Using a Genome-Based PCR Primer Prediction Pipeline to Develop Molecular Diagnostics for the Turfgrass Pathogen *Acidovorax avenae*

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Abstract

Acidovorax avenae is the causal agent of bacterial etiolation and decline (BED) of creeping bentgrass, a poorly understood and often misdiagnosed disease that can result in considerable aesthetic and functional damage to golf course putting greens. Current diagnostics of BED are based on laborious culture-based methods. In this work, we employed a novel alignment-free primer prediction pipeline to design diagnostic primers for turfgrass-pathogenic *A. avenae* using 15 draft genomes of closely related target and nontarget *Acidovorax* spp. as input. Twenty candidate primer sets specific to turfgrass-pathogenic *A. avenae* were designed. The specificity and sensitivity of these primer sets were validated via a traditional polymerase chain reaction (PCR) and

a real-time PCR assay. Primer sets 0017 and 0019 coupled with an internal oligo probe showed optimal sensitivity and specificity when evaluated with the target pathogen, closely related bacterial species, and microorganisms that inhabit the same host and soil environment. Finally, the accuracy of the newly developed real-time PCR assay was evaluated to detect BED pathogens from BED-symptomatic and asymptomatic turfgrass samples. The diagnostic results produced by the real-time PCR assay were consistent with results of a cultural-based method. This assay will allow quicker and more effective detection of the BED pathogen, thus potentially reducing misdiagnoses and unnecessary usage of fungicides.

Acidovorax avenae is the causal agent of bacterial etiolation and decline (BED), a serious disease of creeping bentgrass (*Agrostis stolonifera* L.) on golf course putting greens (Giordano et al. 2010, 2012; Roberts et al. 2018). Often associated with high summer temperature (>30°C), symptoms of this disease appear as irregular-shaped areas of foliar discoloration and stem and blade etiolation, followed by thinning and necrosis of the foliar canopy. First identified in 2009, BED has been consistently detected on golf courses around the United States, including mid-Atlantic, Midwest, and northeastern states, leading to substantial economic losses (Giordano et al. 2012).

Early, accurate diagnosis of BED can lead to proper management recommendations and avoid unnecessary and ineffective fungicide treatments (Sundin et al. 2016). Although an effective management option for BED has yet to be developed, early diagnosis of BED can avoid the misdiagnosis of BED as a fungal disease and, thus, avoid the misuse of fungicides. A recent report by Roberts et al. (2016) revealed that some plant growth regulators show promise in the management of BED.

Accurate diagnosis of BED has been confounded by several factors. First, bacterial diseases on turfgrass are rare. Only a few other

major diseases of turfgrass are caused by bacterial pathogens on annual bluegrass (*Poa annua* L.) and perennial ryegrass (*Lolium perenne*), known as bacterial wilt caused by *Xanthomonas translucens* (Giordano et al. 2015; Mitkowski et al. 2005); thus, turfgrass diagnosticians are typically not adequately equipped for the identification of bacterial pathogens. Second, *Acidovorax* pathogens are often seedborne (Gitaitis and Walcott 2007; Goto 1964; Shakyia and Chung 1983; Walcott and Gitaitis 2000) and, although this has not been confirmed in creeping bentgrass, the pathogen does not always produce distinct symptoms under field conditions. The disease is often very difficult to diagnose and, in such cases, diagnosis must rely on time-consuming isolation and pathogenicity testing. Finally, the turfgrass-pathogenic *Acidovorax avenae* is genetically closely related to other plant-pathogenic members of the *Acidovorax* genus, including *A. citrulli*, *A. cattleyae*, *A. facilis*, and *A. konjaci* (Walcott et al. 2000). Even within *A. avenae* spp., different strains are able to infect different graminaceous hosts, including rice, maize, sorghum, foxtail, and so on, displaying a range of host specificity (Postnikova and Schaad 2002; Stead et al. 2003; Zeng et al. 2017). Our recent study revealed that two distinct phylogroups of turfgrass-pathogenic *A. avenae* strains were equally able to cause the BED symptoms, and both groups are closely related to *A. avenae* strains pathogenic to other graminaceous hosts (Zeng et al. 2017).

Real-time quantitative polymerase chain reaction (real-time PCR) has been widely used in detection and quantification of plant pathogens (López et al. 2010; Pritchard et al. 2012b; Wang et al. 2015). The ability to rapidly amplify and sensitively detect a pathogen DNA by real-time PCR allows diagnosticians to provide definitive diagnostic results within one working day (Scheda et al. 2004). The ability of real-time PCR to deliver sensitive and quantifiable results is dependent on the availability of primer sets that distinguish a target organism or organisms from nontarget organisms (Pritchard et al. 2012a). The design of discriminatory primer sets is typically guided by common sequences in the target organisms that are divergent, or absent, in nontarget organisms. The most commonly used sequences include internal transcribed spacer (ITS) regions, ribosomal DNA (rDNA), “housekeeping” genes, and virulence genes (Grisham et al. 2007; Schaad et al. 2002, 2007). Diagnostic amplification of DNA from members of the *Acidovorax* genus by conventional PCR has been largely based on the sequence of the 16S-23S rDNA, ITS (Song et al. 2002), or unique DNA fragments from profiles using repetitive extragenic palindromic (rep)-PCR (Song et al.

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2004; Walcott et al. 2004). Primers have been described for many plant-pathogenic bacteria, including *Acidovorax* at the genus level (Bahar et al. 2008); however, the *Acidovorax*-specific primers tend to amplify all species and subspecies of *Acidovorax*, and are not specific to strains pathogenic only to grass species, particularly turfgrass (Bahar et al. 2008).

