp (National Center for Biotechnology Information [NCBI] GenBank accession number CP002371) in a circular chromosome which encodes a total of 1,192 putative proteins coding sequence. In all, $\approx 68\%$ of them are predicted to be homologous with known putative functions, and the rest of them are assigned to hypothetical proteins with unknown functions (Table 2). These numbers are similar to the '*Ca. L. asiaticus*' genome. '*Ca. L. solanacearum*' and '*Ca. L. asiaticus*' have a G+C content of 35.24 and 36.48%, respectively, which is considerably lower than the $\approx 60\%$ G+C content observed for most bacterial species in the *Rhizobiaceae* family (16,35,111). Sequence analysis identified three copies of complete rRNA operons (16S, 23S, and 5S), 45 genes encoding for tRNAs, and ≈ 35 genes identified as pseudogenes in the '*Ca. L. solanacearum*' genome (Table 2). No plasmids were found in either *Liberibacter* genome. Comparative analyses of the '*Ca. L. solanacearum*' and '*Ca. L. asiaticus*' genomes revealed that ≈ 884 protein-coding sequences are common to both organisms (58). Notably, 236 coding sequences from '*Ca. L. solanacearum*' have no corresponding orthologs in '*Ca. L. asiaticus*'. On the other hand, 186 coding sequences from '*Ca. L. asiaticus*' have no corresponding orthologs in '*Ca. L. solanacearum*'. Compar-

ative sequence analyses of '*Ca. L. solanacearum*' and '*Ca. L. asiaticus*' genomes show a high level of synteny, suggesting that both circular chromosomes are descendants of a common ancestor. Intriguingly, analyses of two *Liberibacter* genomes revealed that nearly 30% of open reading frames in '*Ca. L. solanacearum*' and '*Ca. L. asiaticus*' have no known function. This implies that a number of biochemical pathways remain to be identified. An incredible biological diversity among novel protein families has been observed in bacterial genomes (87).

Functional determination of these novel genes will improve our knowledge for better understanding of these disease-associated, putative pathogenic bacteria. Recent advances in sequencing technology led to the discovery of a high correlation between the number of genes and the genome size of bacteria. Most bacterial genomes range in size from 0.5 Mbp to upward of 10 Mbp, with the average of 1 kb per gene. Molecular characterization of various microbial genomes has revealed that many pathogenic and mutualistic intracellular bacterial species have smaller genomes than their free-living relatives (5,33,68). The loss of a great number of genes results in the reduction of genome size that is often associated with the adaptation to an obligate lifestyle, in which many molecules can be directly acquired from the host (33,83). The phloem-restricted *Liberibacter* bacteria have small genomes, ≈ 1.2 Mbp. Likewise, the reduction of genome size through gene loss is associated with the evolutionary adaptation to a lifestyle as an obligate intracellular pathogen that largely depends on intermediate metabolites acquired from its hosts.

Bacteriophages, or phages, are bacterial viruses. Two homologous segments of prophage sequences were identified in the '*Ca. L. solanacearum*' genome (P-I and P-II) (Fig. 3) (58). Sequence analysis showed that one of the prophage segments in '*Ca. L. asiaticus*' strain psy62 has a high degree of similarity with the prophage sequences of '*Ca. L. solanacearum*' genome but no circular phage sequence was identified in either *Liberibacter* genome (27,58). More recently, two circular phage genomes (SC1 and SC2) were identified in '*Ca. L. asiaticus*'-infected periwinkle (*Catharanthus roseus*) and dodder plants (*Cuscuta campestris*) (115). In the Asian citrus psyllid, however, both were found integrated in tandem in the '*Ca. L. asiaticus*' UF506 genome as prophage. Sequence analysis showed that SC2 appears to be derived from SC1 but lacks lysis genes and functions as lysogenic conversion. SC1, on the other hand, seemed to be a fully functional, temperate phage with a lytic cycle that appears to be activated when the '*Ca. L. asiaticus*' host infects plants but not when inhabiting psyllids (115). It is not clear whether a lack of a circular phage genome in '*Ca. L. solanacearum*' is due to the fact that sequenced genomes were derived from '*Ca. L. solana-*

TABLE 1
Genetic variations of '*Candidatus Liberibacter solanacearum*' associated with zebra chip^a

Sequencing data on rRNA	Simple sequence repeat	Multilocus sequence typing
Clade 1 or haplotype A	Lineage 2 (type 1)	ST 2
Clade 2 or haplotype B	Lineage 1 (type 2)	ST 1

^a From Glynn et al. (34), Lin et al. (57), Nelson et al. (82), and Wen et al. (110).

TABLE 2
General genome features of '*Candidatus Liberibacter solanacearum*' and '*Ca. L. asiaticus*'

Genome features	' <i>Ca. L. solanacearum</i> '	' <i>Ca. L. asiaticus</i> '
Size (bp)	1,258,278	1,227,204
GC (%)	35.2	36.5
Protein coding genes	1,192	1,136
Hypothetical proteins	405	362
rRNA operons	3	3
tRNA genes	45	44
Pseudogenes	35	32

cearum'-infective psyllids or to some other factor. It remains to be determined experimentally whether such lysogenic and lytic conversions are also the case in an infected host, such as when '*Ca. L. solanacearum*' infects potato plants. Alignment of the two *Liberibacter* genomes suggests that several recombination events have occurred since the divergence of these two species from a common ancestor (58). Phage integration events appear to play a key role in the rearrangements in the *Liberibacter* genomes. In addition to the two prophage genome sequences, there are a number of prophage-like elements and phage remnants distributed throughout the '*Ca. L. solanacearum*' genome, presumably derived from multiple ancestral bacteriophage integration events.

