

A *Serratia marcescens* Strains Involved in Cotton (*Gossypium hirsutum*) Boll Infection by a Prokaryote

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Abstract

A boll infection caused by non-traditional cotton pathogens was first reported to occur in the southeastern U.S. Cotton Belt (year 2000) and has since spread to Texas causing significant yield losses. This study was aimed towards investigating the verde plant bug (*Creontiades signatus*) link between interior boll disease in Texas, USA. Using glasshouse grown bolls, bacteria recovered from locules with disease symptoms from field-grown cotton bolls caged with the piercing-sucking *C. signatus* were analyzed for the capacity to inflict the disease. For pathogenicity testing, spontaneously generated rifampicin resistant (Rif^r) variants were utilized to track the antibiotic resistant bacterium and deter growth of endophytic and contaminating bacteria. To simulate *C. signatus* feeding, a needle (31 gauge) was employed to inoculate bolls at 13 - 15 days after flower bloom. Bacterial suspensions ranged from 10¹ - 10⁶ colony forming units/ml. Field infection symptoms were duplicated after two weeks of bacterial exposure. Infectious strains were best categorized as *Serratia marcescens* based on traditional carbon utilization and enzyme production testing, and a 99% nucleotide sequence identity of 16S ribosomal DNA. Putative *S. marcescens* representatives isolated from rotted bolls exposed to *C. signatus* were shown to reproduce field infection symptoms upon inoculation into greenhouse grown fruit. *Serratia* spp. can inflict disease in alfalfa, cucurbits, and sunflower. The presented data are the first to definitively show that a *Serratia* sp. has the capacity to infect cotton.

Keywords

Verde Plant Bug, Opportunistic Bacterial Infections, Antibiotic Selection

1. Introduction

A recently reported boll disease in south Texas (USA) cotton (*Gossypium hirsutum*) is responsible for yield losses that can reach 15% [1]. In the southeastern Cotton Belt, a similar malady is known [2] [3] [4]. In both situations, early detection is complicated since bolls of affected fields contain necrotic seed and lint yet, are absent of external infection symptoms [5] [6].

In south Texas, an emerging sucking pest called the verde plant bug (*Creontiades signatus*) has been associated with cotton yield losses [1]. The Gulf Coastal region of south Texas is where *C. signatus* naturally occurs. Native weedy plants that are reservoirs of *C. signatus* include London rocket, *Sisymbrium irio* L. (Brassicaceae), pigweed, *Amaranthus* spp. (Amaranthaceae), and nettleleaf goosefoot, *Chenopodium murale* L. (Chenopodiaceae) [7]. *Creontiades signatus* can propagate on cotton [8] [9]. Notably, *C. signatus* is of similar appearance and genetic makeup to *C. dilues* that is autochthonous to Australia and a major plant bug pest of cotton in that country [10] [11].

Here, we report the isolation and putative identification of opportunistic *Serratia marcescens* from immature, diseased cotton bolls from field plots in south TX that were previously exposed exclusively to *C. signatus*. Using glasshouse grown bolls, Koch's postulates were fulfilled demonstrating that *S. marcescens* strains have the capability to produce disease in cotton fruit analogous to those observed in the field.

Serratia marcescens was initially isolated from pond water and considered a non-pathogenic bacterial species that produces a reddish pigment on culture plates. Currently, *Serratia* strains are known to opportunistically infect both humans and certain plants. Specifically, plant pathogenic *Serratia* strains cause disease cucurbits, sunflower, and alfalfa [12]. Our findings are the first that concretely demonstrated a capacity of a *Serratia* species to infect cotton.

2. Materials and Methods

2.1. Bacterial Isolation

Cotton plants grown from Coker cultivar (cv.) seed in field plots at the Texas A&M University—Extension Center-Corpus Christ (USA) were caged with and without field collected *C. signatus* bugs. In August (*i.e.*, late in the regional growing season), unopened green bolls were collected, packaged with frozen gel packs, and transported overnight to the USDA-ARS Insect Control & Cotton Disease Research Unit (ICCDRU) in College Station, Texas. Using 0.5% sodium hypochlorite bolls, were sanitized for eight minutes (1×) followed by sterile water rinses (3×). Boll walls were dissected with a sterile surgical knife. Aprox-

