Recombination of Virulence Genes in Divergent *Acidovorax avenae* Strains That Infect a Common Host

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Bacterial etiolation and decline (BED), caused by *Acidovorax avenae*, is an emerging disease of creeping bentgrass on golf courses in the United States. We performed the first comprehensive analysis of *A. avenae* on a nationwide collection of turfgrass- and maize-pathogenic *A. avenae*. Surprisingly, our results reveal that the turfgrass-pathogenic *A. avenae* in North America are not only highly divergent but also belong to two distinct phylogroups. Both phylogroups specifically infect turfgrass but are more closely related to maize pathogens than to each other. This suggests that, although the disease is only recently reported, it has likely been infecting turfgrass for a long time. To identify a genetic basis for the host specificity, we searched for genes closely related among turfgrass strains but distantly related to their homologs from maize strains. We found a cluster of 11 such genes generated by three ancient recombination events within the type III secretion system (T3SS) pathogenicity island. Ever since the recombination, the cluster has been conserved by strong purifying selection, hinting at its selective importance. Together our analyses suggest that BED is an ancient disease that may owe its host specificity to a highly conserved cluster of 11 T3SS genes.

Crop domestication, a process in which a single species of agriculturally important plants were selected and densely grown on agricultural farmland, represents an important milestone in the history of human civilization (Doebley et al. 2006). This process, while providing human beings with reliable food supplies, reduced genetic diversity and increased host density of the agricultural farmland, by replacing a variety of natural plant species with a single, genetically homogenous cultivar (Stukenbrock and McDonald 2008). The reduction of genetic diversity and increased density of crop species allows

pathogens to evolve from being less virulent to multiple plant species to highly virulent to a single or limited number of plant species (Monteil et al. 2013; Stukenbrock and McDonald 2008). Understanding how a plant pathogen increases in virulence and emerges from a diverse environmental population and the evolutionary mechanisms behind this process can often provide indications for the future mitigation of plant diseases.

There are many recent reports of emergent plant disease that are usually caused by the spread of a clonal pathogen population (Ailloud et al. 2015; Marcelletti and Scortichini 2016; McCann et al. 2013; Menardo et al. 2016). For example the olive quick decline syndrome in southern Italy was caused by a highly clonal population of the bacterial pathogen *Xylella fastidiosa* subsp. *pauca* (Marcelletti and Scortichini 2016). This clonal population probably emerged from a diverse population of *X. fastidiosa* strains that infect coffee plants in central Africa. Putative prophage-like regions were detected in the genomes of four subsp. *pauca* clonal strains. As prophage-like elements play an important role in virulence and adaptation of *X. fastidiosa* to new hosts, it was suggested that these elements play a role in host adaptation and pathogen emergence, although experimental validation is needed. In another study, a new virulent population of the apple scab pathogen *Venturia inaequalis*, which can infect apple (*Malus × domestica*) varieties carrying *Rvi6* resistance, has not emerged from the resident apple pathogen populations but, rather, from a population that infects the wild host *Malus floribunda* (Lemaire et al. 2016).

Here, we are focusing on a subset of *Acidovorax avenae* strains that infect creeping bentgrass (*Agrostis stolonifera* L.). Compared with many food crops (domestication started 4,000 to 10,000 years ago [Balter 2007; Doebley et al. 2006]), domestication of creeping bentgrass is fairly recent, coinciding with the rise in popularity of the game of golf. Originating in the United Kingdom in the 15th century, golf was introduced to the United States in the late 19th century. Increased popularity of golf in the 20th century in the United States not only resulted in the establishment of a large number of golf courses throughout the country (17,672 courses, accounting for 50% of golf courses worldwide) (Ikki-Shuppan 2008) but also the adaptation of specific grass species to putting greens and fairways. Creeping bentgrass has many desirable traits for growth on golf course putting greens. These include the production of vigorous spreading stolons, dense growth, short mowing tolerance, and beautiful coloration. In addition, as one of the cool-season grasses, creeping bentgrass can tolerate cold temperatures in the winter and prefers to grow at a temperature range between

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*The e-Xtra logo stands for “electronic extra” and indicates that five supplementary figures are published online.