Virulence determinants. One of the most powerful approaches for the analysis of pathogen genomes involves computational bioinformatics identifying genes homologous to known virulence factor genes. Sequence-similarity-based searches such as BLAST and position-specific iterated BLAST against a reference database facilitate identification of orthologous genes with the predicted putative functions. Annotation of the '*Ca. L. solanacearum*' genome sequence provides a basis for understanding the host-pathogen interaction that leads to disease development. Many gram-negative plant-pathogenic bacteria possess a type III secretion system (T3SS) to inject effector proteins directly into host cells. T3SSs are encoded by hypersensitive response and pathogenicity genes, which cause disease in susceptible plants but elicit the hypersensitive response in resistant plants (14,59). Interestingly, no T3SS was identified in the '*Ca. L. solanacearum*' genome, nor were offensive virulence factors such as cell-wall-degradation enzymes, cellulose, pectinase, xylanase found (58). Lack of a T3SS apparatus has been found in some insect-mediated transmission and vascular-restricted bacteria, including '*Ca. L. asiaticus*' and *Xylella fastidiosa* (27,102,107). Presumably, this secretion apparatus might not be necessary because the initial infection of this intracellular pathogen is mediated via direct injection into the host cell by its insect vector.

In spite of missing a classical gene-for-gene model in the '*Ca. L. solanacearum*' genome, other genes associated with putative roles in virulence or pathogenicity were identified, including genes involved in ion transport. For example, the zinc transport system is composed of a cluster of three genes (*ZnuABC*) that encode a prokaryotic-type ABC-family protein complex (30). It is predicted that the putative zinc transport system is a high-affinity cation carrier that shuttles zinc ions across the inner membrane of '*Ca. L. solanacearum*' (4). Results of a recent study of '*Ca. L. asiaticus*' suggested that the disruption of this system may present a viable target not only to mitigate HLB symptom severity by making more free zinc available to the plant but also by providing a means to decrease the overall virulence of the bacterium itself (106). ZC-affected potato plants often have yellowing symptoms resembling zinc deficiency, likely associated with the presence of this putative high-affinity zinc carrier. Therefore, the silencing of this gene or the identification of specific inhibitors of this bacterial transporter may be a way to mitigate ZC symptoms. Sequence analysis predicted that the '*Ca. L. solanacearum*' genome encoded iron-transport and accumulation (ITA) systems. The ITA system consists of a five-gene cluster found in the '*Ca. L. solanacearum*' genome and some other pathogenic microbes; however, this system is missing from the citrus HLB-associated '*Ca. L. asiaticus*' (58). It is hypothesized that this gene cluster encodes proteins involved in iron scavenging, transport, and sequestration based on their similarity to known iron transporters and gene arrangement (28,89). Because ZC-affected plants sometimes mimic plants with iron deficiency, perhaps this phenotype is related to a high-affinity iron uptake system possessed by '*Ca. L. solanacearum*'. Thus, functional confirmation of one or more of the genes in the ITA cluster will be necessary to identify the target to develop a gene-based therapy for an effective ZC disease treatment. Another pathogenicity gene that was identified in the '*Ca. L. solanacearum*' genome is a locus encoding an NahG-like salicylate hydroxylase. Overwhelming evidence demonstrates that

