Characterization of a Novel Adhesin-like Gene and Design of a Real-Time PCR for Rapid, Sensitive, and Specific Detection of *Spiroplasma kunkelii*

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ABSTRACT

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Spiroplasma kunkelii, a cell wall-less bacterium, is the causal agent of corn stunt disease. The pathogen is restricted to phloem sieve cells of infected plants and is transmitted by phloem-feeding leafhoppers. Since symptoms of corn stunt disease may not appear until close to flowering time, early detection of the pathogen in disease-transmitting leafhoppers and in symptomless foliar tissues of host plants is critical to disease forecasting and outbreak management. In this study, a field-deployable real-time polymerase chain reaction (PCR) assay was developed for sensitive and specific detection of *S. kunkelii*. Nucleotide sequence from a previously unreported adhesin-like gene was used to design primers and a fluorogenic probe. The assay was able to detect the presence of *S. kunkelii* DNA as low as 5 fg, a sensitivity 100 times more than that of conventional PCR. The assay was found to be highly specific to *S. kunkelii*, as it did not cross-react with one of the most closely related plant pathogenic spiroplasma species, *S. citri*. The assay was successfully applied to rapid field detection of *S. kunkelii* in its plant host and insect vectors.

Additional keywords: diagnostic techniques, mollicutes, sarpin

Corn stunt disease is one of the most devastating diseases of corn (*Zea mays* L.) in Central and South America, and the disease has periodically invaded corn fields in parts of the southern United States (1,6,11,31). Over the past 10 years, the disease occurred yearly in California's Central Valley (31); economic losses from the outbreak in 2001 exceeded \$5 million in Kings County alone (8). *Spiroplasma kunkelii*, commonly known as corn stunt spiroplasma, is the causal agent of this disease (13,14,34). The pathogen is a helical, motile, and cell wall-less bacterium belonging to the class *Mollicutes*. In infected plants, *S. kunkelii* resides exclusively in the phloem sieve cells and is

spread by phloem-feeding leafhoppers, mainly the corn leafhopper, *Dalbulus maidis* (32). Typical symptoms of corn stunt disease include broad chlorotic stripes on the leaves, shortening of the internodes (stunting), and poorly filled ears. In late stages, leaves of diseased plants turn red or brown, and the plants die from the top down. Since foliar symptoms of the disease may not appear until close to flowering time, disease forecasting and early diagnosis are essential components for programs aimed at breeding resistant corn varieties and for the development and implementation of control measures to avoid disease spread and limit economic losses.

Several diagnostic techniques have been used to detect the presence of *S. kunkelii* in diseased plants and in infected insect vectors. These include optical and transmission electron microscopy (14,27), serological methods (1,18,26), and conventional polymerase chain reaction (PCR) (3). Electron microscopy involves not only expensive equipment but also lengthy and complex sample preparation; therefore, it is not feasible for routine diagnostic purposes. Serological techniques such as enzyme-linked immunosorbent assay (ELISA) have been widely used to detect *S. kunkelii*.

Such methods require a supply of consistent and high-quality antibodies and are time-consuming. In recent years, conventional PCR has been employed as an alternative to serological methods for field detection of *S. kunkelii* with increased sensitivity and reduced reagent requirement (3). However, current high disease incidence and its impact on California agriculture demand a more sensitive and rapid method for early detection of corn stunt spiroplasma in the field. Real-time PCR, which was first developed in the 1990s (19), detects amplicons during the exponential phase of the PCR using fluorescence-labeled primers or probes, allowing extremely sensitive, specific, and rapid detection of a given nucleic acid target (17). In addition, since real-time PCR does not require postamplification analysis of amplicons, such as agarose gel electrophoresis, it significantly reduces the likelihood of cross contamination. Amplicons that are inadvertently released into the working environment during postamplification manipulation are a potential source of contamination and consequent false positive results (23). Thus far, real-time PCR has been applied for diagnosis of several plant diseases, including some caused by pathogenic wall-less bacteria known as phytoplasmas (2,9,25,30,33); but prior to the present study, this valuable technique had not been adapted for *S. kunkelii* detection.

