Identification and Pathogenicity of Bacteria Associated with Etiolation and Decline of Creeping Bentgrass Golf Course Putting Greens

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ABSTRACT

Bacterial etiolation and decline has developed into a widespread issue with creeping bentgrass (CBG) (*Agrostis stolonifera*) putting green turf. The condition is characterized by an abnormal elongation of turfgrass stems and leaves that in rare cases progresses into a rapid and widespread necrosis and decline. Recent reports have cited bacteria, *Acidovorax avenae* and *Xanthomonas translucens*, as causal agents; however, few cases exist where either bacterium were isolated in conjunction with turf exhibiting bacterial disease symptoms. From 2010 to 2014, turfgrass from 62 locations submitted to the NC State Turf Diagnostic Clinic exhibiting bacterial etiolation and/or decline symptoms were sampled for the presence of bacterial pathogens. Isolated bacteria were identified using rRNA sequencing of the 16S subunit and internal transcribed spacer region (16S-23S or ITS). Results showed diverse bacteria isolated from symptomatic turf and *A. avenae* and

X. translucens were only isolated in 26% of samples. Frequently isolated bacterial species were examined for pathogenicity to 4-week-old 'G2' CBG seedlings and 8-week-old 'A-1' CBG turfgrass stands in the greenhouse. While results confirmed pathogenicity of *A. avenae* and *X. translucens*, *Pantoea ananatis* was also shown to infect CBG turf; although pathogenicity varied among isolated strains. These results illustrate that multiple bacteria are associated with bacterial disease and shed new light on culturable bacteria living in CBG turfgrass putting greens. Future research to evaluate additional microorganisms (i.e., bacteria and fungi) could provide new information on host-microbe interactions and possibly develop ideas for management tactics to reduce turfgrass pests.

Additional keywords: ecology, environmental microbiology.

Creeping bentgrass (CBG) (*Agrostis stolonifera* L.) is a popular cool season turfgrass species that is often intensely managed throughout much of the transition climate zone, a region of the United States in between the southern region, where warm season turfgrasses are acclimated, and the northern region, where cool season turfgrasses are acclimated (Dunn and Diesburg 2004). The transition zone is not particularly suitable to cool or warm season grasses species, yet both types grow well at different times of each year. Regardless, turfgrass managers are charged with the daunting task of maintaining aesthetics and superior playing quality in an environment with numerous abiotic and biotic stresses. Over the years, many fungal diseases have been described on CBG along with the successful deployment of management practices to reduce their severity. Yet, bacteria have rarely been problematic and are rarely considered important in turfgrass pathology. During the late 1970s, 'Toronto' (C-15) CBG suffered from numerous diseases resulting in a significant decline of the C-15 cultivar, otherwise known as the C-15 problem (Vargas 1981). While fungal diseases were problematic on C-15, this research presented the first report of bacterial disease in turfgrass systems and it was determined that *Xanthomonas translucens* (reported as *Xanthomonas campestris* pv. *graminis*) infection resulted in vascular wilt (Roberts et al. 1981). Bacterial wilt of CBG was described as stunting, wilting from the tip to the crown, and a blue-green color associated with affected plants. Reports of bacterial wilt on CBG decreased in later years as new varieties were developed and planted on golf course putting greens (Roberts 1981). Bacterial wilt (*X. translucens* pv. *poae*) became more problematic on annual bluegrass (*Poa annua* L. f. *reptans* (Haukskn) T. Koyama) resulting in similar symptoms in the eastern United States and Japan (Mitkowski et al. 2005).

In 2009, a severe decline of CBG was observed after the development of widespread chlorotic, abnormal elongation of turfgrass stems and leaves (i.e., etiolation) on golf course putting greens in North Carolina. While etiolation had been previously observed, the etiology of this condition remained unclear until recent reports (Giordano et al. 2010). Previous assumptions as to the cause included fungal infection, nutrient deficiencies, pesticide applications, and/or heat and drought stress. Giordano et al. (2010) demonstrated that *Acidovorax avenae* caused decline of CBG in controlled environmental conditions. Previously, Furuya et al. (2009) reported *Acidovorax* sp. as a cause of bacterial brown stripe on CBG in Japan. Reports of bacterial disease in turfgrass coupled with stressful environmental conditions observed in 2010 and 2011 incited fear among turfgrass managers resulting in numerous site visits and sample submissions to the NC State Turfgrass Disease Diagnostic Clinic. While numerous samples exhibited characteristic bacterial streaming, a diagnostic feature described by Giordano et al. (2010), isolation of *A. avenae* from CBG exhibiting etiolation and decline was not consistent. *Xanthomonas translucens* can cause bacterial etiolation on CBG and the ability of different bacteria to incite similar symptoms in CBG has caused many to question if other bacteria are involved (Roberts et al. 2014).