Although many bioinformatic tools have been developed to design PCR primers for diagnosis of bacterial pathogens, most of them utilize DNA sequences of individual genes as input and predict primers that differentiate target strains from others based on the sequence divergence. This significantly limits the selection of primers as well as their sensitivity and specificity. In this study, we utilized a bioinformatic pipeline that identifies primers at the genome level. Using the 15 draft genomes of turfgrass-pathogenic and other host-pathogenic *Acidovorax* strains as input, we successfully predicted primers that only amplify the turfgrass-pathogenic *A. avenae* strains but not other closely related strains. The specificity and sensitivity of two predicted primer sets were experimentally validated against a panel of *Acidovorax* strains. Employing real-time PCR and conventional PCR assays with primer sets developed herein, we were able to clearly differentiate the turfgrass-pathogenic *A. avenae* from other *Acidovorax* strains pathogenic to other hosts, and microorganisms

that inhabit the same host and environment, with excellent sensitivity, specificity, and rapidity. The molecular methods refined in this study will not only allow for a tool to detect and diagnose infection by the pathogen but also will be useful in ecological studies of the bacterium (i.e., to determine when and where the pathogen proliferates under certain environmental conditions).

Materials and Methods

Bacterial strains and genomes. Turfgrass-pathogenic *A. avenae* isolates were obtained from creeping bentgrass (*Agrostis stolonifera* L.) and colonial bentgrass (*A. capillaris* L.) samples with BED symptoms sent to Michigan State University and the University of Rhode Island turfgrass diagnostic laboratories between 2009 and 2012. The pathogenicity of all *Acidovorax avenae* strains was confirmed by inoculating creeping bentgrass cups as described (Giordano et al. 2012). All other *Acidovorax* isolates from different hosts were obtained from Dr. Ron Walcott at the University of Georgia. Other grass-associated bacteria such as *Ochrobactrum pseudogrignonense*, *Microbacterium binotii*, *Stenotrophomonas maltophilia*, *Herbaspirillum seropedicae*, and *X. translucens* were isolated by the Michigan State University turfgrass diagnostic laboratory from turfgrass samples of golf courses. Bacteria were cultured

Table 1. *Acidovorax* genomes used in this study^a

Strain	16S rDNA identification	NCBI accession	Host of origin	Source
AA78-5	<i>Acidovorax avenae</i>	CP028287	Maize	Ha et al. 2009
AA99-2	<i>A. avenae</i>	CP028289	Maize	Ha et al. 2009
CAT98-1	<i>A. cattleyae</i>	CP028290	Orchid	Ha et al. 2009
COLB1	<i>A. avenae</i>	CP028291	Creeping bentgrass	Zeng et al. 2017
INDB2	<i>A. avenae</i>	CP028292	Creeping bentgrass	Zeng et al. 2017
INV	<i>A. avenae</i>	CP028293	Creeping bentgrass	Zeng et al. 2017
KL3	<i>A. avenae</i>	CP028294	Creeping bentgrass	Zeng et al. 2017
MDB1	<i>A. avenae</i>	CP028296	Creeping bentgrass	Zeng et al. 2017
MOR	<i>A. avenae</i>	CP028297	Creeping bentgrass	Zeng et al. 2017
NCT3	<i>A. avenae</i>	CP028298	Creeping bentgrass	Zeng et al. 2017
QH1	<i>A. avenae</i>	CP028299	Creeping bentgrass	Zeng et al. 2017
QHB1	<i>A. avenae</i>	CP028300	Creeping bentgrass	Zeng et al. 2017
SF12	<i>A. avenae</i>	CP028302	Creeping bentgrass	Zeng et al. 2017
SH7	<i>A. avenae</i>	CP028303	Creeping bentgrass	Zeng et al. 2017
SA2	<i>A. avenae</i>	CP028301	Creeping bentgrass	Zeng et al. 2017

^a rDNA = ribosomal DNA and NCBI = National Center for Biotechnology Information.

Table 2. Twenty polymerase chain reaction primer sets predicted by the primer prediction pipeline that can distinguish turfgrass-pathogenic *Acidovorax avenae* strains^a

Set name	Forward primer (5'–3')	Reverse primer (5'–3')
001	GCATGGAAATGCACGTAGAG	GCTGAAGCAGTACCCTTTCC
002	ATCGATGTGATCACCTGTGG	TTCTTCGTGCGATAACGAAC
003	TCACCTACACTCTGGCGAAG	GTGGAAGGGTGACGAGACTT
004	GGTAGCAGCACTCCACCTCT	ATGGAACCTCTTCAGGGCAGT
005	GCCATATCGCAAGAATTGTG	TTAGCCCAAATCAGCAACTG
006	TTTGACGCTTTGTTCGAGTC	CCTCGACTTCCAAGTTTACA
007	AGATTAGGCGACGGTTGAGT	GGTGCCTCTTATGGGTGATT
008	TTTGACGCTTTGTTCGAGTC	CGACTTCCAAGTTCACATGC
009	CATGGAATGCACGTAGAGC	GCTGAAGCAGTACCCTTTCC
0010	TAGAGCGCTTGCTGGTAGTG	ATCCTATGCCAACAGCACAG
0011	ACCGAAGAGAAGGTCGTCAC	CGACGATCCAGATCACGTAG
0012	AGAGAAGGTCGTCACCATCC	CGACGATCCAGATCACGTAG
0013	CCAAGTTCACATGCTCCAAT	CCGGTACTTTGACCCTTTGT
0014	CTGGAACGCTCTGATGAAGA	AGAGCTTCACACGATTACG
0015	CCTTCGTTGTGTTTCTGGTG	GCCATGAGCATGTAGCTGTT
0016	TTTGACGCTTTGTTCGAGTC	ACCTCGACTTCCAGGTTTAC
0017	GCGCAAAGATGTAGGTTTGA	AGTCCGCCCAATACGATTAC
0018	TTGGAGCAACGAATGATTTT	TTCAACGGTGTATTGCCATT
0019	GGAGCGCAAAGATGTAGGTT	AGTCCGCCCAATACGATTAC
0020	CCGGTACTTTGACCCTTTGT	GCTTCCAAGTTCACATGCTC