Currently, a collaborative research project to sequence the entire genome of *S. kunkelii* strain CR2-3x is near completion, and partially assembled sequence data are publicly available on the internet (<http://www.genome.ou.edu/spiro.html>). In the present study, following a genome-wide search for distinctive *S. kunkelii* sequences as potential targets for improved detection of this pathogen, we identified a gene that encodes a novel adhesin-like protein. Bacterial adhesins are a class of cell surface proteins that are often virulence attributes of pathogenic strains (35,36). In mycoplasmas, adhesins are directly involved in attachment to host cells (21,28) and are able to induce host immune responses (15). Adhesin-like proteins are also found in plant pathogenic

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spiroplasmas, a majority of which are characterized by the presence of a domain designated sarpin (5). Adhesins of the sarpin family, including SARP1 from *Spiroplasma citri* and SkARP1 from *S. kunkelii*, are believed to play an important role in the attachment of spiroplasma cells to gut cells of insect vectors during early stages of infection (5,12,38). Based on the sequence of the unique adhesin-like gene identified in the present study, we designed primers and a fluorogenic (TaqMan) probe, and established a real-time PCR protocol that offers high sensitivity, specificity, and rapidity for reliable field detection of *S. kunkelii* in infected plants and inoculative insect vectors.

MATERIALS AND METHODS

Plant material, insect vector, and strain of *S. kunkelii*. Seedlings, germinated from seeds of the silage corn variety Asgrow RX913, were grown in a greenhouse under ambient lighting at 29 to 32°C/21 to 25°C (day/night). Greenhouse plants of six- to eight-leaf stage were inoculated by placing them in cages for 7 days with 50 adult leafhoppers (*D. maidis*) that had been allowed to feed for 5 days on plants known to be infected with *S. kunkelii* as evidenced by ELISA. Fully expanded leaves of field-grown corn plants and leafhoppers were sampled from locations throughout Kings, Fresno, and Tulare counties and from the University of California Kearney Research and Extension Center in Parlier, where plants and leafhoppers were subject to natural infections. Plant and leafhopper samples collected from the fields were placed in resealable plastic bags and plastic vials, respectively, and kept in an ice chest. Upon returning to the laboratory, the plants samples were stored at 4°C and the leafhopper samples were stored at -20°C until being processed for DNA extraction. *S. kunkelii* strain CR2-3x was provided by Jacqueline Fletcher of Oklahoma State University, Stillwater, and was cultured as previously described (24).

DNA extraction from *S. kunkelii* culture and from plant and insect vector samples. To establish a standard curve for real-time PCR assay, template DNA was extracted from a pure culture of *S. kunkelii* strain CR2-3x as previously described (3). DNA concentration was determined by measuring absorbance at 260 nm, and the quality of the DNA sample was verified on the basis of the A_{260}/A_{280} ratio. A 10-fold serial dilution was made to achieve final DNA concentrations from 50×10^0 ng/ μ l to 50×10^{-9} ng/ μ l. For plant samples, leaf areas with concentrated tubular phloem cells (i.e., midrib portions of the leaf) were thinly sliced and used for DNA extraction. For insect samples, the whole insect body was used. DNA extraction was carried out using REDExtract-N-Amp Plant PCR kit (Sigma Chemical Co., St. Louis, MO) according to the manufacture's instructions.

Real-time PCR. The primers and fluorogenic probe used for real-time PCR analysis in this study were designed based on the sequence of a new adhesin-like gene identified in our annotation of a draft genome sequence of *S. kunkelii* strain CR2-3x. The genome sequence data were accessed at internet site <http://www.genome.ou.edu/spiro.html>. Genome annotation was carried out as described previously (39,40). The forward and reverse primer sequences were as follows: sk104F: 5' CGTTGT TGAACCCG CAGTCAACTT 3' and sk104R: 5' ACAACAAGCATACCCGCA CCTTTG 3'.