imately 1 g of diseased inner boll tissue with *C. signatus* bug feeding punctures was transported into a 1.1-ml microtube (SPEX SamplePrep; Metuchen, NJ) that contained 0.5 ml of PO₄ buffer (0.1 mol/L, pH 7.1) and a stainless steel ball that was sterile (4-mm) in a 1.1 ml tube (96 tubes/rack) for grinding (SPEX SamplePrep; Metuchen, NJ). Another ball was added and the tubes were sealed. The tissue was pulverized (5 min at 1500 strokes/min) with a 2000 Geno/Grinder (SPEX SamplePrep) and diluted with 0.5 ml of PO₄ buffer. The resulting homogenate was 10-fold dilution plated on Luria-Bertani agar (LBA; Difco Laboratories, Detroit, MI). Asymptomatic boll tissue was employed as controls and handled in the same manner as diseased tissue. All plates were incubated at 27°C until colonies were visible yet, not longer than two weeks. Remote colonies were dilution plated on LBA to ensure single cells. For long term storage, purified colonies suspended in LB (1%) broth with diluted glycerol (40%) were held at -80°C.

2.2. Pathogenicity Tests

Greenhouses were used to propagate cotton (cv. Coker) at the USDA-ARS-ICCDRU from seed for use in pathogenicity testing. The material used for planting was standard (dried peat moss, 12 L, vermiculite, 12 L; dolomitic lime, 300 ml; washed sand 18 L; gypsum 1 L; esmigran (Scotts-Sierra Horticultural Products Co., Marysville, OH), 5 ml) and was added to 0.5 kg plastic pots. Reverse-osmosis water was used to saturate the containers. Aerated steam at 74°C for 16 h was used for pasteurization. Seeds were germinated a 48 h at 30°C in moist towels, and then implanted into the planting mixture. Thermostats settings were 30°C and 20°C for cooling and heating, respectively. A fertilizer (150 mg) with chelated minor elements (Peter's Peat-Lite Special 15-16-17; Scotts-Sierra Horticultural Products Co., Marysville, OH) was added weekly.

Bacteria recovered from individual diseased bolls with evidence of *C. signatus* bug feeding (*i.e.*, punctures visible in the inner boll wall) were used in pathogenicity testing. Routine maintenance involved propagation on LBA plates at 27°C incubation. Remote colonies were utilized for all pathogenicity testing sterile Milli-Q (Millipore Sigma; Darmstadt, Germany) water was used to prepare bacterial suspensions from 18 h cultures grown on LBA and adjusted spectrophotometrically ($A_{600} = 1.0$). Ultimate concentrations of 10¹, 10³, or 10⁶ colony forming units (CFU) of a bacterial inoculum were analyzed. As *C. signatus* feeds via a piercing mechanism a 31 gauge needle was used to breach the boll wall suture (*c.* 3 - 5 mm) of bolls at 5 - 9 or 13 - 15 days post-anthesis (DPA). At 1 or 2 wks post-inoculation, bolls were longitudinally-sectioned along the suture and observed for presence/absence of disease symptoms with a set left to natural opening.

Spontaneously generated rifampicin resistant (Rif^r) mutants were generated by successively increased exposure of Rif sensitive remote colonies to the M9 minimal medium [13] amended with Rif reaching 200 µg/ml. Antibiotic resis-

tance to Rif was used for selection to distinguish between Rif^r variants, and both endophytic and contaminating prokaryotes [14] [15]. Strains CC119R, CC113R and CC114R Rif^r were derived from respective parental isolates that were recovered from separate diseased bolls and determined to be capable of producing infection symptoms in initial pathogenicity tests (Table 1). Antibiotic sensitive parental isolates were compared to the growth trajectory of the resistant variants as described before [16].

Infectivity of the Rif^r resistant mutants was compared to the respective parental strains that were sensitive to the antibiotic on green bolls (13 - 15 DPA). In triplicate, a single boll locule was inoculated for pathogenicity determination. Two independent studies were done. Using 18 h cultures, bacterial suspensions in sterile Milli-Q water were prepared and inoculated (method discussed above). Inoculations of 10 µl of sterile water into locules were employed as negative controls. After two weeks, a visual analysis of infection was conducted. In addition, concentrations of bacteria were determined. Using a sodium hypochlorite solution (0.5%), harvested bolls were washed independently for 10 minutes (1×) and then rinsed for 8 min in sterile water (3×). Boll walls were carved off using a sterile surgical knife. Locule tissue (c. 1 g) was placed in phosphate buffer and pulverized (discussed above). Following 10-fold serial dilution, samples were plated on nonselective media (LBA) and selective media (LBA amended added Rif at 200 µg/ml). Following a 24 h incubation at 27°C, colonies of bacterial from the seed and lint tissue were assessed (CFU/g locule tissue). A set of bolls were photographed at maturity (*i.e.*, naturally expose the lint).