^a Primer sequences were predicted by the pipeline, and used in validation assays for the detection of turfgrass-pathogenic strains of *Acidovorax avenae*. Predicted primers in the table include nonredundant pairs with desired specificity selected from the complete set of predicted primers.

in King's B medium (King et al. 1954) and stored at -80°C in 15% of glycerol.

Genomes of 15 strains of *Acidovorax* spp. were acquired from the National Center for Biotechnology Information genome database. This includes 12 strains of *A. avenae* isolated from naturally infected creeping bentgrass (*Agrostis stolonifera* L.), 2 *Acidovorax avenae* strains from maize, and 1 *A. cattleyae* strain isolated from orchid (Table 1). These genomes were used as input in the primer prediction pipeline.

Primer prediction pipeline. The primer design pipeline was adopted from Pritchard et al. (2012a), with minor adjustments for

the purpose of this study. A comprehensive description of the methodology can be found in the supplementary materials of Pritchard et al. (2012a). Briefly, a configuration file specifying the location of input sequences of each isolate in FASTA format, and the species classification of that isolate, was provided to the primer design script (available for download at https://github.com/widdowquinn/find_differential_primers). A single pseudochromosome was compiled for the set of draft contigs belonging to each isolate. Prediction of coding sequences was carried out on the pseudochromosome using PRODIGAL, version 2.6 (Hyatt et al. 2010), where necessary. In total, 20,000 primer sets were predicted for each isolate

Table 3. Polymerase chain reaction amplification from target and nontarget bacterial isolates grouped by primer set at the optimized primer annealing temperature (64°C)

Isolate	Primer Set		Isolate I.D.	Host	Target
	0017	0019			
NCT3			<i>A. avenae</i>	Creeping bentgrass	+
MDB1			<i>A. avenae</i>	Creeping bentgrass	+
URI 17			<i>A. avenae</i>	Creeping bentgrass	+
KL3			<i>A. avenae</i>	Creeping bentgrass	+
INV			<i>A. avenae</i>	Creeping bentgrass	+
MSU 5			<i>A. avenae</i>	Creeping bentgrass	+
MSU 13			<i>A. avenae</i>	Creeping bentgrass	+
MOR			<i>A. avenae</i>	Creeping bentgrass	+
MSU 19			<i>A. avenae</i>	Creeping bentgrass	+
INDB2			<i>A. avenae</i>	Creeping bentgrass	+
URI 1			<i>A. avenae</i>	Creeping bentgrass	+
COLB1			<i>A. avenae</i>	Creeping bentgrass	+
URI 6			<i>A. avenae</i>	Creeping bentgrass	+
URI 10			<i>A. avenae</i>	Creeping bentgrass	+
MSU 14			<i>A. avenae</i>	Creeping bentgrass	+
URI 9			<i>A. avenae</i>	Creeping bentgrass	+
URI 18			<i>A. avenae</i>	Creeping bentgrass	+
MD5			<i>A. avenae</i>	Creeping bentgrass	+
QHB1			<i>A. avenae</i>	Creeping bentgrass	+
MSU 8			<i>A. avenae</i>	Creeping bentgrass	+
SF12			<i>A. avenae</i>	Creeping bentgrass	+
MSU 3			<i>Ochrobactrum pseudogrignonense</i>	Creeping bentgrass	-
negative			n/a	n/a	-
negative			n/a	n/a	-
1 Kb+ ladder			n/a	n/a	-
AA99-2			<i>A. avenae</i>	Maize	-
AA81-1			<i>A. avenae</i>	Maize	-
AA78-5			<i>A. avenae</i>	Vasseygrass	-
FAC96-2			<i>A. facilis</i>	Soil environment	-
FAC94-9			<i>A. facilis</i>	Soil environment	-
CAT98-1			<i>A. cattleyae</i>	Orchid	-
CIT00-1			<i>A. citrulli</i>	Melon	-
CIT96-9			<i>A. citrulli</i>	Melon	-
CIT92-17			<i>A. citrulli</i>	Melon	-
KON			<i>A. konjaci</i>	Konjac	-
MSU 6			<i>Microbacterium binotii</i>	Creeping bentgrass	-
MSU 7			<i>Stenotrophomonas maltophilia</i>	Creeping bentgrass	-
MSU 11			<i>Herbaspirillum seropedicae</i>	Creeping bentgrass	-
XAN-1			<i>Xanthomonas translucens</i> pv. <i>poae</i>	Annual bluegrass	-
XAN-2			<i>Xanthomonas translucens</i>	Perennial ryegrass	-
negative			n/a	n/a	-

Images are from agarose gels that have been aligned against the matching isolate designation (leftmost column) and below the primer names (top row). The image is split so that target isolates of turfgrass-pathogenic *Acidovorax avenae* are located in the top section. The bottom section includes several different nontarget *Acidovorax* spp. as bacterial organisms isolated from the turfgrass phyllosphere and rhizosphere, including two pathogenic *Xanthomonas* spp. The two sections are bound by a 1-kb+ ladder. Both primer sets show results after optimization of annealing temperature at 64°C during thermal cycling.