The primers were custom synthesized by Invitrogen Corp. (Carlsbad, CA). The TaqMan probe, sk104-213 (5' TGGGAC GATATAGTGATACCAGTTGCCC 3'), was custom synthesized by GenScript Corp. (Scotch Plains, NJ) and was labeled with the reporter dye 6-carboxyfluorescein (FAM) at the 5' end and with the quenching dye 6-carboxytetramethylrhodamine (TAMRA) at the 3' end. Real-time PCR was performed using a Smart Cycler (Cepheid, Sunnyvale, CA). In a total volume of 25 μ l, each amplification reaction consisted of 5 μ M each forward and reverse primers, 0.25 μ M probe, 200 μ M dNTP, 1 μ l purified *S. kunkelii* DNA (50×10^0 ng to 50×10^{-9} ng) or 2 μ l plant/insect crude extract, 2.5 μ l GeneAmp 10 $\times$ PCR buffer, and 1.25 U AmpliTaq (Applied Biosystems, Foster, CA). Reactions were carried out for 45 cycles following an initial template denaturation step of 120 s at 95°C. The cycle conditions were: 15 s at 95°C and 45 s at 68°C. The results were analyzed using Smart Cycler Software (Cepheid).

PCR. In addition to real-time PCR assay, the presence of *S. kunkelii* in host plants and insect vectors was independently tested by conventional PCR. The amplification parameters for using primer set CSSF1/CSSR1 were the same as previously described (3). The amplification conditions for using primer set sk104F/sk104R were identical to that for the real-time PCR assay as described above, except without the TaqMan probe. The PCRs were carried out in an automated thermocycler (PTC-200, MJ Research, Waltham, MA). PCR products amplified with primer set CSSF1/CSSR1 and primer set sk104F/sk104R were analyzed by electrophoresis on 0.7 and 2.5% agarose gels, respectively, with TAE buffer under standard conditions (29). DNA bands were visualized using a UV transilluminator after ethidium bromide stain.

RESULTS

Characterization of a novel adhesin-like gene in *S. kunkelii*. During a search of a draft *S. kunkelii* CR2-3x genome sequence for potential targets for improved detection, we identified a gene that encodes a novel adhesin-like protein. This

previously unreported *S. kunkelii* adhesin-like gene was found to be located in an apparent operon consisting of seven open reading frames (ORFs) (Fig. 1A). The ORF of the new adhesin-like gene spans 2,502 nucleotides and is preceded by a putative ribosomal binding site, 5' AG-GAAA 3', 12 bp upstream of the translation initiation codon. Conceptual translation of the ORF yielded a (pre)protein of 833 amino acids with a calculated molecular mass of 92.3 kDa (Fig. 1B). The N-terminus of the preprotein possesses a 23-amino acid signal peptide as suggested by the signal peptide prediction program SignalP V3.0 (4). The N-terminus moiety of the deduced mature protein contains six complete copies and two partial copies of a 39-amino acid repeat (Fig. 1C), a hallmark of the sarpin domain (5). Near the C-terminus of the mature protein, there is a 20-amino acid transmembrane helix, which may anchor the protein to the plasma membrane with a possibility of the N-terminus being located outside of the cell. A BLAST search of the National Center for Biotechnology Information's nonredundant protein database (<http://www.ncbi.nlm.nih.gov/BLAST/>) using the deduced amino acid sequence as a query returned several *S. citri* and one *S. kunkelii* adhesin-like proteins of the sarpin family; therefore, the new *S. kunkelii* adhesin-like protein was designated SkARP2. Over the entire length, SkARP2 shares 68.7 and 58.3% sequence identity with SARP1 from *S. citri* (GenBank accession CAI99877) and SkARP1 from *S. kunkelii* (GenBank accession YP_138230), respectively. The sequence similarity between SkARP1 and SkARP2 is essentially limited to the C-terminus two-thirds of the proteins. The N-terminus one-third of the two proteins (the sarpin domain, which corresponds to amino acid positions 1 to 310 in SkARP2) shares only 8.3% identity. This indicates SkARP1 and SkARP2 are two distinct adhesins that mediate different biological activities and/or bind to different ligands. Nucleotide sequence corresponding to the unconserved sarpin domain region was used to design primers and a fluorogenic (TaqMan) probe for real-time PCR.