To measure the role of the mutant CC119 with Rif resistance (CC119R) on seed and lint infections, three concentrations were inoculated (10¹, 10³, or 10⁶

Table 1. Bacterial strains used in this study.

Strains	Relevant characteristics	Source
<i>Serratia marcescens</i>		
ATCC 13880	Pond water, Type strain, Rif ^s sensitive	American type culture collection
CC119	Diseased Coker cultivar boll from TX ^b field plot, Rif sensitive	This study
CC113	Diseased Coker cultivar boll from TX field plot, Rif sensitive	This study
CC114	Diseased Coker cultivar boll from TX field plot, Rif sensitive	This study
CC119R	Derived from CC119; Rif resistant	This study
CC113R	Derived from CC113; Rif resistant	This study
CC114R	Derived from CC114; Rif resistant	This study
<i>E. coli</i> K12		
ER2267	F ⁺ <i>proA</i> ⁺ <i>B</i> ⁺ <i>lacI</i> ^h Δ (<i>lacZ</i>) <i>M15</i> <i>zff</i> :mini-Tn10 (Kan ^R)/ Δ (<i>argF-lacZ</i>) <i>U169</i> <i>glnV44</i> <i>e14</i> ⁻ (<i>McrA</i> ⁻) <i>rfbD1</i> ? <i>recA1</i> <i>relA1</i> ? <i>endA1</i> <i>spoT1</i> ? <i>thi-1</i> Δ (<i>mcrC-mrr</i>) <i>114::IS10</i>	New England Biolabs, Inc.

^aRif = Rifampicin; ^bTX = Texas, USA.

CFU/ml) into bolls at 13 - 15 after flower as using the methods discussed above. Antibiotic resistance was the basis for tracking of the mutant. CC119R cells heat-killed by exposure to 65°C for an hour were negative controls. Cell death of the samples were verified by plating on LBA.

A couple of independent tests were conducted to analyze disease expression. Including negative controls, both experiments consisted of triplicate inoculations of bacteria. After a two-week period, infection symptoms and/or non-symptoms were determined by visual examination. Seed and lint tissue from inoculated locules (including both live and control samples) were analyzed for the presence of CC119R on non-selective (LBA) and (LBA with added antibiotic) media. The analysis involved the measurement of bacterial concentrations (CFU/g locule tissue) base plating 10-fold serial dilutions on both selective and nonselective media. Bolls inoculated with either CC11R or water (*i.e.*, controls) were harvested after a two-week period and assessed for disease. As above, bacterial concentrations from both seed and lint tissue were based on plating all samples on non-selective media following serial dilutions (10-fold).

2.3. Pathogen Characterization Based on Phenotype

Representative bacteria (CC119, CC113, and CC114) recovered from separate diseased field bolls with similar symptoms and colony types, and a demonstrated capacity to produce disease in greenhouse grown bolls with comparable symptoms to the original field-grown bolls were further characterized as described before [16] [17]. In brevity, testing consisted of Gram staining, growth in anaerobic conditions, production of oxidase and catalase along with characteristic color of a colony on LBA. The control used in all testing was the *Serratia marcescens* type strain (ATCC 13880). Those isolates generally identified as Enterobacteriaceae were subsequently putatively categorized to species. For expanded phenotypic characterization, Analytical Profile Index test strips (API 20E; bioMerieux, Inc., Durham, NC) were used following the manufacturer's protocol along with incorporating the *S. marcescens* type strain.