Due to the rarity of bacterial diseases in turfgrass systems, little is known regarding how bacteria, both beneficial and pathogenic, persist and survive in turfgrass ecosystems. Sundaram et al. (1988) previously isolated N₂ fixing *Azospirilla* bacteria from Kentucky bluegrass (*Poa pratensis* L.) seeds and roots. Elliot et al. (2004) also showed that soil in the southeastern United States contained numerous culturable bacteria and actinomycetes that were stable over time and location and that CBG roots had different microbial populations compared with bermudagrass roots. Other studies have shown that microbial antagonists can reduce fungal diseases like summer patch (*Magnaporthe poae* Landschoot and Jackson) and brown patch (*Rhizoctonia solani* Kuhn) (Giesler and Yuen 1998;

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Kobayashi et al. 1995). While these and other studies present considerable information regarding the soil microflora and the efficacy of biological controls in turfgrass systems, research is needed to determine what bacteria are present in relation to etiolation and decline of CBG. Based on the sudden emergence of suspected bacterial disease, the objectives of this research were to (i) isolate and identify bacteria from turfgrass exhibiting symptoms of bacterial etiolation and/or decline and (ii) characterize frequently isolated bacteria for pathogenicity to CBG.

MATERIALS AND METHODS

Isolation of bacteria. From 2010 to 2014, bacteria isolates were obtained from CBG samples submitted to the NC State

TABLE 1. Identification of bacterial isolates from 2010 to 2014 using 16S rRNA sequencing homology compared with known sequences in the Basic Local Alignment Search Tool for nucleotides (BLASTn)

Genus	Predominant species isolated within each genera	Number of isolates within genus
<i>Acidovorax</i>	<i>avenae</i> (18) ^z	18
<i>Acinetobacter</i>	<i>baumannii</i> (2), <i>calcoaceticus</i> (2), and <i>oleivorans</i> (1)	5
<i>Aeromonas</i>	<i>veronii</i>	1
<i>Agrobacterium</i>	<i>vitis</i>	2
<i>Agrococcus</i>	<i>jejuensis</i>	1
<i>Arthrobacter</i>	<i>defluvii</i>	1
<i>Bacillus</i>	<i>aerophilus</i> (1), <i>cereus</i> (2), <i>mycoides</i> (1), <i>pumilus</i> (2), and <i>thuringiensis</i> (6)	12
<i>Brevibacillus</i>	<i>agri</i>	1
<i>Burkholderia</i>	<i>cepacia</i> (2) and uncultured (1)	3
<i>Chryseobacterium</i>	<i>hugamense</i> (1), <i>rigui</i> (2), <i>taichungense</i> (1), and <i>taiwanense</i> (4)	8
<i>Cronobacter</i>	<i>dublinensis</i>	1
<i>Curtobacterium</i>	<i>flaccumfaciens</i> (2) and <i>pusillum</i> (1)	3
<i>Enterobacter</i>	<i>cloacae</i> (11) and <i>hermannii</i> (1)	13
<i>Escherichia</i>	<i>coli</i> (1) and <i>hermannii</i> (2)	3
<i>Exiguobacterium</i>	<i>acetylicum</i>	1
<i>Flavobacterium</i>	<i>johnsoniae</i>	1
<i>Herbaspirillum</i>	<i>huttiense</i> (4) and <i>seropedicae</i> (1)	5
<i>Labrys</i>	<i>okinawensis</i>	1
<i>Luteibacter</i>	<i>rhizovicinus</i> (1) and <i>yeojujensis</i> (4)	5
<i>Mesorhizobium</i>	<i>ciceri</i>	1
<i>Microbacterium</i>	<i>binotii</i> (1), <i>oleivorans</i> (4), <i>hydrocaroxydans</i> (1), <i>paradoxans</i> (2), and <i>testaceum</i> (9)	17
<i>Niabella</i>	<i>yanshanensis</i>	1
<i>Paenibacillus</i>	<i>humanensis</i> (5) and <i>illinoisensis</i> (1)	6
<i>Pantoea</i>	<i>agglomerans</i> (3), <i>ananatis</i> (18), and <i>eucria</i> (6)	27
<i>Pedobacter</i>	<i>heprinus</i> (1) and <i>saltans</i> (1)	2
<i>Pseudomonas</i>	<i>alcaligenes</i> (1), <i>cichorii</i> (11), <i>fluorescens</i> (1), <i>fulva</i> (9), <i>mendocina</i> (1), <i>montellii</i> (1), <i>oryzihabitans</i> (1), <i>peli</i> (1), <i>pseudoalcaligenes</i> (1), <i>putida</i> (2), <i>stutzeri</i> (6), and <i>trautweinei</i> (1)	37
<i>Rheinheimera</i>	<i>tangshanensis</i>	1
<i>Rhizobium</i>	<i>pseudoryzae</i> (1) and <i>taibaishanense</i> (1)	3
<i>Sphingobacterium</i>	<i>multivorum</i> (2), <i>siyangensis</i> (2), and <i>yabuuchiae</i> (1)	5
<i>Sphingomonas</i>	<i>sanguinis</i> (1) and <i>yabuuchiae</i> (1)	2
<i>Stenotrophomonas</i>	<i>acidaminiphila</i> (1) and <i>maltophila</i> (24)	25
<i>Variovorax</i>	<i>paradoxus</i>	2
<i>Xanthomonas</i>	<i>translucens</i>	14
Total-33		228 isolates