Within the adhesin operon, two other genes, *traE* and *mob*, also encode proteins that are likely associated with cell surface structures (Fig. 1A). Deduced *traE* and *mob* gene products contain sequence identities and/or domain architectures similar to VirB4 (TraE) and VirD4 (Mob) proteins, respectively. In pathogenic bacteria, VirB4 and VirD4 orthologs are components of type IV secretion/conjugation systems and are involved in cross-membrane trafficking of macromolecules such as DNA and proteins (10). The remaining four ORFs in the operon (*orf2*, *orf3*, *orf5*, and *orf7*) encode hypothetical proteins of unknown functions (Fig. 1A). Interestingly, paralogs of all the genes except *orf7* in this adhesin

operon are present in a previously described *S. kunkelii* plasmid (12). While the chromosomal location of the adhesin operon identified in this study has yet to be determined, it cannot be ruled out that it resides on an extrachromosomal element, as multiple adhesin paralogs of the sarpin family were reported to be in different plasmids in *S. citri* (16).

Standard curve. TaqMan probes derive their fluorescence signal from the hydrolysis of the labeled probe by *Taq* polymerase's 5' to 3' exonuclease activity. The hydrolysis frees the reporter dye (FAM in this study) from the quenching dye (TAMRA in this study) and results in an increased fluorescence signal (22,37). Based on this principle, a DNA dilution series (from 50×10^0 ng/ μ l to 50×10^{-9} ng/ μ l) was used as templates for real-time PCR, and a standard curve was generated by plotting the threshold cycle number for each serially diluted DNA sample versus the logarithm of the corresponding DNA concentration (Fig. 2). This threshold cycle (C_T) was the point where the fluorescence signal exceeded the background level. The standard curve had a slope of -0.248 and a correlation coefficient of 0.999 , which indicated that the standards fit to a straight line nearly perfectly. Replicate curves, generated from three independent experiments, were virtually identical (data not shown).

Sensitivity and specificity of the real-time PCR. According to the standard curve (Fig. 2), the effective linear detection range was between C_T 14.99 and C_T 39.97. The detection limit of the real-time PCR method was compared with that of conventional PCR using the same dilution series of the DNA template. In these tests, the real-time PCR assay was able to detect *S. kunkelii* DNA at the dilution of 50×10^{-7} ng/ μ l (i.e., 5 fg template DNA, Fig. 2), whereas conventional PCR detected *S.*

kunkelii DNA at the dilution of 50×10^{-5} ng/ μ l (i.e., 500 fg template DNA) regardless of whether primer set CSSF1/CSSR1 (Fig. 3A) or primer set sk104F/sk104R (Fig. 3B) was used. In our real-time PCR assays, no fluorescence signal was detected from DNA samples isolated from in vitro cultured *S. citri*, from healthy corn, or from aster yellows phytoplasma ('*Candidatus* Phytoplasma asteris'—related strain)—infected plant materials.