Table 1. Genomes and *Acidovorax* strains used in this study

Isolate	Bacteria	Origin	Year	Host	Phylogroup
COLB1	<i>A. avenae</i>	Texas	2010	Creeping bentgrass 'Penn A4'	2
INDB2	<i>A. avenae</i>	Indiana	2010	Creeping bentgrass ^b	2
INV	<i>A. avenae</i>	Ohio	2010	Creeping bentgrass	1
KL3	<i>A. avenae</i>	Ohio	2010	Creeping bentgrass	1
MD5	<i>A. avenae</i>	Illinois	2010	Creeping bentgrass	2
MDB1	<i>A. avenae</i>	Maryland	2010	Creeping bentgrass	1
MOR	<i>A. avenae</i>	Ohio	2010	Creeping bentgrass	2
NCT3	<i>A. avenae</i>	North Carolina	2011	Creeping bentgrass 'Penn G2'	1
QH1	<i>A. avenae</i>	North Carolina	2009	Creeping bentgrass 'Penn G2'	2
QHB1	<i>A. avenae</i>	North Carolina	2009	Creeping bentgrass 'Penn G2'	1
Sa2	<i>A. avenae</i>	Pennsylvania	2011	Creeping bentgrass 'Alpha'	1
SF12	<i>A. avenae</i>	New York	2011	Creeping bentgrass	2
SH7	<i>A. avenae</i>	Rhode Island	2010	Colonial Bentgrass 'Greenwich'	2
URI_9	<i>A. avenae</i>	Massachusetts	2010	Creeping bentgrass	1
MSU_2	<i>A. avenae</i>	Texas	2010	Creeping bentgrass 'Penn A4'	2
MSU_8	<i>A. avenae</i>	Indiana	2010	Creeping bentgrass	2
MSU_13	<i>A. avenae</i>	North Carolina	2011	Creeping bentgrass 'Penn G2'	1
MSU_14	<i>A. avenae</i>	North Carolina	2011	Creeping bentgrass 'Penn G2'	2
MSU_15	<i>A. avenae</i>	Idaho	2012	Creeping bentgrass	1
MSU_19	<i>A. avenae</i>	Michigan	2012	Creeping bentgrass	1
URI_1	<i>A. avenae</i>	Connecticut	2010	Creeping bentgrass	1
URI_2	<i>A. avenae</i>	Massachusetts	2010	Creeping bentgrass	2
URI_4	<i>A. avenae</i>	New Jersey	2010	Creeping bentgrass	2
URI_6	<i>A. avenae</i>	Virginia	2010	Creeping bentgrass 'L-93'	1
URI_10	<i>A. avenae</i>	North Carolina	2010	Creeping bentgrass	2
URI_16	<i>A. avenae</i>	Michigan	2011	Creeping bentgrass	1
URI_17	<i>A. avenae</i>	Rhode Island	2011	Creeping bentgrass	2
URI_18	<i>A. avenae</i>	New York	2011	Creeping bentgrass	2
AA78_5	<i>A. avenae</i>	Georgia	1978	Maize	n/a
AA81_1	<i>A. avenae</i>	Georgia	1981	Maize	n/a
AA99_2	<i>A. avenae</i>	Georgia	1999	Maize	n/a
CAT98_1	<i>A. cattleyae</i>	California	n/a	Orchid	n/a
ATCC19860	<i>A. avenae</i>	United States	1909	Maize	n/a
AAC00-1	<i>A. citrulli</i>	United States	1990	Watermelon	n/a
AA_30003	<i>A. avenae</i>	Japan	n/a ^c	Rice	n/a
AA_30015	<i>A. avenae</i>	Nigeria	n/a	Rice	n/a
AA_30044	<i>A. avenae</i>	Nigeria	n/a	Millet	n/a
AA_30179	<i>A. avenae</i>	Brazil	n/a	Sorghum	n/a
AA_30296	<i>A. avenae</i>	Japan	n/a	Rice	n/a
AA_30297	<i>A. avenae</i>	Japan	n/a	Rice	n/a
AA_30298	<i>A. avenae</i>	Japan	n/a	Rice	n/a
AA_30305	<i>A. avenae</i>	United States	n/a	Vasey grass	n/a
KONJ	<i>A. konjaci</i>	Japan	n/a	Konjac	n/a
CIT92_17	<i>A. citrulli</i>	Georgia	n/a	Melon	n/a
CIT92_300	<i>A. citrulli</i>	Georgia	n/a	Melon	n/a
CIT96_9	<i>A. citrulli</i>	Georgia	n/a	Melon	n/a

(continued on next page)