^z Numbers in parentheses designate to the number of individual species isolated within each genus.

Turfgrass Diagnostic Clinic exhibiting symptoms of etiolation and/or unexplained (i.e., fungal pathogens not observed) chlorosis and decline. While environmental data were not collected from individual samples, the majority of samples submitted over the 4-year period were sent from golf courses experiencing environmental stress (i.e., extended daytime temperatures >32°C and drought). To isolate bacteria, individual turfgrass plants were extracted from the turfgrass stand surface, root and senescent tissue were removed and discarded, and the remaining plant material was surface disinfested in diluted sodium hypochlorite solution (0.6% NaOCl; 10% Clorox) for 5 min. The resulting tissue was then rinsed in sterile dH₂O, placed on a sterile glass slide in a drop of sterile dH₂O, and cut to allow bacteria to stream out into the sterile H₂O for at least 2 min. The slide was examined for bacterial streaming and the resulting bacterial suspension was transferred to three individual petri dishes containing nutrient agar (NA). After 24 to 48 h, the 3 most prevalent individual colonies exhibiting distinct colony morphology were isolated using a transfer loop to T-streak each bacterium on a fresh NA plate (Harley 2004). Once bacteria were isolated, cultures were transferred to nutrient broth and allowed to grow for at least 48 h before being transferred to 20% glycerol and placed in cold storage at -80°C. Bacteria were regenerated from cold storage for experimental use by streaking a transfer loopful of frozen material on nutrient agar.

Identification of isolates. For identification, rRNA was extracted from bacterial samples by placing a 3- to 5-μl volume of bacteria cells scraped from growth on a NA plate in a 1.5-ml capacity microcentrifuge tube containing 50 μl of sterile dH₂O. The resulting suspension contained in each microcentrifuge tube was heated in boiling water for 10 min to extract bacteria rRNA. After boiling, 1 μl of the product was added to a polymerase chain reaction (PCR) of 15 μl in volume that was prepared having concentrations of 1.5 mM 10× PCR Buffer (Bioline USA, Taunton, MA), 0.8 mM dNTP mix (Bioline), 0.2 μM primer 63f (CAG GCC TAA CAC ATG CAA GTC), 0.2 μM primer 1387r (GGG CGG WGT GTA CAA GGC), and 2.5 U of *Taq* polymerase (Bioline) (Marchesi et al. 1998). The 16S subunit amplification was performed using thermal cycling conditions consisting of an initial denaturation step at 94°C for 2 min; followed by 30 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s; and a final extension step at 72°C for 5 min. Positive PCR amplification was confirmed by gel electrophoresis before purifying samples using ExoSAP-IT (Affymetrix, Santa Clara, CA); 1.5 μl of ExoSAP-IT was added to 6 μl of PCR product and subjected to thermal cycling conditions of 37°C for 30 min followed by 80°C for 20 min. Purified samples were sequenced using BigDye v3.0 obtained from the Duke Center for Genomic and Computational Biology. Sample reactions (10 μl in volume) were prepared and sequencing reactions were performed using thermal cycling conditions of an initial denaturation step at 96°C for 2 min, followed by 40 cycles of 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min. The resulting reaction volume was submitted to the Duke University Genome Sequencing Laboratory for sequencing. Sequencing results for each sample submission were assembled into a consensus 16S rRNA sequence using both forward and reverse reactions with CLC Genomics Workbench (CLC-GW) v7.5 (CLC bio: A QIAGEN Company, Boston, MA). Preliminary identification was obtained using the Basic Local Alignment Search Tool (BLASTn) utility in the National Center for Biotechnology Information (NCBI) Database (<https://www.ncbi.nlm.nih.gov/>). Reference sequences of identified bacteria genera and species were obtained using the NCBI Reference Sequence database and all 16S sequences were aligned using the native analysis (settings: slow and accurate) in CLC-GW. Phylogenetic analysis of aligned sequences was performed by a neighbor-joining tree construction using the Jukes-Cantor nucleotide distance measure with a 10,000 bootstrap replicate analysis (Jukes and Cantor 1969). The resulting tree was converted to an unrooted, color-coded by genera, circular cladogram using the Interactive Tree of Life (Letunic and Bork 2011).