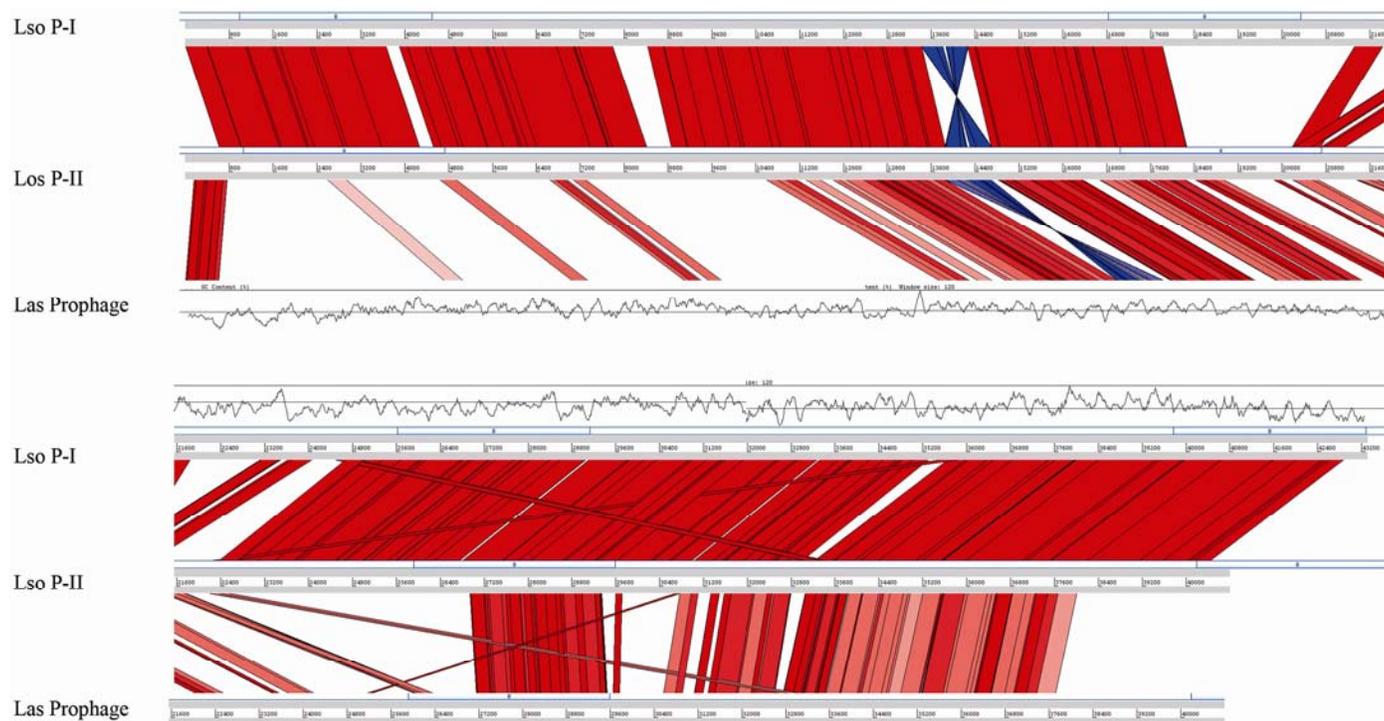


FIGURE 3

Syntenic conservation between prophage sequences of '*Candidatus Liberibacter solanacearum*' (P-I and P-II) and '*Candidatus Liberibacter asiaticus*' (Las Prophage). Colored bands represent tBLASTx similarity: collinear (red) and inverted (blue).

salicylic acid (SA) accumulation is essential for expression of multiple modes of plant disease resistance (9). Transgenic tobacco and *Arabidopsis thaliana* expressing the bacterial enzyme salicylate hydroxylase cannot accumulate SA, because it is rapidly degraded to catechol by the introduced enzymatic activity (7,113). This defect not only makes the plants unable to induce systemic acquired resistance (SAR) but also leads to increased susceptibility to viral, fungal, and bacterial pathogens. Therefore, a functional defect of the salicylate hydroxylase gene in '*Ca. L. solanacearum*' may enhance host SAR in response to '*Ca. L. solanacearum*' infection. *Liberibacter* spp. have not been successfully cultured in vitro. Consequently, conventional gene knock-out techniques cannot be applied to determine gene function. Elucidation of the function of these virulence genes will likely provide further fundamental insights into how this pathogenic bacterium survives with its hosts and elicits disease development, as well as facilitate development of new gene-based therapeutic treatment strategies for this potato disease.

Metabolic properties. '*Ca. L. solanacearum*' is environmentally restricted to the sieve cells of phloem vessels of potato plants or in the salivary glands of potato psyllids. Thus, its lifestyle reflects adaptive features to nutritional niches in both hosts. The complete genome sequence of '*Ca. L. solanacearum*' allows reconstruction of detailed predictive metabolism models based on bioinformatics. Annotation of the '*Ca. L. solanacearum*' genome revealed that it encodes a large number of membrane-transporter-related proteins. Membrane transports are essential for the movement of substrates across a permeable barrier of the cell membrane. Transporters enable the acquisition of organic nutrients and inorganic elements, efflux of toxin compounds, ion homeostasis, signal sensing, energy production, and various important cellular functions (84). '*Ca. L. solanacearum*' contains a nearly complete set of genes encoding enzymes in glycolytic pathways and enzymes required to convert pyruvate to acetyl-coA, an important biochemical molecule for fatty acid metabolism and entry into the tricarboxylic acid cycle (58). Apparently, these enable it to effectively utilize carbon sources available in phloem sieve cells of the host for catabolic and anabolic reactions (58). Although there are a few genes for production of enzymes found in the '*Ca. L. solanacearum*' genome involving de novo biosynthesis of amino acids, at least three complete transport systems were identified. These transport systems function as a broad-range amino acid transport capability, including the L-amino acid ABC transporter, proline glycine-betaine ABC transporter system, and DctA-family dicarboxylate transporter. All of these transporter systems are also present in the members of *Rhizobiaceae*. Because both '*Ca. L. solanacearum*' and '*Ca. L. asiaticus*' lack the key enzymes involved in the terminal stages of oxidative phosphorylation pathway, it is concluded that both '*Ca. L. asiaticus*' and '*Ca. L. solanacearum*' have limited capacity for aerobic respiration, which seems to be consistent with oxygen-limited phloem cells where they have become adapted. Additionally, genes encoding for sulfur metabolism are also absent in both genomes, suggesting that both bacteria may use other alternative energy-generating pathways under anaerobic conditions. Indeed, genome analyses identified a gene encoding ATP/ADP translocase of the NttA family in both bacteria (27,58). Functional analysis confirmed that this transport protein was able to import ATP directly from the host cell's cytosol and translocate it into the bacterial cell. Thus, it can be concluded that NttA provides the '*Ca. Liberibacter* sp.' cell with energy (105). This unique feature has been found in other obligate intracellular bacteria such as the intracellular bacteria *Rickettsia prowazekii*, *Chlamydia psittaci*, and *Lawsonia intracellularis* (40,97). Surprisingly, orthologs of the NttA transport family are absent from all other *Rhizobiaceae*. Given the reduced size of the '*Ca. Liberibacter* sp.' (1.2 Mb) genome, it appears that this transport was acquired through hori-

zontal gene transfer early in the evolution of '*Ca. Liberibacter*' spp. Although the biochemical function of the translocase was confirmed, the biological role has yet to be determined. The capability of acquiring ATP from the host could play an important role in this obligate intracellular bacterium in planta and this feature may be exploited to facilitate a means for growing an axenic culture of this bacterium in vitro (105).