pseudochromosome using EPRIMER3 (Rice et al. 2000), keeping only the primer sets that exist entirely within a predicted coding sequence. PRIMERSEARCH (Rice et al. 2000) was used to identify cross-amplification of each isolate. A filter for off-target amplification was implemented by querying primer sets against sequenced Enterobacteriaceae and other β -Proteobacteria (but not *Acidovorax*) genomes using BLASTN. Primer sets were classified according to their ability to amplify host (turfgrass)-specific *Acidovorax* spp. The results were written to a summary output file. Commands and configuration files used in this study were deposited to GitHub (https://github.com/wjidea/AVX_Diagnostic_Find_Primer). Thermodynamics specified for amplification primers during the prediction phase included a T_m (melting temperature) between 58 and 60°C (59°C optimal) and a GC content of 30 to 80%, with no more than two G+C in the last five nucleotides of the 3' end. During the prediction process, runs of identical nucleotides were avoided, and sequence constraints were applied to design sets of amplicons of 150 to 250 bp in length. Primer sets contained flanking primer sequences only, or flanking primer sequences and an internal oligomer for post-amplification hybridization. Hybridization probes were specified to have a length of 13 to 30 bp with a T_m between 68 and 70°C. Non-redundant, putative turfgrass-pathogenic *A. avenae*-specific primer sets are listed in Table 2.

Primer evaluation in conventional PCR. Seven bacterial isolates were first screened with each of the 20 putative specific primer sets: two target isolates of *A. avenae* COLB1 and UR11 and five nontarget isolates, including *A. avenae* AA81-1 and AA99-2 (nontarget *A. avenae*), *A. cattleyae* CAT98-1 (nontarget *Acidovorax* sp.), *A. citrulli* CIT00-1 (nontarget *Acidovorax* sp.), *A. konjaci* KON (nontarget *Acidovorax* sp.), and *X. translucens* pv. *poae* PAR5 (nontarget turf pathogen). Once screened, primers with specificity to the target turfgrass isolates were further tested against a broader range of target and nontarget bacterial isolates (Tables 1 and 3). Prior to all experiments, bacteria were grown on King's B medium and DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer's protocol. PCR of 25 μ l contained DNA template at 5 to 20 ng/ μ l, 0.2 μ M final concentration of each primer, and 12.5 μ l of a 2 $\times$ MeanGreen PCR Master Mix (Syzygy Biotech). Annealing temperature was set to 60°C due to the proposed optimal

annealing temperature for each primer being predicted near 60°C by the pipeline. Standard cycling conditions included 1 cycle at 94°C for 5 min; 30 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 1 min; and 1 cycle of 72°C for 5 min. Products were resolved on a 1% agarose gel in Tris-borate-EDTA buffer at 0.5 $\times$ concentration, alongside a 1-kb+ ladder (Life Technologies). The images were captured under standard conditions in a gel documentation system at 300 dpi and saved in TIFF format. Image manipulations (combination of multiple gel results into single figures) were carried out using Adobe Illustrator.

Further conventional PCR assay optimization was carried out with selected primer sets (0003, 0010, 0017, and 0019) utilizing a gradient range of annealing temperatures. Briefly, 10 ng of genomic DNA from target (QHB1, COLB1, MSU14, and MOR) and nontarget (AA78-5, CAT98-1, CIT00-1, and FAC96-2) isolates was used in PCR preparations according to the aforementioned protocol. The PCR was run using a gradient of four annealing temperatures (58, 60, 64, and 66°C) using the following cycling conditions on an Eppendorf Mastercycler nexus thermal cycler: initial denaturing at 94°C for 90 s; followed by 35 cycles of 94°C denaturing for 15 s, gradient temperature (°C) annealing for 20 s, and 72°C extension for 30 s; with a final cycle of 72°C for 7 min. Gel electrophoresis and band imaging were performed as mentioned above. Further testing of all target and nontarget isolates on hand was carried out with the primer sets 0017 and 0019 and performed under the protocol described above with the selected annealing temperature of 64°C during PCR thermal cycling.

Real-time PCR evaluation. Two primer sets (0017 and 0019) with specificity to turfgrass-pathogenic *A. avenae* via conventional PCR were selected for further evaluation in real-time PCR assay development. The Primer Express 3.0 software (Applied Biosystems) was used for the TaqMan probe design. Candidate probe sequences were queried against the GenBank database for Enterobacteriaceae using BLASTN as well as the genomic sequences derived in this study, and *A. avenae*-specific oligonucleotides were selected. The TaqMan probe 6FAM-TACGGGTACGCCACAA-MGBNFQ was synthesized by Life Technologies (<http://www.thermofisher.com/>) and consists of a minor DNA groove-binding quencher. The ZEN probe 6FAM-ACCGTCGCTTACCATCACCGTAGATTC

Table 4. Detection of turfgrass-pathogenic *Acidovorax avenae* with designed primers and probes from frozen and fresh turfgrass samples