Bacteria genera that were isolated in high frequency and previously determined pathogenic to CBG were also identified using rRNA sequencing of the 16S-23S or internal transcribed spacer (ITS) region using the primers 1493f (AGT CGT AAC AAG GTA GCC GT) and 23r (GTG CCA AGG CAT CCA CC) (Schaad 2001; Schaad et al. (2008)). Purification and sequencing reactions were performed using the techniques described above. Reference sequences cited in previous literature of multiple species from each genera were obtained from the NCBI Reference Sequence database and all sequences were aligned in CLC-GW along with phylogenetic analysis using the Jukes-Cantor nucleotide distance measure and 10,000 bootstrap replicates.

Pathogenicity testing. With the exception of *Stenotrophomonas* spp., only frequently isolated bacteria (i.e., >10 isolations) were examined for pathogenicity to CBG seedlings using a modified method developed by Roberts (1981). A 'G-2' CBG seed was planted in seed starter trays filled with calcined clay (Turface Allsport; Profile Products LLC, Buffalo Grove, IL) and allowed to grow in a greenhouse at ~30°C daytime and ~20°C nighttime that received irrigation two times daily. Seedlings were fertilized every 7 days by applying 1/2 strength Hoagland's solution (Hoagland and Arnon 1950) plus iron and algaecide (VWR) at a rate calibrated to deliver 4.9 kg N ha⁻¹. For each treatment, a bacterial suspension was prepared by flooding fully colonized NA plates with 100 mM phosphate + 1% peptone buffer (pH = 7.0) and scraping cells loose with a sterile inoculating loop. The resulting suspension was then diluted to 10⁹ colony forming units (CFUs) per milliliter. Plants were inoculated after 4 weeks growth (i.e., three to five leaf stage) by removing each plant from the soil, trimming the leaf tissue to 2 cm, and submerging each of the four plants (to serve as replicates) into the bacterial suspension of each treatment (*A. avenae*, *Enterobacter cloacae*, *Pantoea ananatis*, *Microbacterium testacium*, *Pseudomonas putida*, *Pseudomonas fulva*, *X. translucens*, and a noninoculated control) for 5 min on a shaker bench at 100 rpm. Sterile phosphate buffer plus peptone was used for the non-inoculated control. After inoculation, each of the four individual plants were replanted in 3.8 × 21 cm cone-tainers (Ray Leach Cone-tainers, Stuewe and Sons Inc., Tangent, OR) filled with calcined clay and placed back on the greenhouse bench. Plant quality was

rated on a scale of 1 to 9 (1 = completely dead and 9 = healthy) every other day for signs of chlorosis and decline. All data were subjected to analysis of variance using the MIXED procedure in SAS 9.4 and means were separated using Tukey's honest significant difference.