The sequencing of bacterial genomes has given insight into the genomic inventory that enables prediction of putative biochemical information about '*Ca. L. solanacearum*'. The precise substrate specificity for some significant pathways could not be determined with certainty without functional confirmation. However, this type of analysis provides a good overview of the biochemical and metabolic capability of this organism, which is particularly important for unculturable bacteria where conventional bacteriological approaches are often limited or not applicable.

Aspects of the etiology and epidemiology of ZC. The etiology and epidemiology of any plant disease is likely to be complex when it involves an insect vector, and ZC of potato is no different. Several important aspects of the disease will be reiterated here for clarity. The putative pathogen of ZC is '*Ca. L. solanacearum*' (21,52,53,55) and it is vectored by the potato psyllid, *B. cockerelli* Šulc. The situation is made more complicated by the implication that another bacterium, '*Ca. L. psyllauros*', is the cause of a similar, yet distinct potato disease, psyllid yellows (PY) (Fig. 1D to F) (39,110). It was suggested that '*Ca. L. psyllauros*' is a secondary endosymbiont of the potato psyllid because it was demonstrated to be both vertically and horizontally transmitted through several life stages of the insect, raising additional questions regarding the ancestry of this bacterium (39). It is not a universally held belief, however, that PY is caused by '*Ca. L. psyllauros*' (101); instead, it may be caused by a toxin injected by the potato psyllid (17,29). It is interesting to note that the association of the potato psyllid in the development of ZC (71,74) predated the identification of '*Ca. L. solanacearum*'/'*Ca. L. psyllauros*' as putative pathogens of potato (39,52,53,55). Molecular characterization and phylogenetic analysis of the full-length 16S rRNA from the new species of '*Ca. Liberibacter*' further confirmed the association of ZC disease of potato and the potato psyllid vector (56). The etiological cause of ZC was further confirmed by grafting ZC-affected plant tissue to healthy plants, which developed typical symptoms of ZC, and the etiological agent was visualized using electron microscopy, as previously indicated (20,99). Using the available PCR technology, '*Ca. L. solanacearum*' has been confirmed in ZC-affected potato and tomato plants from Texas (1,99,110), California (20), and Mexico (76,77,110). Using a wide array of PCR primers, '*Ca. L. solanacearum*' was found to be associated with ZC-affected plants from throughout Texas in archived ZC-affected potato samples collected in 2005 to 2007 (110). The similarity among all of these 'isolates' of '*Ca. L. solanacearum*'/'*Ca. L. psyllauros*' was >99% (110). These studies also confirmed the presence of '*Ca. L. solanacearum*'/'*Ca. L. psyllauros*' in Colorado, Kansas, and Nebraska in 2008 and in seed potato tubers produced from Wyoming in 2007 (110). Of much greater concern is the outbreak of ZC in the major potato-producing states of Idaho, Oregon, and Washington in 2011 (18,22). Although economic losses in these later states were confined, it does appear that ZC is spreading and is likely to be present wherever the insect vector *B. cockerelli* is found.

The vector of ZC-associated '*Ca. L. solanacearum*', *B. cockerelli*, is known to be a spatially and temporally variable insect in the United States and New Zealand (15,61,70). Research has been ongoing to determine the phenology and seasonal migration of the psyllid throughout the native range, especially where it is known to be associated with ZC. It has been clearly demonstrated that psyllid populations differ in their ability to

transmit the ZC-associated ‘*Ca. L. solanacearum*’ in potato (69). Although the precise explanation for this variability is not clearly understood, it may be due to genetic differences among psyllid populations, haplotypes or biotypes of the ZC-associated bacterium, or both. Previously, genetic variation of potato psyllids associated with ZC was determined at both the biotype level and the population level using either cytochrome oxidase I or inter-SSR analysis (45). The goal of this type of analysis has been to compare and contrast psyllids collected at different locations and at different times of the year and ultimately determine the movement patterns of this insect. It is epidemiologically important to determine whether there are biotypes of the potato psyllid that are more efficient vectors of ‘*Ca. L. solanacearum*’. This information would further our understanding of insect genotypic diversity and possibly help predict the annual severity of ZC expression. It has been known for some time that there are at least two biotypes of *B. cockerelli*, a western and a central U.S. biotype (61). More recently, a “northwestern” biotype of *B. cockerelli* in the United States has been identified (101) and implicated in the ZC epidemic in the Pacific Northwest (18,22). The impact that these different biotypes have on the epidemiology of ZC is not yet known but studies to determine the impact that psyllid populations have on disease development are ongoing.