2.4. Categorization Based on 16S Ribosomal DNA Sequence

A Polymerase Chain Reaction (PCR) primer set designed previously [16] to generate a 16S ribosomal product was utilized. The predicted 1.4 K base pair fragment based on *E. coli* positioning was ligated into the XbaI-NotI sites of the pDrive cloning vector (New England Biolabs Inc., Beverly, MA) then transformed into *E. coli* ER2267 (Table 1) by the CaCl₂ method [13]. Sequencing was performed at the Institute of Developmental and Molecular Biology, Gene Technologies Laboratory at Texas A&M University on a contract basis. Derived 16S rDNA gene sequence data from both strands were edited and assembled using Sequencher 4.8, and then compared to sequences in the GenBank database using the BLAST program available at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/BLAST/>). A Qiagen kit (Valencia,

CA) was used for all PCR experiments with an amplification protocol that consisted of an initial denaturation step at 96°C for 5 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min with a final extension at 72°C for 5 min using a PTC-200 DNA Engine Cycler (MJ Research Inc., Waltham, MA).

The generated 16S rDNA sequence from isolate CC119 was compared with the type strains of several α subclass of the Proteobacteria species including *Serratia marcescens* ATCC 13880 (AB681729.1). The phylogenetic analysis was performed using MEGAX [18] Juke-Cantor calculated distances, and a tree was constructed using the Unweighted Pair Group Method Average (UPGMA). The tree was assembled to scale, with branch lengths (above the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Bootstrapping was conducted for 1000 replicates with confidence levels greater 50% indicated at the tree internodes.

3. Results

3.1. Bacterial Isolation

The bolls feed upon by the insects resulted in disease whereas, infection symptoms were not apparent in bolls from plants not exposed to insects. Bacteria were isolated from 10-fold serial dilution plating of pulverized homogenate from discolored seed and lint. Colony morphology of a majority of the bacteria isolated from diseased seed and lint tissue was mucoid with smooth, entire margins, an umbonate elevation, and a reddish pigmentation on LBA. Preliminary identification of isolated and suspected pathogens indicated that they were best classified as *Serratia* species. Yeasts were not detected from any of the rotted bolls in the pilot field study. Bacteria were not detected in the asymptomatic tissue samples.

3.2. Pathogenicity Tests

Three isolates designated CC119R, CC113R, and CC114R were used in all tests to measure the optimal inoculation method. Disease symptom development at one week post-inoculation occurred in tissue surrounding the injection (*c.* 3 mm) with spotty yellowish pink discoloration of the seed coat. In contrast, seed and lint necrosis was visible throughout the inoculated locule two, three and four weeks post-inoculation. Each of the three representative isolates tested by wound inoculation consistently resulted in disease and locule rot.

The antibiotic resistance marker was employed to fulfill Koch's postulates and measure bacterial densities in inoculated fruit. The spontaneously derived Rif resistant mutants for the isolates (CC119R, CC113R, and CC114R) were determined to be capable of causing seed and boll rot. Rifampicin resistance for CC119R, CC113R, and CC114R was stable for the two week testing period. Based on dilution plating and colony counts on LBA, growth patterns between

the Rif resistant mutants and their respective parental strains (CC119, CC113, and CC114) were essentially equal.

Simulated *C. signatus* feeding was utilized to compare the infection potential of the Rif^r and respective Rif sensitive parental strains. An amendment to the technique consisted of injecting bacteria with a syringe and 31 gauge needle at a final concentration of 10^3 CFU/ml. Bacteria were not recovered from asymptomatic control locules (**Figure 1**) that were injected with sterile water and plated on either selective or non-selective media two weeks post-inoculation. Following the two-week incubation period, locules inoculated with parental strains were consistently diseased with bacterial concentrations of 10^8 CFU/g tissue on LBA, whereas no bacteria were detected on parallel Rif amended LBA plates. Disease symptoms and bacterial populations (10^8 CFU/g) in locules inoculated with the Rif mutants were comparable to those of parental strains. Notably, the Rif mutants were detected at equivalent levels on LBA with or without Rif. Locules from bolls that were injected with sterile water two weeks post-anthesis, and then grown to maturity had scar tissue just about the site of the initial inoculation (**Figure 1(C)**). Matured open bolls with locules that were inoculated with either the Rif^r mutants or the parental types developed necrosis of the entire locule.