Real-time PCR results for primer sets and probe types ^a					Sample description	Host	Isolation ^b
Sample	0017		0019				
	TaqMan	ZEN	TaqMan	ZEN			
HTRC_2012	25.7 (+)	24.6 (+)	23.9 (+)	23.5 (+)	Research plot, putting green turf, inoculated with turfgrass-pathogenic <i>A. avenae</i> , etiolation, streaming (+), frozen	Creeping bentgrass	+
HTRC_2013	19.6 (+)	19.2 (+)	18.5 (+)	20.6 (+)	Research plot, putting green turf, inoculated with turfgrass-pathogenic <i>A. avenae</i> , etiolation, streaming (+), frozen	Creeping bentgrass	+
LVL12	20.4 (+)	19.8 (+)	18.9 (+)	21.7 (+)	Golf course, putting green turf, etiolation, decline, streaming (+), frozen	Creeping bentgrass	+
IL_HIN	22.0 (+)	21.4 (+)	20.8 (+)	22.4 (+)	Golf course, putting green turf, etiolation, decline, streaming (+), frozen	Creeping bentgrass	+
GLET10	37.4 (−)	37.1 (−)	38.8 (−)	ND (−)	Golf course, putting green turf, etiolation, streaming (−), frozen	Creeping bentgrass	−
BNTSD1	37.5 (−)	37.2 (−)	37.6 (−)	ND (−)	5-week-old seedlings, greenhouse grown, no symptoms, streaming (−), fresh	Creeping bentgrass	−
GLNRM	37.0 (−)	36.9 (−)	38.6 (−)	ND (−)	Golf course, putting green turf, adjacent to etiolated area, no symptoms, streaming (−), frozen	Creeping bentgrass	−
BCCX	31.8 (−)	31.0 (−)	31.1 (−)	31.3 (−)	Golf course, fairway turf, etiolation, streaming (+), isolated and confirmed <i>Xanthomonas campestris</i> pv. <i>translucens</i> , frozen	Perennial ryegrass	−

^a Mean real-time polymerase chain reaction (PCR) cycle threshold (Ct) value of two technical replicates. Samples consisted of 15 to 20 mg of fresh or frozen plant material. All Ct values were derived from two technical replicates of DNA preparations. TaqMan probe was a standard hydrolysis probe with an MGB quencher to inhibit fluorescence. Zen probe was a double quenched TaqMan probe with an additional internal ZEN quencher. ND = not detected due to lack of amplification.

^b Isolation of *A. avenae*. If bacterial infection was suspected, isolation attempts took place using serial dilutions of macerated tissue onto selective media for *A. avenae* spp.; + represents isolation and identification of *A. avenae* via 16S ribosomal DNA sequencing and – represents no isolation.

G-/3IABkFQ/ was synthesized by Integrated DNA Technologies (<http://www.idtdna.com>) and incorporates an additional internal ZEN quencher. Real-time PCR assays for detecting *A. avenae* were performed in 25- μ l reaction mixtures containing 2 μ l of template DNA (5 to 20 ng/ μ l), 20 μ M each primer, 10 μ M TaqMan probe, and 2.5 μ l of TaqMan Universal PCR Master Mix (Applied Biosystems). Each sample was run as three technical replicates. Amplifications were carried out on an ABI Step One Plus real-time PCR system (Applied Biosystems) using the program of an initial denaturing step at

95°C for 10 min followed by 40 cycles, each consisting of denaturing at 95°C for 15 s and extension at 60°C for 60 s. Standard regression curves were plotted for 10-fold serial dilutions of *A. avenae* genomic DNA against cycle threshold (Ct) values. Every set of reactions also included a water control to verify no contamination of reagents with template DNA.

Real-time PCR validation with turfgrass samples. To further examine and validate assay detection, specificity, and sensitivity in vivo, eight samples of turfgrass from various sources (Table 4) were

Fig. 1. Flowchart of the primer design procedure and output.

subject to diagnostics using both a traditional cultural method and the real-time PCR assay developed in this study. The general fitness of the grass samples and presence or absence with the typical BED symptoms were documented. Grass samples were dissected and tested for bacterial streaming using the method described before (Giordano et al. 2010, 2012). Colonies were isolated on Luria-Bertani agar plates and the identification of these colonies was determined by 16S rDNA sequencing. For the real-time PCR diagnostics, grass samples were prepared for DNA extractions. Briefly, 15 to 20 mg of stem and leaf tissue from one to two grass plants was macerated in bead mill homogenizing tubes on a mini bead beater for 1 min in 1.5 ml of lysis (API) buffer (Qiagen). DNA was prepared using the Qiagen DNeasy Plant Mini Kit according to the manufacturer's instructions. Concentrations of DNA were measured using a Nanodrop-1000 spectrophotometer (ThermoFisher Scientific) and DNA sample preps were used directly in real-time PCR assays. Real-time assays were conducted according to the specifications indicated above.

Results

Primer prediction, and PCR validation. A flowchart describing the primer design procedures and outputs is illustrated in Figure 1. The 15 draft genomes of *Acidovorax* strains were used as input in the primer prediction pipeline with their hosts of origin (i.e., target classification) indicated in Table 1. Contigs of each strain were concatenated into a pseudochromosome and the coding regions of the pseudochromosome from turfgrass-pathogenic *A. avenae* were subject to primer design. Approximately 20,000 primers were predicted from each target strain (Fig. 1). Two independent rounds of screens for potential cross-amplification of nontarget microorganisms were conducted using PRIMERSEARCH and BLASTN against the local draft genome database, and against all Enterobacteriaceae chromosome and plasmid sequences from GenBank database ([ftp://ftp.ncbi.nih.gov/genomes/bacteria](http://ftp.ncbi.nih.gov/genomes/bacteria)), respectively. Each target strain contains approximately 244 strain-specific primers (Fig. 1). After the two rounds of screening, 65 primer sets were predicted as putative discriminatory diagnostic primers for turfgrass-pathogenic *A. avenae* isolates. After discarding redundant primer sets, 20 pairs remained for further bench experimental testing (Table 2).