Initially, nothing was known about acquisition and transmission of ‘*Ca. L. solanacearum*’ and environmental effects. The successful transmission of an insect-vector plant pathogen is largely dependent on vector transmission efficiency, and important pieces of the host–pathogen–vector relationship have been established. For example, adult psyllids have been shown to be more efficient than nymphs in transmitting ‘*Ca. L. solanacearum*’ (12). Acquisition success is influenced by feeding site and is highest when psyllids have access to the whole plant (64). Interestingly, inoculation access period had virtually no influence on the overall incidence of ZC, severity of disease development, and yield loss (11); however, the rate of successful inoculation increased with higher vector numbers, and plants inoculated with multiple psyllids had higher ‘*Ca. L. solanacearum*’ titers (64). Disease progress and tuber yield is clearly independent of psyllid density (12,64). A single adult potato psyllid is capable of successfully transmitting ‘*Ca. L. solanacearum*’ to potato in as little as 6 h (12). Air temperatures immediately following inoculation with *Liberibacter*-infective psyllids have been reported to affect ZC development (75), although more recent studies suggest that air temperatures following infection have no effect on latent period or symptom development (A. Rashed and C. Rush, *personal communication*). The stark difference in these results may be due to differences in the haplotype of the ‘*Ca. L. solanacearum*’ or the psyllid vector used in the respective experiments; however, that information is not currently known. Temperatures as low as 17°C have been reported also to slow development of ‘*Ca. L. solanacearum*’ as do temperatures >32°C. Interestingly, very similar effects of temperature have been observed on the insect vector (17,60,109). The optimum temperature for psyllid development is estimated to be ≈27°C. Egg laying, egg hatch, and nymph development also slow at 32°C and cease at 35°C (60). Therefore, vector dynamics and pathogen development appear to be similarly reduced at temperatures of ≥32°C.

The source or sources of ‘*Ca. L. solanacearum*’ from which *B. cockerelli* acquires the bacterium is still a source of speculation. Potato psyllids have long been thought to overwinter in the Lower Rio Grande Valley on wild *Lycium* spp. (36,94). This solanaceous host also has been found to be infected with ‘*Ca. L. solanacearum*’, albeit at very low frequencies (110). Therefore, it is suspected but not empirically proven that the potato psyllid can acquire the ZC bacterium while overwintering, which may explain why frequencies of ‘*Ca. L. solanacearum*’ are traditionally higher early in the growing season than later (19). Titers of ‘*Ca.*

L. solanacearum’ in potato psyllids have been demonstrated to be substantially reduced in starved potato psyllids (95), which may explain why migrating populations of *B. cockerelli* have lower ‘*Ca. L. solanacearum*’ frequencies than those coming off overwintering hosts. Additionally, ‘*Ca. L. solanacearum*’ is known to have a negative effect on the population growth rate of the potato psyllid by reducing fecundity and survival of nymphs (80). It is apparent that more detailed information on pathogen–vector relationships and interactions is necessary if risk assessment models are to be effective in predicting ZC development.

Because the potato psyllid migrates from overwintering sites, it makes epidemiological sense that the development of ZC is more intense along the edges of an affected field rather than further infield (112). Disease progress was best explained by a second-degree polynomial (quadratic) equation. Perhaps more interesting was the determination that ZC infections in potato fields did not develop spatially in a completely random pattern but, rather, in clusters, with ZC-infected plants situated in close proximity to each other (41). However, analysis of the infection clustering did not support secondary spread of the pathogen from an initial infection point. This is in direct contrast to earlier observations of ZC development in the field in Texas, where the disease developed in “holes” or pockets (Fig. 4). It is not known whether these holes are the result of secondary spread of the pathogen from individual focal points (71) or whether these clusters of infection have formed from plant-to-plant movement of competent vectors. A very fortunate attribute of ZC development is that seedborne ‘*Ca. L. solanacearum*’ does not appear to be epidemiologically important (42). Although the vegetative propagule used as “seed” to plant potato can harbor ‘*Ca. L. solanacearum*’ (42,86), it has not yet been demonstrated to be a source of inoculum in development of an epidemic. Seedborne ZC does have a detrimental effect on seed quality, however, largely through the reduction of germination rates and subsequent seedling vigor (42,86).

Host responses. ZC causes significant losses in tuber yield (11,42). The development of ZC symptoms in tubers occurs ≈3 weeks after infection, after which tuber development ceases (10). Concomitantly, tuber solids began to decrease as soon as ZC symptoms began to appear. The rapid development of ZC tuber symptoms in the aforementioned studies supports previous studies on movement of ‘*Ca. L. solanacearum*’ in potato and tomato (49). In these studies, the ‘*Ca. L. solanacearum*’ bacterium moved in the phloem and was more readily detected in the upper- and middle-tier leaves, before it was detected in fully developed leaves, 2 to 3 weeks after initial exposure to ‘*Ca. L. solanacearum*’-inoculative psyllids (49). It was concluded that ‘*Ca. L. solanacearum*’ translocation within potato and tomato plants is similar to what has been previously described for phloem-limited viruses and phytoplasmas.

A number of studies have investigated the physiological response of the host to infection at the cellular and molecular level. For example, there is evidence of cell death in potato tubers affected by ZC (66) and a build-up of host phenolics and plant defense-related proteins (3,81,108). Total protein content increases threefold, which includes increases in gene expression regulatory factors such as cyclophilin and tuber storage proteins such as patatin (3). Tyrosine has been suggested to be the primary cause of enzymatic browning in potato tubers (81). Glucose and sucrose levels also are elevated in ZC-diseased tubers, reducing process quality in potato chips due to the Maillard reaction that occurs during high-temperature frying (12,31). The characteristic development of this potato chip defect was the ultimate source of the name given to this potato disease, “zebra chip” (Fig. 5). Initial investigations have indicated that potato plants are more susceptible to the pathogen phenologically early in the plant’s life cycle

than later (31), although temperatures at the time of infection may affect the infection process, development of disease symptoms, and the bacterium–vector relationship (75). More recent studies indicate that plant age at the time of infection, over a broad range of temperatures, has no effect on the development of initial ZC symptoms or on the rate of symptom severity (64) (C. Rush, *personal communication*). These conflicting reports may be due to the specific interaction of ‘*Ca. L. solanacearum*’ haplotypes and potato cultivar and the conditions with which the studies were performed.