Seedling inoculations that resulted in significant decline were examined in further detail by inoculating multiple plants grown in cone-tainers to form small turf stands similar to a putting green. CBG 'A-1' was seeded in cone-tainers (3.75 × 20 cm) filled with a sterilized mixture of sand and peat (85% sand/15% peat). The CBG was allowed to grow on a greenhouse bench at ~28°C daytime and ~20°C nighttime for 8 weeks receiving irrigation two times daily and fertilization with 1/2 strength Hoagland's solution applications calibrated to deliver 4.9 kg N ha⁻¹ every 7 days. Turf was cut at ~1 cm to maintain a dense turf canopy similar to a putting green surface. Bacterial suspensions (*A. avenae*-1, *A. avenae*-2, *P. ananatis*-1, *P. ananatis*-2, *P. ananatis*-3, *P. ananatis*-4, *P. ananatis*-5, *E. cloacae*, and *E. hermannii*, and nontreated control) were prepared as previously discussed to obtain bacterial suspensions containing 10⁹ CFUs ml⁻¹. *Acidovorax* isolates were included as positive controls. Inoculation was performed by cutting the turf surface similar to normal trimming with scissors dipped in bacterial cell suspensions of each isolate. Scissors were dipped after each cutting and all replicates were trimmed with inoculated scissors prior to flame sterilization to move to successive isolates. The remaining cell suspension for each isolate was applied to the turf surface as a drench using an atomizer bottle. After inoculation, plants were randomized within a cone-tainer rack with a 1-cone-tainer buffer between blocks. The rack was placed in a sealed clear plastic bin (Cambro Co., Huntington Beach, CA) for 48 h to maintain high humidity and then transferred to the greenhouse bench for observation. Plants were inoculated using the same method twice 7 days apart (30 May and 6 June) and symptoms of etiolation were rated when observed by counting abnormally long and chlorotic leaves within each replicate. Turf quality was rated every few days using a scale of 1 to 9 (1 = completely dead and 9 = healthy) and turf density and color were all components of turf quality. Data were subjected to analysis of variance using the MIXED procedure in SAS 9.4 (SAS Institute Inc., Raleigh, NC) and means were separated using Tukey's honest significant difference.

Fig. 1. Phylogenetic relationships of bacteria associated with bacterial etiolation and/or decline isolated from 2010 to 2014. Alignment was performed using rRNA sequences of the 16S subunit in CLC-Genomics Workbench7.5 and the unrooted tree was generated using the Jukes-Cantor method and 10,000 bootstrap replicates in CLC-GW and the Interactive Tree of Life (iTOL) online tool.

RESULTS

Isolation and identification of bacteria associated with turfgrass bacterial disease. A total of 228 isolates were collected from 78 samples submitted from 62 locations over a 4-year period. Samples were obtained from Arkansas, California, Georgia, Illinois, Kentucky, Missouri, New Jersey, North Carolina, Ohio, Pennsylvania, Tennessee, Virginia, and West Virginia. Results from BLASTn alignments of individual isolates identified 33 different genera encompassed by various species within each genus (Table 1). When comparing 16S rRNA sequences from isolates obtained in our study in the BLASTn, 211 isolates exhibited 98 to 100% similarity to known sequences, 13 isolates were 95 to 97% similar, 3 isolates were 90 to 94% similar, and 4 isolates had <90% similarity. Instances of lower percent homology to a specific match were supported with sequence data from ITS sequences to positively identify each bacterial isolate (data not shown). Phylogenetic analysis of the 16S rRNA sequence data proved similar to the BLASTn results, which formed 13 distinct clusters encompassed within the Actinobacteria, Bacteroidetes, Firmicutes, α -Proteobacteria, β -Proteobacteria, and γ -Proteobacteria (Fig. 1). Of all bacteria isolated, γ -Proteobacteria exhibited the greatest diversity with six different clusters as observed in the phylogenetic tree. Closely related genera were not easily differentiated within the 16S rRNA phylogenetic tree, such as genera within the family *Enterobacteriaceae*, as *Pantoea* and *Enterobacter* species grouped together instead of in defined groups.

Sequencing of the ITS rRNA region was also performed for 18 *Acidovorax* isolates and 12 *Xanthomonas* isolates (Figs. 2 and 3). These sequences were often more variable across genera and sometimes difficult to amplify when compared with the 16S subunit. Multiple sequence copies of variable length were occasionally present in a few amplifications, which resulted in lower quality sequence data in these samples. The vast majority of our isolates collected from turfgrass grouped well with known strains of *A. avenae*. *Acidovorax* 10895-A and *Acidovorax* 11415-C2 were more similar to *A. citrulli* and *A. facilis* than *A. avenae* subsp. *avenae* (Fig. 2). Comparing ITS sequences in *X. translucens* isolates to known reference sequences also showed multiple relationships across these bacteria. Of the 12 isolates sequenced, seven showed strong similarity to *X. translucens* pv. *poae*; one isolate was similar to *X. translucens* pv. *graminis* while the additional four isolates were grouped outside of the known grass pathovars with *translucens* and *undulosa* (Fig. 3).