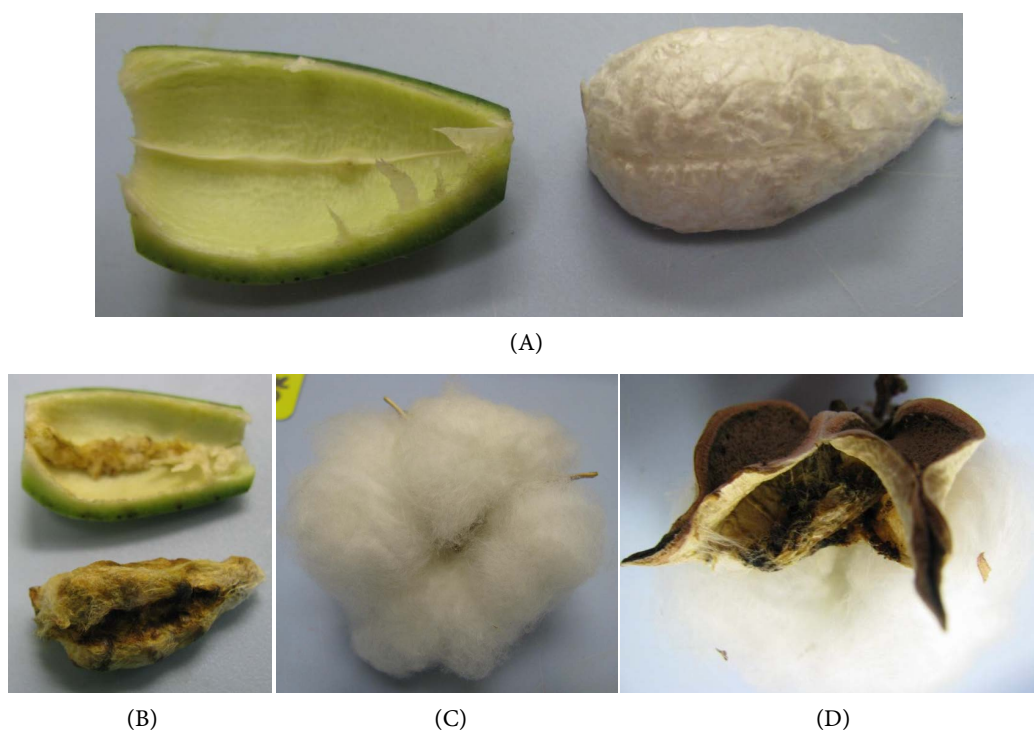


Figure 1. Differences between control and diseased greenhouse grown fruit with a locule inoculated two weeks post-anthesis using a 31 gauge needle. (A) A locule injected with 10 μ l of sterile water showing no apparent infection symptoms two weeks post-inoculation. (B) A locule injected with strain CC119R showing disease symptoms two weeks post-inoculation. (C) An open boll showing a locule injected with 10 μ l of sterile water two weeks post-anthesis with a minimal scar tissue from the initial puncture wound. (D) An open boll showing a locule injected with strain CC119R two weeks post-anthesis showing a matted, deteriorated boll.

Seed from diseased locules were sectioned and contained shriveled and brown embryos. Irrespective of use of parental strains or the Rif resistant mutants, disease occurred only in locules that were injected with bacteria.

The disease produced by either the parental or Rif^r variants resulted in similar infection symptoms. Thus, the Rif resistant mutant CC119R was exclusively used at a range of concentrations to examine potential effects of inoculum on disease symptom expression and bacterial populations (**Table 2**). As in field samples, none of the inoculated concentrations used produced exterior boll rot symptoms. Injections of CC119R at either 10³ or 10⁶ CFU/ml resulted in locule symptoms analogous to field infected boll samples with bacterial concentrations detected at 10⁸ CFU/g two weeks following the inoculations (**Table 2**). Disease symptoms were contained to the inoculated locule two weeks after injecting bolls with 10⁸ CFU/ml of the mutant. The locule tissue was completely withered, and bacterial concentrations reached 10⁹ CFU/g of diseased tissue. In contrast, locules injected with either heat-killed cells or sterile water appeared healthy (**Table 2**). Growth was not detected from cell suspensions of either 10¹, 10³, or 10⁶ CFU/ml on LBA following inoculation of heat-killed cells.

Table 2. Detection of strain CC119R from locule tissue of inoculated greenhouse grown cotton fruit.