Although all of the impacts proteobacterial pathogens have on affected plants are not understood, it is likely that the presence of the ZC-associated ‘*Ca. L. solanacearum*’ in the phloem is similar to another proteobacterium, a ‘*Ca. Phlomobacter* sp.’, and its effect on the roots of sugar beet (100). We know that ‘*Ca. L. solanacearum*’ infection of potato phloem disrupts carbohydrate flow to belowground portions of the plant, causing a number of the symptoms associated with ZC (11,31). The lack of carbohydrate flow to the roots will likely cause them to starve and decline in function, thereby causing the aboveground portions of the plant to also slowly decline and eventually die (Fig. 1A and B).

Host range. From the initial suggestion of ‘*Ca. L. solanacearum*’ as the putative pathogenic cause of ZC, it has been

known that ‘*Ca. L. solanacearum*’ is also associated with a number of other solanaceous hosts in addition to potato and tomato (52,53,55). In New Zealand, ‘*Ca. L. solanacearum*’ has been associated with a new disease of *Capsicum* sp. and tomato while also causing a potato disease very similar to ZC in the United States (54,55). In addition, ‘*Ca. L. solanacearum*’ also has been found to infect *S. betaceum* (tamarillo) and *Physalis peruviana* (Cape gooseberry) in New Zealand (53,54). ‘*Ca. L. solanacearum*’ has been associated with a psyllid-associated disorder of carrots in several countries in Europe (2,72,73,78,79). These are the first reports of a nonsolanaceous host for ‘*Ca. L. solanacearum*’. The association of ‘*Ca. L. solanacearum*’ in carrot and with two other psyllid species raises interesting questions about the diversity of ‘*Ca. L. solanacearum*’ and its specific relationship with insect vectors, which is discussed elsewhere in this review.

In the United States, a survey of ‘*Ca. L. solanacearum*’ in solanaceous hosts in Texas found that silverleaf nightshade (*S. elaeagnifolium* Cav.), wolfberry (*Lycium barbarum* L.), black nightshade (*S. ptychanthum* Dun.), and jalapeno pepper (*Capsicum annuum* L.) were hosts for the ZC-associated bacterium (110). The ‘*Ca. Liberibacter*’ spp. detected in all of these solanaceous plant hosts had similarity indices of 99.8% to the partial 16S rRNA gene sequences compared with the sequences of the



FIGURE 4

Development of zebra chip (ZC) in a cluster of infections in the field, which initially suggested secondary spread of the ZC pathogen from an initial focal point.

same bacterium detected in ZC-affected potato plants (110). The role that these alternate hosts such as perennial solanaceous plants wolfberry and silverleaf nightshade have on the epidemiology of '*Ca. L. solanacearum*' is not known but further studies are warranted and should be performed, as they have been for HLB caused by '*Ca. L. asiaticus*' (23,25). For any of these plant hosts to be identified as epidemiologically significant reservoirs of '*Ca. L. solanacearum*' inoculum, they will have to be demonstrated to retain the ZC-associated '*Ca. L. solanacearum*' in an infectious state over an extended period of time and also be an important colonization host for *B. cockerelli*.

Molecular diagnosis. Detection of '*Ca. L. solanacearum*' in its insect vector is essential for studying its etiology and epidemiology and in developing effective disease management strategies (36). Because ZC is associated with a prokaryotic bacterium, most of the PCR methods developed for '*Ca. L. solanacearum*' detection have utilized a conventional PCR format, with primers to amplify 16S and 23S ribosomal DNA sequences (39,50,52,54,91,99,110). Although a number of forward primers have been empirically designed based on the 16S rRNA sequence of '*Ca. L. solanacearum*' (NCBI GenBank accession number EU980389 (50,55,110), the reverse primer most commonly used for the PCR reaction is the Liberibacter-universal primer OI2c (47). As a result, it is not surprising that some of the primers developed to detect '*Ca. L. solanacearum*' also amplify some strains of '*Ca. L. asiaticus*' and '*Ca. L. africanus*' (92,110). Fortunately, all of the

PCR primers developed to date are specific for '*Ca. Liberibacter*' spp. associated with the ZC complex and do not detect other prokaryotic plant pathogens that affect potato (110). In addition to PCR primers using 16s and 23s rDNA genes, more recent primers have utilized genomic DNA such as a conserved bacterial housekeeping gene, adenylate kinase (91). These primers have been demonstrated to a detection limit of ≈ 0.65 ng (91). Genomic-based PCR primers also have been developed to facilitate detection of '*Ca. L. solanacearum*' in the potato psyllid and have been found to be valuable in determining the frequency of the bacterium in its vector (19).

Few studies have investigated the effectiveness of detecting '*Ca. L. solanacearum*' in infected potato plant parts. In one study, the overall efficiency of the current PCR technology associated with the detection of true positives was demonstrated to be as low as 60% (110). Detection efficiencies have been generally determined to be higher for belowground portions of plants, such as stolons, roots, and tubers, as opposed to leaves, leaf petioles, and aboveground stems (50,110). However, these results might be contradicted by more recent studies which found '*Ca. L. solanacearum*' detection frequencies higher in upper- and middle-tier leaves than in lower aboveground tissues; however, no attempt was made to assay belowground tissues (49). The presence of PCR inhibitors in tuber tissues can be problematic and caution needs to be exercised when using tuber tissue to detect '*Ca. L. solanacearum*' infections (110).