^a GC = guanine-cytosine; NCBI = National Center for Biotechnology Information.^b If cultivar information is not labeled, it means the cultivar of the host is unknown.^c n/a = not applicable or not available.^d S. Lucas, A. Copeland, A. Lapidus, J.-F. Cheng, L. Goodwin, S. Pitluck, O. Chertkov, B. Held, J. C. Detter, C. Han, R. Tapia, M. Land, L. Hauser, N. Kyrpides, N. Ivanova, G. Ovchinnikova, I. Pagani, S. Gordon, and T. Woyke.^e A. Copeland, S. Lucas, A. Lapidus, K. Barry, J. C. Detter, T. Glavina del Rio, E. Dalin, H. Tice, S. Pitluck, H. Kiss, T. Brettin, D. Bruce, C. Han, R. Tapia, P. Gilna, J. Schmutz, F. Larimer, M. Land, L. Hauser, N. Kyrpides, E. Kim, D. Stahl, and P. Richardson.^f PAMDB = Plant-Associated Microbes Database.

15 and 24°C. Due to these reasons, creeping bentgrass has become the most widely used turfgrass species on golf course putting greens in the United States (Bonos and Murphy 2009).

In 2009 to 2012, a new turfgrass disease was reported on creeping bentgrass from many golf courses in the United States (Giordano et al. 2010). This disease occurred over a wide range of geographical regions in the United States, including 15 states mostly located in the mid-Atlantic, southeast, and midwest regions. Infected turfgrass displayed symptoms of chlorosis, elongation of the leaf blades, necrosis, and decline (Giordano et al. 2010, 2012). This disease often occurred on grass that was intensively maintained under hot, stressful weather conditions in the summer. The causal agent was later diagnosed to be the bacterium *Acidovorax avenae* (formerly known as *Acidovorax*

avenae subsp. *avenae* and *Pseudomonas avenae*) (Giordano et al. 2010, 2012). The pathogenic *A. avenae* isolates can infect and colonize the vascular tissue of creeping bentgrass (Giordano et al. 2012). The host range of the pathogens is mostly restricted to creeping bentgrass (*Agrostis stolonifera*), with only mild pathogenicity to other grass hosts (Giordano et al. 2012). This disease was named, after the causal agent (bacterium) and the symptoms (etiolation and decline), bacterial etiolation and decline (BED). In a previous study, 28 *A. avenae* specimens were isolated from infected creeping bentgrass with BED symptoms and the virulence of these isolates was confirmed in growth-chamber pathogenicity tests (Giordano et al. 2012).

Diseases caused by *A. avenae* have been reported for many plants in the Poaceae family since the beginning of the 20th

Table 1. (continued from previous page)

No. contigs	Largest contigs	Total length	GC ^a (%)	N50	NCBI accession	Reference
191	208,047	5,517,506	68.4	76,320	SRR4096853	This study
279	158,622	5,679,539	68.1	45,561	SRR4096854	This study
269	166,406	5,750,708	68.2	61,274	SRR4096855	This study
296	148,999	5,499,225	68.4	38,955	SRR4096856	This study
1	5,93,846	5,593,846	68.4	5,593,846	SRR4096857	This study
161	522,546	5,663,181	68.3	107,765	SRR4096858	This study
2,909	213,018	7,943,278	58.8	26,289	SRR4096819	This study
221	260,470	5,519,745	68.4	65,391	SRR4096820	This study
169	205,103	5,541,121	68.3	69,933	SRR4096821	This study
173	274,288	5,726,390	68.3	95,527	SRR4096822	This study
171	162,211	5,599,143	68.2	74,243	SRR4096823	This study
338	234,522	5,668,144	67.7	60,032	SRR4096824	This study
244	140,427	5,575,105	68.3	47,637	SRR4096825	This study
1	5,470,205	5,470,205	68.6	5,470,205	SRR4096850	This study
n/a	n/a	n/a	n/a	n/a	n/a	Giordano et al. 2012
n/a	n/a	n/a	n/a	n/a	n/a	Giordano et al. 2012
n/a	n/a	n/a	n/a	n/a	n/a	Giordano et al. 2012
n/a	n/a	n/a	n/a	n/a	n/a	Giordano et al. 2012
n/a	n/a	n/a	n/a	n/a	n/a	This study
n/a	n/a	n/a	n/a	n/a	n/a	This study
n/a	n/a	n/a	n/a	n/a	n/a	Giordano et al. 2012
n/a	n/a	n/a	n/a	n/a	n/a	Giordano et al. 2012
n/a	n/a	n/a	n/a	n/a	n/a	Giordano et al. 2012
n/a	n/a	n/a	n/a	n/a	n/a	Giordano et al. 2012
n/a	n/a	n/a	n/a	n/a	n/a	Giordano et al. 2012
n/a	n/a	n/a	n/a	n/a	n/a	This study
n/a	n/a	n/a	n/a	n/a	n/a	This study
n/a	n/a	n/a	n/a	n/a	n/a	This study
424	157,587	5,335,484	68.5	25,071	SRR4096817	This study
1	5,751,786	5,751,786	68.6	5,751,786	SRR4096818	This study
208	166,632	5,224,194	68.8	60,802	SRR4096851	This study
488	89,429	5,563,628	68.4	23,751	SRR4096852	This study
1	5,482,170	5,482,170	68.8	5,482,170	NC_015138	Unpublished ^d
1	5,354,379	5,354,379	68.5	5,354,379	NC_008752	Unpublished ^e
n/a	n/a	n/a	n/a	n/a	n/a	PAMDB ^f
n/a	n/a	n/a	n/a	n/a	n/a	PAMDB
n/a	n/a	n/a	n/a	n/a	n/a	PAMDB
n/a	n/a	n/a	n/a	n/a	n/a	PAMDB
n/a	n/a	n/a	n/a	n/a	n/a	PAMDB
n/a	n/a	n/a	n/a	n/a	n/a	PAMDB
n/a	n/a	n/a	n/a	n/a	n/a	PAMDB
n/a	n/a	n/a	n/a	n/a	n/a	PAMDB
n/a	n/a	n/a	n/a	n/a	n/a	n/a
n/a	n/a	n/a	n/a	n/a	n/a	n/a
n/a	n/a	n/a	n/a	n/a	n/a	n/a