Inoculation (CFU ml ⁻¹)	Boll appearance ^a	Bacteria recovered ^b (LBA)	Bacteria recovered ^b (LBA + Rif ^r)
CC119R			
10 ⁶	No exterior symptoms; entire locule infected and rotted	1 × 10 ¹⁰ CFU/g	1 × 10 ¹⁰ CFU/g
10 ³	No exterior symptoms; entire locule infected and rotted	5 × 10 ⁸ CFU/g	5 × 10 ⁸ CFU/g
10 ¹	No exterior symptoms; entire locule infected and rotted	5 × 10 ⁸ CFU/g	5 × 10 ⁸ CFU/g
CC119R ^d (heat-killed)			
10 ⁶	Puncture mark visible; no discoloration of locule	<10 ¹ CFU/g	<10 ¹ CFU/g
10 ³	Puncture mark visible; no discoloration of locule	<10 ¹ CFU/g	<10 ¹ CFU/g
10 ¹	Puncture mark visible; no discoloration of locule	<10 ¹ CFU/g	<10 ¹ CFU/g
H ₂ O	Puncture mark visible; no discoloration of locule	<10 ¹ CFU/g	<10 ¹ CFU/g

^aSuspensions of bacteria in sterile water were injected using a 31-gauge needle into a single locule per boll two weeks post-anthesis. ^bBolls were harvested two weeks following inoculations and dilution plated on both Luria-Bertani agar (LBA) medium and LBA amended with rifampicin (200 µg/ml) reported in colony forming units (CFU). ^cRif = Rifampicin. ^dCells were exposed to 65°C for 1 h.

Strain CC119R was further employed to examine effects of boll age at inoculation on both the severity of rot symptoms and bacterial densities (Table 3). Bolls injected less than six DPA were aborted, cavitated, or had split prematurely. Bolls inoculated with CC119R at 6 - 8 DPA had extensive disintegration of the locules leaving only a small mummified, sooty black residue. Thus, bacterial densities were not determined (Table 3). Control bolls 6 - 8 DPA were prone to fungal contamination. Locules from bolls inoculated 13 - 15 DPA with CC119R exhibited symptoms that resembled the infected field cotton bolls (Figure 2) from which the CC119R parental strain was isolated. Disease symptoms from the inoculated locule such as dark spots or stripes that developed immediately and contiguously under the suture were apparent throughout the tissue. The lint was stained brown, decomposed, and not viscous. The non-inoculated locules from the same bolls did not show disease symptoms. Levels of CC119R in infected locules were consistent with densities determined from studies that compared the Rif mutants and the parental strains (10^8 CFU/g). Bolls inoculated at 20 - 28 DPA with derivative CC119R expressed symptoms that extended only to the area immediately surrounding the injection point. Bacterial densities from locules inoculated 20 - 28 DPA were detected at 10-fold lower levels than populations from bolls inoculated 13-15 DPA.

3.3. Phenotypic Characterization and 16S Ribosomal DNA Sequencing of the Pathogens

Isolates CC119, CC113, and CC114 were Gram negative, rod-shaped, facultative anaerobes, and catalase positive. With the exception of the gelatinase test, the API 20E results for the three unknown isolates were identical to the reactions of the *S. marcescens* type strain (Table 4). Isolate CC113 and *S. marcescens* ATCC 13880 did not liquefy the charcoal gelatin, whereas isolates CC119 and CC114 diffused the substrate after 24 h. A 1.4 kb portion of the 16S rDNA gene for isolate CC119 was cloned and sequenced. The percentage of identities between sequence from the tested isolates was 99% when compared to the 16S rDNA of *S. marcescens* strain SM2617 and SM2618 (Figure 3).

Table 3. Disease symptoms resulting from wound inoculation of cotton cv. Coker at different boll ages with strain CC119R.

Boll age at inoculation	CC119R ^a		Control ^b	
	Boll appearance ^c	Bacteria ^d recovered	Boll appearance	Bacteria recovered
6 - 8 days post-anthesis	rot of all locules; boll abscission	not done	mark at inoculation point (c. 1 mm)	not done
13 - 15 days post-anthesis	entire locule infected and rotted	5×10^8 CFU/g	mark at inoculation point (c. 1 mm)	$<10^1$ CFU/g
20 - 22 days post-anthesis	necrosis at inoculation point (c. 3 mm)	1×10^7 CFU/g	mark at inoculation point (c. 1 mm)	$<10^1$ CFU/g

^aA bacterial suspension in sterile water was inoculated through the capsule of a single locule at a final concentration of 10^3 CFU/ml using a 31-gauge needle. ^bMock inoculations of bolls involved injecting sterile water (20 μ l) into the capsule of a single locule using a 31-gauge needle. ^cBolls were harvested 14 d after the inoculation date. ^dSamples were plated on Luria-Bertani agar (LBA) amended with rifampicin (200 μ g/ml) reported in colony forming units (CFU).