FIGURE 5

Range of symptoms of the zebra chip defect in potato chips. The development of these symptoms is due to a build-up of glucose in medullary ray tissue that caramelizes during high-temperature frying due to the Maillard Reaction.

There has been interest in quantifying the development of '*Ca. L. solanacearum*' in hosts once the plant becomes infected. In order to accomplish this, the expected amplicon for the PCR reaction was cloned in a plasmid and the number of copies of DNA estimated using the number of base pairs in the sequence and the average molecular mass of a base pair in double-stranded DNA (50). This method was useful in determining that 20 copies of the target 16S rDNA of '*Ca. L. solanacearum*' could be detected in the DNA extract of a ZC-affected plant using a quantitative (q)PCR format. Using this methodology and applying it to naturally infected potato plants from the field, it was determined that the majority of plants harbored an '*Ca. L. solanacearum*' population of 10^5 to 10^6 cells/g of tissue (50). As expected for a phloem-restricted pathogen, '*Ca. L. solanacearum*' populations were three times higher in belowground than aboveground tissue, which likely explains why detection efficiencies are much higher when belowground plant tissue is used (50,110). In this study, 92% of all field samples tested positive for '*Ca. L. solanacearum*' using qPCR whereas only 40 to 46% tested positive using conventional PCR, depending on the primers used (50).

Molecular methods of pathogen detection can only be efficient when using high-quality DNA. Thus, it is extremely important to verify that the DNA is of sufficient quality to be amplified in a PCR reaction. When PCR assays utilize only a single set of primers to detect a microbe, a negative result does not distinguish between the absence of the target DNA sequences, the presence of PCR inhibitors, or poor DNA quality. As a result, a multiplex PCR platform that combines pathogen-specific primers with an internal control that utilizes a plant-based DNA sequence target could be beneficial. Two such multiplex PCR methods have been developed to detect '*Ca. L. solanacearum*' in plants, one utilizing a potato β -tubulin gene sequence (110) in a conventional PCR format, the other using a plant mitochondrial cytochrome oxidase gene sequence in a qPCR format (50). The use of such an internal control determined that 19% of the DNA samples tested gave a negative '*Ca. L. solanacearum*' result due to poor DNA quality, clearly demonstrating the usefulness of such an approach (110).

Despite recent improvements in PCR detection technology (19,91), there is still a need for improvement. For example, the development of a multiplex PCR format that would distinguish '*Ca. L. solanacearum*' haplotypes (57) while simultaneously determining potato psyllid biotypes (45,103) could be very useful in epidemiological studies. Further improvements in qPCR also could facilitate epidemiological studies of ZC, as demonstrated with HLB-associated *Liberibacter* spp. in citrus (43,51,104). For example, qPCR has been found to be extremely useful in determining infestation rates of '*Ca. L. asiaticus*' in the Asian citrus psyllid in Florida (51), which were 10 to 100%. PCR is currently being used to detect '*Ca. L. solanacearum*' in potato psyllids collected throughout the United States as part of a ZC disease management plan (19,21). In contrast to the HLB situation, only $\approx 2\%$ of these potato psyllids have tested positive for '*Ca. L. solanacearum*' (21).

Disease management. In the absence of obvious genetic resistance, it is predictable that an agricultural industry will seek the use of insecticides in an attempt to effectively manage an insect-vector disease. ZC is a relatively new disease in the United States (21,37) and, in the absence of other effective disease management strategies and tactics, efficacious insecticides have become the primary means by which the vector and, therefore, the disease are managed (32,36,62,63). Most of these studies have concentrated on the efficacy of various insecticides on the life stages of the potato psyllid. For example, thiamethoxam and abamectin were found to be effective in significantly reducing adult populations of *B. cockerelli*, although residual effects on adult mortality were relatively short (≈ 24 h) (32).

Insecticides such as spiromesifen were found effective against nymphs (36). Unfortunately, there is a lack of evidence that the use of insecticides to manage psyllid populations will have any effect on reducing the incidence and severity of ZC disease development under conditions where potato psyllid populations harbor high frequencies of '*Ca. L. solanacearum*' infection. For that reason, a few studies involving tomato and the potato psyllid have investigated the potential to improve host resistance and insecticides to effectively manage both the vector and the disease (62,63).

Current pest management practices rely on the use of insecticides to increase psyllid mortality but they can also be used to effectively manage potato psyllid populations by impacting each life stage in ways that do not affect mortality. For example, some insecticides such as imidacloprid, abamectin, and pymetrozine have been shown to reduce probing durations while concomitantly increasing the amount of time adult psyllids spend off the leaf surface, suggesting that these insecticides also act as repellents (13). Perhaps more importantly, two of the insecticides used to manage psyllid populations, imidacloprid and abamectin, also significantly lowered the transmission of '*Ca. L. solanacearum*', which may help reduce further infections (13). However, the development of insecticide resistance compromises the ability of any chemical treatment to effectively limit the rate of disease development, and some populations of potato psyllid have already been documented with resistance to imidacloprid (64).