Primers that amplified each of the initial two target turfgrass strains (i.e., COLB1 and URI_1) with adequate amplification intensity and little to no amplification of the six nontarget bacterial DNA were subjected to a broader range of isolate testing via conventional PCR. COLB1 and UR_I were used in the initial screen because they belong to each of the two phylogroups of *A. avenae*, as described previously (Zeng et al. 2017). When using 10 ng of bacterial genomic DNA as template in each PCR, primer sets 0017 and 0019 produced positive PCR results with each of the 22 evaluated target strains, yielding a single band of the expected product size of 200 bp (Fig. 2). Under the predetermined annealing temperature of 60°C, both primer sets produced faint positive bands with other *Acidovorax* spp. and subspecies, as well as other bacterial organisms assessed in this study (Fig. 2). Gradient thermal cycling with annealing temperatures ranging from 58° to 66°C determined specific binding to only target DNA in turfgrass isolates to take place at the annealing temperature of 64°C. At 66°C, sensitivity of the PCR assay was lost in several of the target isolates (data not shown). Further confirmation of specificity of both the 0017 and 0019 primer sets at 64°C showed only amplification of DNA from target isolates, with complete absence of banding from nontarget bacterial isolate DNA (Table 3). Adjustment of the annealing temperature provided the desired specificity of the primers to turfgrass-pathogenic strains of *A. avenae* while limiting nonspecific or phantom banding from the nontarget bacterial hosts (Table 3).

Development of real-time PCR detection of turfgrass-pathogenic *A. avenae*. The two primer sets (0017 and 0019) produced by the bioinformatics pipeline and confirmed with conventional PCR were used to develop real-time PCR detection assays of turfgrass-pathogenic *A. avenae*. Two probes with different fluorescence chemistries, TaqMan and ZEN, were used in real-time PCR assay development. Detection limits for real-time PCR were

determined with 10-fold serial dilutions of 100 ng of initial *A. avenae* genomic DNA under conditions described above. The regression curves between the DNA amounts and the Ct values were linear: for 0017/TaqMan, $y = -3.4x + 46$ ($R^2 = 0.989$), with a PCR efficiency of 96.84% and, for 0017/ZEN, $y = -3.1x + 48$ ($R^2 = 0.978$), with a PCR efficiency of 110.17% (Fig. 3). For 0019/TaqMan, $y = -3.3x + 47$ ($R^2 = 0.983$), with a PCR efficiency of 100.92% and, for 0019/ZEN $y = -3.5x + 52$ ($R^2 = 0.989$), with a PCR efficiency of 93.07% (Fig. 3). Although the real-time PCR assays could detect concentrations as low as 0.1 pg of pure *A. avenae* DNA at Ct values of 35 or higher, we considered samples with Ct values greater than 31 as *A. avenae* negative based on our preliminary tests to ensure detection specificity, thus eliminating false-positive results. Therefore, the actual detection limit for the real-time PCR assay is between 10 and 100 pg of *A. avenae* DNA, based on formula of efficiency.

To evaluate the specificity of the real-time PCR assay, we used the developed real-time PCR protocol to detect a panel of 8 turfgrass-pathogenic *A. avenae* and 15 other *Acidovorax* strains or other bacterial species (Table 5). The eight turfgrass-pathogenic *A. avenae* strains tested not only represent the two phylogroups of turfgrass *A. avenae* (group 1 = INV and URI1 and group 2 = the rest of strains) but also represent a large geographical area where the strains were isolated, including the northeast (URI1, URI18, and SF12), Midwest (MOR, INDB2, and INV), mid-Atlantic (MSU14), and southern (COLB1) United States. Both assays (0017 and 0019 primer sets) conducted using the ZEN probe produced positive results with all eight target *A. avenae* strains from turfgrass (Ct < 31) and negative results with all other bacterial species tested (Ct > 31) (Table 5). The assays conducted with the TaqMan probe, however, showed reduced specificity to some of the target isolates, particularly for isolate URI_1 (Table 5). Nonetheless, overall Ct values in all of the assays remained below the determined limit of detection (Ct < 31) for target isolates and above the limit of detection for nontarget organisms.

Detection of turfgrass-pathogenic *A. avenae* from symptomatic turfgrass tissues by real-time PCR validation. In vivo detection of turfgrass-pathogenic *A. avenae* in symptomatic and asymptomatic turfgrass tissues was assessed using the above real-time PCR assays. *A. avenae* was positively detected (Ct < 31) from four of four suspected infected samples (HTRC_2012, IL_HIN, HTRC_2013, and LVL12) using all four primer-probe assay combinations (Table 4). In positive detection experiments, Ct values correlated with visual disease symptoms, intensity of microscopic bacterial streaming, and confirmation of *A. avenae* via selective medium (King's B) plating and 16S rDNA sequencing (i.e., lower Ct values from heavily infected samples). Negative results (Ct > 31) were obtained from four samples (GLET10, BNTSD1, GLNRM, and BCCX), which were included based on their symptomology and origin. The GLET10 sample displayed symptoms of etiolation and chlorosis; however, it failed to produce signs of bacterial streaming or result in isolation of *A. avenae* onto selective medium. The BNTSD1 sample was from newly established creeping

Fig. 2. Polymerase chain reaction amplification of target and nontarget DNA from bacterial isolates using select primer sets designed from whole genome sequences of different *Acidovorax* spp. All reactions took place using the annealing temperature specified by the primer design pipeline (60°C). **A**, Amplification of target isolates (left) and nontarget isolates (right) using the primer set 0017. **B**, Amplification of target isolates (left) and nontarget isolates (right) using the primer set 0019. Note the intensified banding on target isolates and subtle banding on nontarget isolates.

bentgrass seedlings and showed no symptoms or signs of bacterial infection. The GLNRM sample was from an adjacent, asymptomatic area of a golf course putting green displaying no etiolation symptoms. The BCCX sample originated from a perennial ryegrass golf course fairway displaying symptoms of etiolation and decline. Heavy bacterial streaming was detected and isolations of *X. translucens* were achieved; however, no *A. avenae* was isolated from this sample.