century. *Acidovorax avenae* was first described by Manns (1909) as the causal agent of leaf blight on oats in Ohio. In 1922, *A. avenae* was found to cause a similar disease on foxtail in Arkansas (Rosen 1922). Later *A. avenae* was commonly found as the causal agent of bacterial leaf blight of corn in the southern United States (Sumner and Schaad 1977). In Asia, *A. avenae* has caused bacterial leaf streak and bacterial brown stripe on rice (Kadota 1996; Shakya et al. 1985). Other sporadic infections caused by *A. avenae* in North America have also been observed on millet, barley, and sugarcane (Clafin et al. 1989; Elliott 1923; Gitaitis et al. 1978; Martin and Wismer 1989; Schaad et al. 2008). A single case of bacterial infection on creeping bentgrass caused by *A. avenae* (identified by 16s rDNA sequencing) was reported in Fukuoka Prefecture, Japan in 2000 (Fuyuya et al. 2009). However, large-scale occurrence of BED on creeping bentgrass caused by *A. avenae* was never been reported prior to 2009. The emergence and spread of BED in North America is not well understood. Knowledge about origin and diversity of *A. avenae* are crucial to mitigate the spread of the disease in creeping bentgrass and may also help us to understand and

prevent the emergence of other plant diseases. Management practices, including applying growth regulators and alleviating general stress of the grass with reduced mowing, have been shown effective in reducing the severity of this disease (Roberts et al. 2015, 2016).

In this study, we used comparative genomics approaches to characterize the first culture collection of the turfgrass pathogen *A. avenae* in North America. We provide evidence that the causal agents of BED on creeping bentgrass in the United States belong to two distinct phylogroups in the *A. avenae* species complex. Despite the genetic differences of the two pathogen lineages, the nucleotide sequences of a cluster of 11 genes located in the type III secretion system (T3SS) pathogenicity island are highly conserved in these two lineages. Finally, we provide evidence that recombination within the turfgrass *A. avenae* isolates played an important role during pathogen evolution. We demonstrate that the formation of the conserved T3SS sequences among turfgrass *A. avenae* isolates can be traced back to three recombination events. Together, our results allow us to propose a testable hypothesis about the evolution of host specificity in *A. avenae*.

RESULTS

Turfgrass-pathogenic *A. avenae* strains from the United States belong to two distinct phylogroups.

Between 2009 and 2012, 28 isolates of *A. avenae* were collected from creeping bentgrass with BED symptoms on golf courses across the United States (Table 1). Strains included in this study were collected from 15 states in the southeast, mid-Atlantic, midwest, Gulf coast, and northwest regions of the United States (Table 1). To understand the relationship of turfgrass-pathogenic *A. avenae* to other species in the *Acidovorax* genus, we also included a selection of other plant-pathogenic members.