Characterization of pathogenicity of bacteria frequently associated with bacterial disease in CBG. Seedling inoculations were performed to obtain a quick and reliable method to select bacteria for additional screening on turfgrass stands. Replicated inoculations were similar across experiments and are presented together in Figure 4. *A. avenae*, *P. ananatis*, and *X. translucens* substantially reduced seedling quality 10 days after inoculation, whereas *E. cloacae*, *M. testaceum*, *P. putida*, and *P. fulva* inoculations did not affect seedling quality (Fig. 4). Following the seedling inoculations, *P. ananatis* was reisolated from symptomatic tissue, thus fulfilling

Fig. 2. Phylogenetic relationships of *Acidovorax avenae* bacteria associated with bacterial etiolation and/or decline isolated from 2010 to 2014. Alignment was performed using rRNA sequences of the 16S-23S (ITS) subunit in CLC-Genomics Workbench7.5 and the tree was generated using the Jukes-Cantor method and 10,000 bootstrap replicates in CLC-GW. Sequences designated as RefSeq* were obtained from the NCBI database on the basis of Schaad et al. (2008) and *Comamonas testosteroni* strain BCRC 10956 was used as an outgroup in the analysis.

Fig. 3. Phylogenetic relationships of *Xanthomonas translucens* bacteria associated with bacterial etiolation and/or decline isolated from 2010 to 2014. Alignment was performed using rRNA sequences of the 16S-23S (ITS) subunit in CLC-Genomics Workbench7.5 and the tree was generated using the Jukes-Cantor method and 10,000 bootstrap replicates in CLC-GW. Sequences designated as RefSeq* were obtained from the NCBI database on the basis of Chaves and Mitkowski (2013) and *Stenotrophomonas maltophilia* strain DL10 was used as an outgroup in the analysis.

Fig. 4. Impact of bacteria on turf quality of 'G-2' creeping bentgrass seedlings. Quality was rated on a scale of 1 to 9 (1 = completely dead and 9 = completely healthy) and results are presented as inoculated versus noninoculated, which were combined from multiple experiments. Error bars represent Tukey's honest significant difference at the 0.05 probability level.

Koch’s postulates. Further examination of *P. ananatis* isolates on more established turfgrass resulted in quality reductions that varied across the experiments. Inoculation of positive controls (*A. avenae*-1 and -2) did result in etiolation symptoms 6 days postinoculation, whereas all *P. ananatis* strains did not (Fig. 5). In the same experiment, turf quality was reduced by *P. ananatis*-5 and -6 and *A. avenae*-1 at 6 days after the initial inoculation (Table 2). All other species and isolates tested did not reduce turf quality compared with the noninoculated control (Table 2). Six days following the second inoculation, isolates of *A. avenae*-1 and -2, and *P. ananatis*-1, -5, and -6 all reduced turf quality. At the same time, turf quality of plants inoculated with *E. cloacae*, *P. ananatis*-2, -3, and -4 were not different from the noninoculated control. Reductions in turf quality were observed as chlorosis, dieback from the leaf tip, and thinning.

DISCUSSION

It should be noted that results from our research only included culturable bacteria. Bacteria exist in many different ecosystems and many species are considered nonculturable using traditional techniques (Pham and Kim 2012). Future research incorporating newer

metagenomic approaches, such as sequencing of environmental DNA, will yield more information regarding the presence of bacteria and additional microorganisms associated with turfgrass disease. Regardless, this research improves our knowledge of the bacteria isolated in conjunction with etiolation and decline of CBG. Examination of submitted turf samples over a 4-year period showed that *A. avenae* and *X. translucens* were not frequently isolated from declining turfgrass. Previous reports indicate that *A. avenae* was the sole cause of etiolation and decline and yet our work does not demonstrate constant association with these symptoms in the field. Sequencing of 16S rRNA can provide identification to genus in most cases and of the 78 samples submitted to the NC State Turf Diagnostic Clinic exhibiting symptoms of bacterial etiolation and/or decline, *Acidovorax* was isolated from 10 locations and *Xanthomonas* was isolated from 12 locations; samples from two locations contained both *A. avenae* and *X. translucens*. In reality, only 26% of the samples submitted suspecting bacterial decline and etiolation yielded either *A. avenae* or *X. translucens*. Although research from Giordano et al. (2012), Roberts et al. (2015a, b), and this work demonstrate decline and/or etiolation in pathogenicity assays, it remains unclear why

Fig. 5. Etiolation of ‘A-1’ creeping bentgrass 6 days postinoculation with bacteria. Number of etiolated plants represent averages over four cone-tainer replicates and error bars represent Tukey’s honest significant difference at the 0.05 probability level.