Detection of *S. kunkelii* in plant host and insect vector. To apply the real-time PCR method to field detection of *S. kunkelii* in its plant host and insect vector, double-blind tests were performed using corn and leafhopper samples collected from areas of disease occurrence in California's Central Valley. The results from real-time PCR and conventional PCR assays of plant

and insect samples are shown in Table 1. For each PCR-positive sample, an effective C_T value could always be obtained when the sample was subjected to a real-time PCR assay using either the original or a 10-fold diluted DNA preparation. On the other hand, for all conventional PCR-negative samples, real-time PCR assays also produced negative results (i.e., did not generate fluorescence signals above the threshold value) except for two plant samples (04/184FC2 and 04/230FC1) with low *S. kunkelii* titers that gave positive real-time PCR results, a manifestation of the superior sensitivity of the real-time PCR method (see discussion below).

DISCUSSION

Rapid and accurate detection of infectious agents in field samples and agricul-

Fig. 2. Standard curve derived from the real-time polymerase chain reaction amplification of the *Spiroplasma kunkelii* adhesin-like gene segment. The template was a 10-fold serial dilution of genomic DNA isolated from in vitro cultivated *S. kunkelii* strain CR2-3x. The amount of the template DNA per reaction ranged from 50×10^0 to 50×10^{-9} ng. Vertical axis represents log concentration of template DNA; horizontal axis represents number of threshold cycles.

Fig. 1. Structure of the adhesin-like gene *SkArp2*. **A**, *SkArp2* is located in an operon with six other genes, four of which encode hypothetical proteins of unknown functions. The other two genes, *traE* and *mob*, encode membrane proteins that are components of type IV secretion/conjugation system. **B**, *SkArp2* encodes a putative adhesin-like protein with an N-terminal signal peptide (gray box), a transmembrane helix (hatched box), and a sarpin domain containing tandem repeats (arrows). **C**, Amino acid sequence alignment of the six complete and two partial repeats in the sarpin domain.

Fig. 3. Conventional polymerase chain reaction amplification of targeted *Spiroplasma kunkelii* gene sequences, using the same template DNA dilution series described in Figure 2. **A**, 0.7% agarose gel electrophoresis of amplicons (505 bp) generated with primer set CSSF1/CSSR1. **B**, 2.5% agarose gel electrophoresis of amplicons (213 bp) generated with primer set sk104F/sk104R. Lanes M, 1 kb-Plus DNA ladder (Invitrogen). Lanes 1 to 10, DNA template from 50×10^0 to 50×10^{-9} ng.

Table 1. Real-time polymerase chain reaction (PCR) detection of *Spiroplasma kunkelii* in its host plant *Zea mays* and insect vector *Dalbulus maidis*

	C _T ^a with real-time PCR assay (DNA dilution series)		
Sample code	10 ⁰	10 ⁻¹	PCR result
Plant samples			
41T1	0	0	–
46T2	26.56	28.72	+
46T3	22.70	24.91	+
61T2	25.52	25.14	+
62T2	30.67	31.30	+
72T3	0	0	–
80T1	25.43	28.14	+
80T2	25.36	25.39	+
80T3	24.59	26.20	+
96T1	0	0	–
04/184FC2	0	39.50	–
04/230FC1	0	39.47	–
Insect samples			
D1	0	38.23	+
D2	36.70	38.78	+
D4	0	0	–
D8	0	0	–
D16	0	0	–
D18	0	0	–
D19	28.59	29.58	+
D20	0	39.97	+

^a C_T = threshold cycles.

tural products is essential to monitor disease incidence, prevent pathogen spread, and control economic losses. Real-time PCR as a quick diagnostic technique has been broadly employed for diagnosis and detection of plant diseases and quantification of the respective pathogens that cause these diseases (25,30). Recently, the development and application of real-time PCR technology has been extended to detect and quantify phytoplasmas, a group of wall-less bacteria that parasitize plant hosts and insect vectors (2,9,25,33). In the present study, a real-time PCR assay was devised, for the first time, to detect a plant pathogenic spiroplasma. This assay offers very high sensitivity with a detection limit of approximately 5 fg of *S. kunkelii* genomic DNA, which is equivalent to 2.5 genome copies or colony forming units (CFU). This level of sensitivity is 100-fold greater than that of conventional PCR (3).