We first inferred a phylogenetic tree of the newly identified turfgrass-pathogenic *A. avenae* isolates to other closely related members of genus *Acidovorax* with a seven-gene multilocus sequence analysis (MLSA). Two methods, the maximum likelihood (ML) and Bayesian method of phylogenetic inference, produced a phylogenetic tree with identical topology (Fig. 1). While some nodes are not well supported by the bootstrap values in the ML tree, they are strongly supported by high posterior probability values in the Bayesian tree (Fig. 1). The phylogeny showed that the turfgrass-pathogenic *A. avenae* strains are not monophyletic but fall into two distinct phylogroups

(turfgrass groups 1 and 2) (Fig. 1). Each turfgrass phylogroup is more closely related to strains pathogenic to other monocot hosts (such as maize or rice) than to the other turfgrass phylogroup.

BED disease in turfgrass is caused by a set of highly diverse bacterial strains.

Seven turfgrass group 1 isolates, seven turfgrass group 2 isolates, three maize isolates, and one orchid isolate were selected for genome sequencing. Fifteen high-quality draft genomes and three complete genomes were generated (Table 1). We first determined the population diversity of the turfgrass *A. avenae* isolates. We identified a total of 165,645 single nucleotide polymorphisms (SNPs) across a total alignment length of 3,154,840 bp (avg. pairwise identity = 98.1%) in a whole-genome alignment of 14 turfgrass genomes. The overall diversity of the 14 turfgrass *A. avenae* genomes was 0.0279, as determined by Watterson's theta (Watterson 1975), about three times higher than the value observed in the kiwifruit pathogen *Pseudomonas syringae* pv. *actinidiae* (Watterson's theta = 0.008) (McCann et al. 2013). Interestingly, although the *Acidovorax* strains are more diverse than the *P. syringae* pv. *actinidiae* strains, genome rearrangements are less common. The *A. avenae* strains display a high level of synteny with significantly fewer translocations and

Fig. 1. Phylogeny of *Acidovorax* strains pathogenic to turfgrass and other plant hosts, inferred by multilocus sequence analysis. A phylogenetic tree generated from a seven-gene concatenated nucleotide sequence alignment using maximum likelihood (ML) with a general time reversible (GTR) model. A 50% cutoff was used to create the bootstrap consensus tree, and bootstrap support values greater than 60% are indicated at each node. The same alignment was also used in a Bayesian method of phylogenetic inference analysis, which generated a converged tree with the same topology as the ML-GTR method. Numbers in parentheses indicate posterior probabilities. Colors indicate hosts of origin.

Table 2. Genome rearrangement events between maize strain AA81-1 and turfgrass strains MD5 and URI9

	Total SNPs ^a	Breakpoints	Translocations	Inversions	Insertions/deletions
AA81-1:MD5	123,743	880	67	12	407
AA81-1:URI9	174,727	998	54	10	456
MD5:URI9	157,075	1,042	68	14	466

^a SNPs = single nucleotide polymorphisms.

inversions, which indicates lower rates of recombination between and within strains in *A. avenae* (Table 2, Supplementary Fig. S1). Together, these results suggest that BED pathogen strains isolated between 2009 and 2012 are highly divergent (about half of that previously observed for *Escherichia coli* [Touchon et al. 2009; Bertels et al. 2014]) but, comparatively, have not been affected much by recombination.

The phylogeny of the 20 representative *Acidovorax* strains was also inferred, based on the SNPs identified at the genome level (Bertels et al. 2014). Consistent with the phylogeny from the seven-gene MLSA analysis, the whole-genome phylogenetic tree also grouped most turfgrass *A. avenae* strains into two phylogroups (Fig. 2, turfgrass groups 1 and 2). Sa2, which grouped with turfgrass group 1 in the MLSA tree (Fig. 1), was grouped separately in the whole-genome phylogenetic tree (Fig. 2). Furthermore, the nodes that are not well-supported (low bootstrap values) in the MLSA phylogeny (Fig. 1) are supported by high bootstrap values in the whole genome phylogeny (Fig. 2).

Geographical distribution of turfgrass *A. avenae* strains.

To test whether the phylogenetic relationships are shaped by geography, we also measured the distances between isolates and correlated them with genetic distances. Figure 3 shows that there are no significant correlations between genetic distance and geographic distance, which suggests that dispersal rates of *A. avenae* are too high to maintain spatially structured populations.