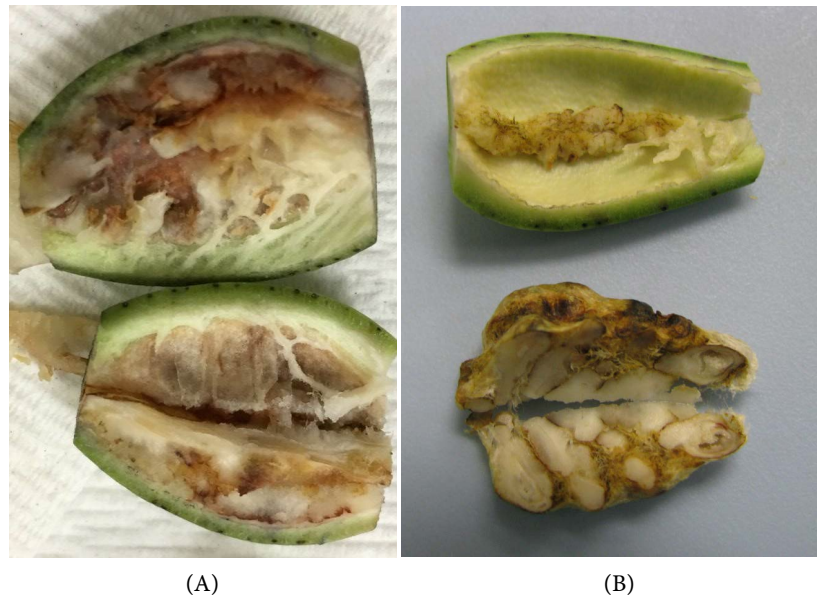


Figure 2. Symptom expression of diseased cotton fruit. (A) Field infected cotton boll samples exposed to *C. signatus*. (B) Greenhouse grown fruit injected two weeks post-anthesis with strain CC119R using a 31 gauge needle, and then harvested two weeks post-inoculation.

Table 4. Phenotypic and biochemical characteristics of isolates CC119, CC113, and CC114 that included the *Serratia marcescens* type strain ATCC 13880.

Test	CC119	CC113	CC114	<i>S. marcescens</i>
Reddish pigment on LBA	+	+	+	+
Growth at 37°C	+	+	+	+
Motility	+	+	+	+
Production of ^b :				
Beta-galactosidase	+	+	+	+
Arginine dihydrolase	-	-	-	-
Lysine decarboxylase	-	-	-	-
Ornithine decarboxylase	-	-	-	-
H ₂ S	-	-	-	-
Deaminase	-	-	-	-
Indole	-	-	-	-
Acetoin (Voges-Proskauer reaction)	+	+	+	+
Gelatinase	+	-	+	-
Citrate utilization ^a	-	-	-	-
Urea hydrolysis ^a	-	-	-	-
Acid production from ^a :				
Glucose	+	+	+	+
Mannitol	+	+	+	+
Inositol	-	-	-	-

Continued

Sorbitol	-	-	-	-
Rhamnose	+	+	+	+
Sucrose	+	+	+	+
Melibiose	-	-	-	-
Amygdalin	+	+	+	+
Arabinose	+	+	+	+
Oxidase	-	-	-	-

^aTesting results were determined using the API 20E system.