TABLE 2. Turf quality of ‘A-1’ creeping bentgrass after inoculation with bacteria species associated with bacterial etiolation

Bacteria	Turf quality ^z								
	(1 to 9; 9 = best)								
	30 May	1 Jun	2 Jun	3 Jun	5 Jun	9 Jun	12 Jun	16 Jun	23 Jun
<i>Acidovorax avenae</i> 1	6.3	6.0	5.5	5.0	4.5 cd	3.5 b	3.1 d	2.8 b	3.5
<i>A. avenae</i> 2	7.0	6.8	6.5	6.5	5.3 abcd	5.5 ab	4.1 cd	5.0 ab	4.8
<i>Enterobacter cloacae</i>	6.8	6.5	6.8	6.8	5.5 abcd	6.8 a	6.0 abc	5.1 ab	4.8
<i>Pantoea ananatis</i> 1	7.0	7.0	7.0	6.8	6.5 ab	4.8 ab	4.0 cd	3.5 ab	4.3
<i>P. ananatis</i> 2	7.0	6.8	6.8	6.5	5.5 abcd	5.8 ab	5.5 abc	4.8 ab	5.0
<i>P. ananatis</i> 3	6.8	6.8	6.5	6.5	5.5 abcd	6.0 ab	6.0 abc	4.9 ab	5.1
<i>P. ananatis</i> 4	6.8	7.0	7.0	6.5	6.3 abc	6.0 ab	5.6 abc	5.3 a	5.6
<i>P. ananatis</i> 5	6.5	6.3	6.5	6.0	4.3 d	4.5 ab	4.1 cd	3.8 ab	4.3
<i>P. ananatis</i> 6	6.3	5.8	5.8	5.0	4.3 d	5.8 ab	4.5 bcd	4.3 ab	4.3
Nontreated	7.0	7.0	7.3	7.0	7.0 a	7.0 a	6.9 a	5.4 a	5.5
Tukey’s HSD	1.2	1.7	2.0	2.2	2.0	2.8	2.1	2.5	2.6
Source of variation									
Isolate	NS ^q	NS	NS	*	***	**	***	*	NS

^z Turf quality was rated on a scale of 1 to 9 (1 = completely dead and 9 = completely healthy) periodically after inoculation with bacteria (10⁹ CFU ml⁻¹) on 30 May and 6 June 2014. *, Significant at the 0.05 probability level; **, significant at the 0.01 probability level; ***, significant at the 0.001 probability level; and NS, not significant.

etiolation/decline is frequently observed. Moving forward, turfgrass managers finding one of the bacteria listed above should be cautious in drastically changing management practices nor should the absence of confirming the presence of these organisms exclude the possibility of bacterial infection and colonization.

Isolated bacteria associated with turfgrass samples were diverse belonging to four distinct phyla (Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria), 7 classes, 11 orders, 22 families, and 33 genera. A high percentage of isolates exhibited strong similarity (>95%) to known isolates using BLASTn. Yarza et al. (2008) state that 16S rRNA gene sequences hold a reasonable threshold value of 95% homology for similar genera while Janda and Abbott (2007) state that 99% sequence similarity is required for species identification. When comparing isolates in a phylogenetic analysis, we did observe some resolution problems with sequences believed to be in the *Enterobacter*, *Escherichia*, and *Pantoea* genera; however, Janda and Abbott (2007) previously mention that groups within the *Enterobacteriaceae* can have resolution difficulties when using 16S rRNA to identify genera and species. *Stenotrophomonas* and *Xanthomonas* isolates also clustered close together but this was expected with the similarities among these bacteria sequences. Based on these results, additional sequencing is necessary to confirm species identification within these genera.

While multiple copies of ITS can exist between similar strains of the same species, use of this sequence can be used to provide better resolution at the species and subspecies level (Barry et al. 1991). Similar results were observed with ITS rRNA sequences of *Acidovorax* and *Xanthomonas* isolates examined in our study. *Acidovorax* isolates obtained in our study showed strong similarity with multiple known *Acidovorax* isolates that were previously used in reclassification of subspecies of *A. avenae* (*cattleyae*, *citrulli*, and *oryzae*) into *A. cattleyae*, *A. citrulli*, and *A. oryzae* (Schaad et al. 2008). Giordano et al. (2010, 2012) previously reported *A. avenae* subsp. *avenae* as the cause of bacterial etiolation and decline on CBG on the basis of percent similarity to reference 16S rRNA sequences; however, the analysis of 16S and ITS sequences obtained in our study do not provide a clear resolution of subspecies within the *Acidovorax* genus. Based on research presented here and previous work utilizing 16S sequencing, it should be noted that the specific species and subspecies of *Acidovorax* pathogens occurring in turfgrass systems is currently unresolved. Previous reports were able to differentiate these bacteria on the basis of DNA/DNA reassociation testing (Schaad et al. 2008; Willems et al. 1992), which may be useful in the future if more specific primers are not developed.