In our double-blind tests of plant and insect samples collected from disease-affected areas in California, the detection of *S. kunkelii* using this real-time PCR method produced results consistent with those obtained from conventional PCR assays using primer sets CSSF1/CSSR1 and sk104F/sk104R, but was more sensitive. For all PCR-positive samples, the real-time PCR assay gave effective C_T values (Table 1). On the other hand, for a majority of PCR-negative samples, real-time PCR also produced negative results, except for two plant samples, 04-189FC2 and 04-230FC1 (Table 1), which gave C_T values of 39.50 and 39.47, respectively. The C_T values for these two plant samples were at the lower margin of the linear detection range of the real-time PCR method, whereas such low levels of target DNA were beyond the detection limit of the conventional PCR. This demonstrates that the real-time PCR assay developed in the present study was reliable and much more sensitive than conventional PCR in field applications and is an effective tool for detection of low populations of *S. kunkelii* in its host plants and insect vectors.

In addition to enhanced sensitivity, a reliable diagnostic method must also be designed to achieve maximal specificity. The primer and probe set used in this real-time PCR-based detection method possesses unique *S. kunkelii* nucleotide sequences that share no significant homology with any other known gene sequences deposited in the GenBank as of this writing. The target of the primer and probe sequences in the *S. kunkelii* genome is a 5' portion of a previously unreported adhesin-like gene. The existence of adhesin-like protein SARP1 in *S. citri* (5,38) and SkARP1 in *S. kunkelii* (12) has been reported. The detection target, adhesin-like gene *SkArp2*, shares 58% identity with the previously reported *SkArp1* gene at both nucleic acid and deduced amino acid levels (GenBank accessions AY528560 and YP_138230).

The conserved region between the two adhesin-like genes is limited to the 3' portion of the genes (C-terminus portion for the encoded proteins). The portion of the *SkAtp2* gene that is recognized by the primer and probe set shares no homology with either *SkAtp1* or *SATP1*. To test whether the real-time PCR-based detection assay would cross-react with other wall-less plant pathogenic bacteria, DNA preparations from *S. citri* and a '*Candidatus* Phytoplasma asteris'-related phytoplasma strain were subjected to a real-time PCR assay. The results were negative. This demonstrates the high specificity of the assay, as it does not cross-react even with one of the most closely related plant pathogenic spiroplasma species, *S. citri*.

The adoption of Sigma's REDExtract-N-Amp kit for DNA extraction from plant and leafhopper samples significantly contributed to the rapidity of the detection process. However, as with any other rapid DNA extraction protocol, the presence of trace amounts of impurities in the DNA preparation could lead to inhibition of subsequent real-time PCR reactions. As shown in Table 1, some PCR-positive samples (for example, 04/184FC2) failed to generate fluorescence signals in the real-time PCR assay when undiluted DNA extract was used. This problem was overcome by a 10-fold dilution of the original DNA extracts; presumably the dilution sufficiently reduced the concentration of inhibitors present in the DNA preparation. Inhibitory plant materials, especially encountered with aged or stressed plant tissues, have been reported previously (2,7,20). In field tests, attention should be given to details such as amounts and types of plant and insect tissues to be extracted and included in the reaction mixture of real-time PCR. For detection of *S. kunkelii* as well as other phloem-inhabiting pathogens, use of leaf areas with concentrated phloem cells (the midrib) would not only enrich for the target pathogens but also decrease the amount of green tissues that could contain inhibitors.

In conclusion, we have designed primers and TaqMan probe and established a real-time PCR-based protocol, which makes the detection of *S. kunkelii* easier and faster than ever before. The entire detection process, including sample preparation, can be completed in approximately 1 h. Since the detection method is highly sensitive, specific, and reliable, it provides a useful tool for both field diagnosis and laboratory research.

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