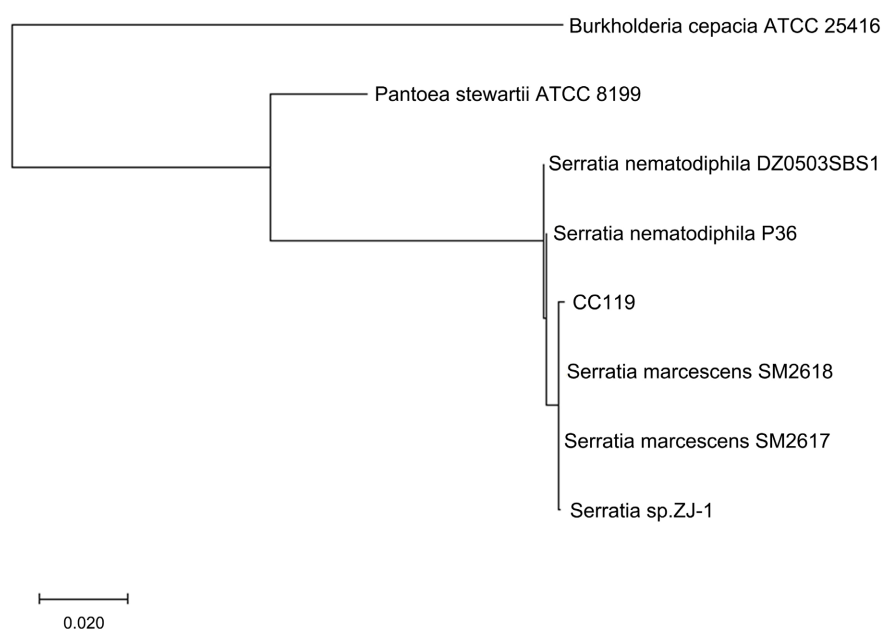


Figure 3. Phylogenetic tree of 16S rDNA showing the relationships among the boll infected isolates, and type strains for several α subclass of the Proteobacteria species. The sequences were aligned in Clustal W. Juke-Cantor distances were calculated and the tree was constructed in molecular evolutionary genetics analysis (MEGAX) using the Unweighted Pair Group Method Average (UPGMA) [18]. Bootstrapping was done for 1000 replicates and confidence levels greater than 50% are indicated at the internodes.

4. Discussion

Diseased fruit resulted from caging of field green bolls with field collected *C. signatus* bugs that feed using a sucking method. Conversely, disease was not detected in insect-free bolls suggesting association between *C. signatus* bug feeding and pathogen transmission. Other boll pests that feed via a piercing-sucking mechanism and are responsible for vector-borne infections include both the brown and southern green stink bugs [19] [20] [21].

The predominant bacterial colony morphology type consisted of a reddish hue. Representatives were shown to be capable of inflicting disease in greenhouse grown bolls with symptoms that resembled infected field boll samples

(**Figure 2**). Infection spread throughout the inoculated locule by two weeks post-inoculation (**Figure 1**). Consistent with cracked diseased field fruit pathogen inoculated greenhouse grown bolls were matted at maturity with lint and seed stained brown (**Figure 1**).

Resistance to Rif was introduced into isolates determined to cause boll disease for use as a selection marker to differentiate between Rif sensitive contaminants and the inoculated Rif^r mutants. The rifampicin mode of action consists of a broad bacterial host range with irreversible binding to the β -subunit of RNA polymerase; therefore, blocking bacterial transcription [22]. The stability of Rif^r in generated variants was used to study root colonization in field work [23]. Here, Rif^r was determined to holdfast in resistant derivatives propagated under non-selective conditions and thus, used to fulfill Koch's postulates.

5. Conclusions

In the current report, we showed that infection of immature bolls of greenhouse grown cotton produced symptoms similar to those observed in field grown bolls exposed to *C. signatus*. Bolls infested by the southern green stink bug (*Nezara viridula* L.) have reportedly caused an increase in the incidence of "hard-locks." Injury due to insect feeding was not consistently detected. Further, reductions in disease incidence result from the application of insecticides to cotton fields with a history of inner boll rot. Hence, bacterial recovery from diseased field grown boll continues in efforts to study a possible spectrum of opportunistic species and prospective seasonal variations.

The whole genome of *Serratia* strain CC119 was recently sequenced for the purpose of identifying gene products potentially involved in boll infection. Several potential pathogenicity and virulence genes were revealed including porins and invasins. Disease determinants shall be used to develop a PCR-based system to directly detect the pathogen from field samples. The molecular technique will simplify the costly and laborious process that now encompasses traditional culture and classification procedures that involve isolating and identifying bacteria followed by disease testing to distinguish between saprophytic and pathogenic strains.

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Author Contributions

EGM conceived the study and conducted the bacteriology work. JPG and MJB

conducted field work in Corpus Christi, TX. AAB conducted glasshouse work in College Station, TX.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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