Discussion

Because BED is a newly emerged disease in turfgrass, efficient and accurate diagnostic tools for the detection of BED pathogens are unavailable. Lack of proper identification and diagnosis of turfgrass-pathogenic *A. avenae* not only results in delayed accurate management recommendations but also hinders foundational epidemiological research of BED. The techniques employed herein exemplify the utility of draft genome sequences in the development of discriminatory diagnostic primer sets. Manual design of diagnostic primers is not only time consuming but also heavily relies on the availability of known common genes with divergent DNA sequences. For example, previous research in developing diagnostic primers of *A. citrulli* were derived from unique bands amplified from rep-PCR (Bahar et al. 2008) fingerprinting profiles (Ha et al. 2009). This not only limits the selection of available primers but also limits the resolution of the detection technique. Primers derived from these fingerprinting profiles often can differentiate pathogens at the species level (e.g., *A. citrulli* from *A. avenae*) (Bahar et al. 2008) but may not be effective in differentiate pathogens at subspecies level. In contrast,

genome-based primer prediction methods such as the pipeline employed by this study enlarge the search pool to the coding regions of the entire genome, thus providing a better chance to identify primers with higher discriminatory potentials. Primers developed from this study were able to identify and discriminate a diverse population of turfgrass-pathogenic *A. avenae* (strains belonging to two distinct phylogroups) (Zeng et al. 2017) from *A. avenae* pathogenic to other hosts and environmental nontarget bacteria. Furthermore, compared with the alignment-based primer design method such as KPATH (Slezak et al. 2003), the alignment-free primer prediction method used in this study avoids limitations resulting from the need to identify a conserved signature of significant length and common to all targets prior to primer design.

The sets of primers in the output from the Python script pipeline all showed some level of discriminatory ability and specificity to the predefined group of input sequences. Validation of select primers against a wide array of target and nontarget bacterial isolates confirmed specificity of the primer sets, which can separate very closely related strains. Isolates of *A. avenae* from corn, rice, sorghum, and other grass family hosts as well as other *Acidovorax* spp. were not amplified during PCR assays under optimal primer annealing conditions. Some differences in the intensity of the PCR bands were evident among target turfgrass-pathogenic *A. avenae* isolates tested, although the vast majority were strongly positive, indicative of a high concentration of amplified product.

An essential aspect of this project was the diagnostic capability of the designed discriminatory primers, and the subsequent development

Fig. 3. Real-time polymerase chain reaction (PCR) standard curves of the log of *Acidovorax avenae* (COLB1) input genomic DNA concentration versus the corresponding cycle threshold (Ct) values. The DNA concentration was 0.1 pg to 100 ng. Standard errors are labeled for each data point. **A**, Standard curve for primer set 0017 with the ZEN probe. **B**, Standard curve for the primer set 0017 with the TaqMan probe. **C**, Standard curve for the primer set 0019 with the ZEN probe. **D**, Standard curve for the primer set 0019 with the TaqMan probe.

of a reproducible assay to be used in the turfgrass industry for specific detection of BED pathogen *A. avenae*. Real-time PCR has become a popular diagnostic tool for rapid and sensitive detection and quantification of pathogens in various host samples (Gruber et al. 2001; Hogg et al. 2007; Lievens et al. 2006; Winton et al. 2002). The primers and probes designed in this study allowed us to detect *A. avenae*, the causal agent of BED, in stems and leaves of creeping bentgrass plants.

Specificity and sensitivity are two major factors that need to be taken into consideration during the development of real-time PCR diagnostic assays. Both primer sets (0017 and 0019) in combination with TaqMan and ZEN probes displayed desirable sensitivity and specificity (Table 5). In regards to sensitivity, primers in combination with ZEN probes (average Ct values of 24.2 and 24.7 for 0017 and 0019, respectively) displayed higher sensitivity than TaqMan probes (average Ct values of 27.4 and 28.0 for 0017 and 0019, respectively) (Table 5), given the same amount of target genomic DNA. In regards to specificity, the 0017/ZEN primer set (Ct value of target organism = 24.72 and average Ct value of nontarget organism = 37.27) displayed higher specificity than the 0019/ZEN primer set (average Ct value of target organism = 24.20 and average Ct value of nontarget organism = 35.89) (Table 5). Furthermore, compared with the 0017/ZEN primer set, the assays conducted with the 0019/ZEN primer set also showed higher specificity to some of the target isolates, particularly for isolate URI_1 (Table 5). Thus, we recommend using the 0017/ZEN primer set in the standard diagnostic assay for BED. This specific assay was able to detect as little as 10 pg of pathogenic *A. avenae* genomic DNA in turfgrass samples, equivalent to 1,956 *A. avenae* cells. This level of detection (detecting 10 pg of target DNA with Ct < 31) is comparable with the detection levels reported previously for *A. citrulli* (detecting 0.5 to 5 pg of target DNA with Ct between 26 and 30) (Cho et al. 2015). The PCR amplification efficiencies were calculated and provided (Fig. 3), which could be used for the purpose of quantification of the pathogens. The PCR amplification efficiencies of four assays developed were all above 93% (Fig. 3).