The use of ITS sequencing for resolving *X. translucens* pathovars also showed variability among isolates. These results were not surprising as previous literature by Chaves and Mitkowski (2013) showed that ITS can be used to differentiate both *graminis* and *poae* pathovars of *X. translucens*. While isolates in our study were taken from CBG, it appears that both *X. translucens* pv. *poae* and *X. translucens* pv. *graminis* can be isolated from CBG turf. Moreover, additional isolates of *X. translucens* showed greater similarity to pathovars *translucens* and *undulosa* meaning that additional work is needed to resolve *Xanthomonas* species occurring in CBG turf. Previous research on *Xanthomonas* in turfgrass has been limited to phytopathogens (Chaves and Mitkowski 2013; Giordano et al. 2015; Mitkowski et al. 2005; Roberts et al. 2014). Strong variability is known to exist within the xanthomonads and Vauterin et al. (2000) state that additional opportunistic *Xanthomonas* spp. may exist with many different plants therefore also contributing to population heterogeneity. Future work that includes additional strains and sequence data using species-specific primers, such as DNA gyrase B (*gyrB*), may provide better resolution to species and subspecies or pathovar designations in both *Acidovorax* and *Xanthomonas* bacteria occurring in turfgrass systems (Dauga 2002).

Characterization of pathogenicity with selected isolates from frequently isolated bacteria was quickly differentiated using the seedling technique previously developed by Roberts (1981). Seedling

inoculations support recent reports of *A. avenae* and *X. translucens* pathogenicity on CBG (Furuya et al. 2009; Giordano et al. 2012, 2010; Roberts 1981; Roberts et al. 1981). Although not presented here, additional greenhouse experiments performed at the same time of this research also illustrated the ability of *X. translucens* to cause bacterial etiolation on CBG turf (Roberts et al. 2014). Further inoculations with *P. ananatis* showed variable results across different strains. While *A. avenae* isolates within our greenhouse inoculation served as a positive control, only 50% *P. ananatis* isolates reduced turf quality compared with noninoculated CBG across multiple experiments. None of the *P. ananatis* isolates caused etiolation as only chlorosis and tip dieback occurred after inoculation. Etiolation was also uncommon on turf samples with *P. ananatis* as chlorosis and thinning were more often problematic in submitted field samples infected with that bacterium alone. Reports of plant pathogenic *P. ananatis* have become more frequent in recent years with infections occurring on maize (*Zea mays* L.) (Alippi and López 2010), onion (*Allium cepa* L.) (Kim et al. 2012), and rice (*Oryza sativa* L.) (Lee et al. 2010). From agricultural crops to forest tree species, *P. ananatis* is a ubiquitous epiphyte that is regarded as an emerging pathogen and can colonize diverse environments worldwide (Coutinho and Venter 2009). In our research, the frequency of *P. ananatis* isolations were more frequent than *A. avenae* and *X. translucens* combined. To our knowledge, this is the first report of *P. ananatis* as a pathogen of turfgrass, although inoculation results showed differences in virulence among strains isolated. Future research to include field inoculations is needed to better understand the impact of various *P. ananatis* strains on CBG as results from recent field inoculations with *A. avenae* have varied from greenhouse studies. Inoculations performed by Giordano et al. (2012) resulted in necrosis of multiple CBG cultivars in the greenhouse whereas field inoculations by Roberts et al. (2015a, b) caused etiolation symptoms without decline.

In summary, our research shows that multiple bacteria are capable of infecting CBG turfgrass putting greens; however, the degree and severity of symptoms varies with each of the bacteria tested. Moving forward, turfgrass managers should avoid equating etiolation and decline solely with *A. avenae*. While research surrounding management of etiolation and decline has offered some solace to turfgrass managers, current recommendations are not conclusive. Additional research to continue screening bacterial species may demonstrate that even more bacteria are capable of infecting turfgrass species. Identification and characterization of all bacteria associated with bacterial etiolation and decline will ultimately improve our understanding of the etiology of this phenomenon and may result in improved management techniques for control. Moreover, this research sheds new light on the presence of bacteria in turfgrass putting greens. Future research to understand how microbes survive and interact with plant hosts in turfgrass systems in a community structure may provide new means for beneficial bacteria that support plant growth or serve as biological controls for a variety of diseases.

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