Growers also use a variety of other management tactics to limit the economic impact of ZC as it affects their potato crop. Laboratory testing of psyllids obtained from a trapping network has confirmed the suspicions of growers that the highest frequencies of '*Ca. L. solanacearum*' in migrating populations occur early in the growing season (19). This may be because the potato psyllid can acquire the ZC-associated '*Ca. L. solanacearum*' from an overwintering host, such as *Lycium* spp. or other solanaceous hosts, that are capable of harboring the bacterium (110). It has also recently been demonstrated that the potato psyllid can overwinter on *S. dulcamara* L. in Idaho (A. Jensen, *personal communication*). The role that this *Solanum* sp. may have on the epidemiology of '*Ca. L. solanacearum*' is also warranted. As previously stated, it has also been demonstrated that colonies of *B. cockerelli* lose infectivity when starved (95), which may occur when psyllids migrate, thereby reducing the risk of bacteriferous psyllids initiating a serious epidemic. As a direct result, potato growers in a number of potato-production areas of Texas where ZC is endemic have resorted to delaying planting so as to avoid psyllid populations less likely to be vectoring '*Ca. L. solanacearum*' (N. C. Gudmestad, *unpublished data*). Because psyllids move into fields from the periphery (112), potato growers also treat the edges of fields with insecticides so as to reduce the opportunity of the insect vector to become established in the entire field.

Other disease management tactics being investigated include the development of traps that attract male and female adult psyllids through the use of volatile chemical attractants (38). Entomopathogenic fungi may also provide additional components useful in an integrated pest management strategy (48) in addition to the use of kaolin particle films and other biorational insecticides which appear to be useful in repelling all developmental stages of the potato psyllid (85,114). These potential alternative methods of ZC management are a priority and are being intensively investigated.

The future for disease management likely lies with disease-resistant potato cultivars (13). Although all potato cultivars currently grown in the United States are susceptible to ZC, a number of potato genotypes have been identified in which there was a significant reduction in '*Ca. L. solanacearum*' transmission compared with susceptible controls. However, the exact nature of

the host–parasite interaction contributing to the reduced transmission is still unclear (13). The development of ZC-resistant germplasm is a priority and is ongoing.

Conclusion and future perspectives. Since the first identification of ZC as a new disease of potato in the United States and New Zealand, a significant effort to determine the cause and, ultimately, the management of the disease has taken place. In addition to determining the cause of ZC, we now have a much deeper understanding of the putative proteobacterium involved. Although ‘*Ca. L. solanacearum*’ remains a relatively primitive pathogen in a genomic sense, it possesses a number of unique attributes that potentially allow it to be a successful plant pathogen. Although ‘*Ca. L. solanacearum*’ lacks substantial enzymatic capability and a T3SS (58), its genome does have other orthologous virulence-related means at its disposal. These need to be explored further because some hosts infected with ‘*Ca. L. solanacearum*’ become severely diseased, such as potato and tomato, while others such as eggplant and pepper can harbor dense populations of ‘*Ca. L. solanacearum*’-infected psyllids with little or no ill effect (N. C. Gudmestad, *unpublished*). Furthermore, studies have shown that phosphonate fungicides induce SAR in potato (6), which has been demonstrated to significantly reduce ‘*Ca. L. solanacearum*’ titers when plants are treated prior to infection (93). It is not known whether this is related to the functional suppression or defect in the salicylate hydrolase gene in ‘*Ca. L. solanacearum*’. Nevertheless, there are genomic data available that may provide the basis for the development of disease management tactics in the future.

The ‘*Ca. L. solanacearum*’ vector, *B. cockerelli*, adds an additional level of complexity to the ZC disease of potato. Although it has been long recognized as an insect pest of potato in certain regions (17,29,94,109), its recent association with a putative bacterial pathogen is as perplexing as it is agriculturally problematic. If ‘*Ca. L. psyllauros*’ is truly a secondary endosymbiont of *B. cockerelli* (39), then the question remains whether or not there are avirulent wild-type ancestors that still exist, perhaps in some other solanaceous plant host. Recent studies suggest there may be ‘*Ca. L. solanacearum*’ haplotype–*B. cockerelli* haplotype interrelationships that could affect ZC disease development. For example, PCR testing of the western *B. cockerelli* haplotype revealed only infections of haplotype A ‘*Ca. L. solanacearum*’ whereas central U.S. haplotypes of the potato psyllid are predominately infected with haplotype B ‘*Ca. L. solanacearum*’ (A. Wen and N. C. Gudmestad, *unpublished*). Although we have substantial information on acquisition, transmission and effect of psyllid densities on ZC development (11,12), there are no data to suggest that this is true for all haplotypes of the vector and the ZC-associated ‘*Ca. L. solanacearum*’.

Although there has been a great deal of progress made in our understanding of the ZC disease complex, it is apparent that there is much more to be learned before this disease can be managed long term in a sustainable manner. There are still substantial gaps in our knowledge of disease epidemiology, particularly as it affects vector survival and overwintering, as well as pathogen dynamics. There are also substantial gaps in our understanding of disease resistance mechanisms, which ultimately will be the primary means by which this disease is effectively and sustainably managed. Despite that, the depth of our understanding, relative to the genetic diversity of the putative pathogen and its vector, the migration of the vector, and the frequency of ‘*Ca. L. solanacearum*’ infection, coupled with effective insect and disease management integrated pest management strategies in the short term, have been impressive for a recently developed potato disease. The expectation is that future studies will continue to improve our understanding of this pathogen–host–vector interaction, while concomitantly reducing the economic impact of ZC in the United States and elsewhere.

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