Further validation of the real-time PCR assay involved testing DNA from samples of live and frozen turfgrass stems and leaves collected from symptomatic and asymptomatic turfgrass between 2012 and 2013. Results were compared with visual estimates of disease severity at the time of collection, microscopic observations of bacterial streaming, and isolation onto selective medium and molecular identification via 16S rDNA sequencing. The real-time PCR assays detected *A. avenae* in four of the eight samples. These results agreed not only with the symptoms of etiolation and decline observed among the samples but also with presence of bacterial streaming from tissues determined by microscopic detection and isolation of *A. avenae* on selective medium from the same four of eight samples. The one exception was the BCCX sample from perennial ryegrass, with Ct values around 31. This sample showed heavy bacterial streaming from stems and leaves and advanced symptoms of etiolation and decline. However, when isolation measures were taken, only a *Xanthomonas* pathogen could be recovered and characterized out of the tissue. It is likely that *A. avenae* is capable of cocolonizing turfgrass plants with *Xanthomonas* spp. and other pathogenic and non-pathogenic bacterial organisms. Trace amounts of *A. avenae* within that sample could have resulted in reduced Ct values and elevated amplification. Roberts et al. (2018) recently reported the isolation and characterization of a *Xanthomonas* sp. associated with creeping bentgrass etiolation in tandem with *A. avenae* in the southeastern United States. Therefore, it is likely that this sample of perennial ryegrass could harbor both the suspected primary *Xanthomonas* causal agent as well as the target organism *A. avenae*. We only tested this detection method using DNA extracted from bacteria and grass samples as template and have not used fresh plant samples as detection template. When using raw samples (plant extracts), dilution of the sample may be needed before adding it into the PCR to reduce PCR inhibition caused by plant polyphenols and polysaccharides (Schrader et al. 2012).

In summary, we present here a real-time PCR assay that is sensitive and specific for turfgrass-pathogenic *A. avenae* detection. Real-time PCR assays utilizing either the 0017 or 0019 primer set with

Table 5. Isolate name and sensitivity and specificity of select real-time polymerase chain reaction (PCR) primers and probes on DNA preparations from target and nontarget bacterial organisms

Real-time PCR results for primer sets and probe types ^a							
Isolate	0017		0019		Species ^b	Host or source	Target
	TaqMan	ZEN	TaqMan	ZEN			
INV	22.8	22.1	23.6	22.7	<i>Acidovorax avenae</i>	Creeping bentgrass	+
URI_1	34.1	29.7	30.3	26.4	<i>A. avenae</i>	Creeping bentgrass	+
MOR	27.9	23.7	29.8	25.5	<i>A. avenae</i>	Creeping bentgrass	+
INDB2	28.6	24.3	30.0	25.6	<i>A. avenae</i>	Creeping bentgrass	+
COLB1	28.6	24.7	28.8	25.0	<i>A. avenae</i>	Creeping bentgrass	+
MSU_14	28.0	23.9	30.8	26.5	<i>A. avenae</i>	Creeping bentgrass	+
URI_18	20.9	20.4	20.8	20.2	<i>A. avenae</i>	Creeping bentgrass	+
SF12	28.6	24.8	29.6	25.9	<i>A. avenae</i>	Creeping bentgrass	+
AA99-2	ND	38.7	ND	ND	<i>A. avenae</i>	Maize	—
AA81-1	ND	38.7	ND	ND	<i>A. avenae</i>	Maize	—
AA78-5	35.8	34.6	36.4	37.8	<i>A. avenae</i>	Vasseygrass	—
CAT98-1	39.5	36.5	ND	ND	<i>A. cattleyae</i>	Orchid	—
CIT00-1	ND	39.0	ND	ND	<i>A. citrulli</i>	Melon	—
CIT96-9	36.7	35.3	39.2	35.6	<i>A. citrulli</i>	Melon	—
CIT92-17	36.7	36.5	ND	39.5	<i>A. citrulli</i>	Melon	—
FAC94-9	37.1	35.2	39.2	36.3	<i>A. facilis</i>	Soil	—
FAC96-2	34.1	32.8	34.6	33.5	<i>A. facilis</i>	Soil	—
KONJ	ND	ND	ND	ND	<i>A. konjaci</i>	Konjac	—
MSU_3	33.9	32.0	34.2	33.0	<i>Ochrobactrum pseudogrignonense</i>	Creeping bentgrass	—
MSU_6	38.6	37.1	39.8	38.4	<i>Microbacterium binotii</i>	Creeping bentgrass	—
MSU_7	32.1	31.1	32.9	31.4	<i>Stenotrophomonas maltophilia</i>	Creeping bentgrass	—
XAN-1	39.4	37.9	ND	ND	<i>Xanthomonas translucens</i> pv. <i>poae</i>	Annual bluegrass	—
XAN-2	34.9	32.9	35.2	33.6	<i>Xanthomonas translucens</i>	Perennial ryegrass	—

^a Mean real-time PCR cycle threshold value of two technical replicates. TaqMan probe was a standard hydrolysis probe with an MGB quencher to inhibit fluorescence and Zen probe was a double quenched TaqMan probe with an additional internal ZEN quencher. ND = not determined due to lack of amplification.

^b Species were originally identified via 16S ribosomal DNA sequencing (Giordano et al. 2012).

ZEN probe produced the most reliable results, and can be employed in turfgrass diagnostic laboratories for BED detection. This assay allows for the detection of *A. avenae* directly from infected turfgrass plants within 5 h and, therefore, represents a rapid and simple method to identify BED in the field and evaluate the effectiveness of treatments used for its control. This assay also has many other potentially useful applications in plant breeding, disease quarantine, and a wide array of epidemiological studies.

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