Turfgrass and maize *A. avenae* isolates display host specificity and preference.

Although the turfgrass-pathogenic *A. avenae* and maize-pathogenic *A. avenae* were isolated from two different hosts, these two groups of pathogens are genetically closely related. To determine if the turfgrass and maize *A. avenae* strains exhibit host specificity, we compared the virulence of turfgrass and maize *A. avenae* strains on maize and creeping bentgrass. When inoculated on maize, maize *A. avenae* strains display a strong

virulence phenotype, whereas the turfgrass strains we tested (from both group 1 and group 2) are nonpathogenic to maize (Supplementary Fig. S2). Host specificity is also supported by our pathogen population assays, which show that maize isolates establish significantly larger populations than turfgrass strains when inoculated on maize (Fig. 4A). Conversely, on turfgrass, both turfgrass *A. avenae* and maize *A. avenae* can cause some necrosis and plant death (Fig. 4B) but the disease symptom is much more severe on turfgrass inoculated with turfgrass *A. avenae* than the ones inoculated with maize *A. avenae*. Additionally, turfgrass *A. avenae* strains establish significantly larger populations on turfgrass (Fig. 4A) and caused more plant death (Fig. 4B) compared with maize *A. avenae* strains. Together, these analyses show that turfgrass *A. avenae* and maize *A. avenae* specifically and preferentially infect the original hosts they were isolated from.

The two phylogroups of turfgrass *A. avenae* contain conserved sequences in the T3SS pathogenicity island.

Despite the significant divergence of the two turfgrass *A. avenae* phylogroups, both groups showed high specificity at infecting turfgrass. To identify a genetic cause for this specificity, we performed a genome-wide search for genes that are more closely related within turfgrass-infecting *A. avenae* than they are to their homologs in the maize-pathogenic strains. Nucleotide sequence alignments of 4,179 genes were individually generated and were used to infer the phylogeny of the turfgrass and maize isolates. Of the 4,179 trees, 26 grouped the two turfgrass phylogroups and the turfgrass-infecting Sa2 strain together, separate from the maize phylogroup (Figure 5 shows four examples and the full list of phylogenetic trees is available in Supplementary Figure S3). The identified genes encode functions such as virulence, chemotaxis, iron transportation, metabolism, and unknown functions (Table 3). Interestingly, 11 of these genes are located in the T3SS pathogenicity island as a gene cluster (Fig. 6B). The fact that the two distantly related phylogroups of turfgrass *A. avenae* both share high sequence similarity

Fig. 2. Phylogeny of *Acidovorax* strains, inferred from a whole-genome alignment. The phylogenetic tree was generated using a merged whole-genome alignment generated by REALPHY. The merged tree was rooted using Cat98-1 and AAC00-1 as outgroups. A 50% cutoff was used to create the bootstrap consensus tree, and bootstrap support values greater than 60% are indicated at each node. Turfgrass pathogenic *A. avenae* strains were grouped into two phylogroups (turfgrass groups 1 and 2) with the exception of Sa2.

in this virulence-related gene cluster suggests that these genes may contribute to host specificity in *A. avenae*.

To further define the sequence conservation in the T3SS pathogenicity island, we generated a 32-kb nucleotide alignment of 34 genes in the T3SS pathogenicity island (Acav_0510-Acav_0543) from four maize and 14 turfgrass *A. avenae* strains. Within the alignment, one region showed a particularly low genetic variability (approximately 17,500 to 27,500 bp) (Fig. 6C). This region completely overlaps with the location of the 11 host-specific genes identified with our phylogenetic method (Fig. 6B, highlighted in green).

Recombination occurred between the two turfgrass phylogroups but not between the turfgrass phylogroups and the maize phylogroup.

The clustering of turfgrass-infecting *A. avenae* strains in the T3SS pathogenicity island in turfgrass *A. avenae* could be caused

by various mechanisms; however, recombination seems to be the most likely. To test for recombination, we applied a Phi test (Bruen et al. 2006) to a total of six *A. avenae* genomes (two group 1 turfgrass strains, INV and NCT3, two group 2 turfgrass strains, QH1 and SF12, and two maize strains, AA78-5 and AA81-1), which predicts high recombination rates between these genomes with significant statistical support ($P < 0.0001$). To determine the extent and the exact location of the recombination events in turfgrass and maize *A. avenae* strains, we applied GENECONV (Sawyer 1989). In turfgrass strains, high levels of gene conversion were detected between strains of the same phylogroup (Fig. 7A, boxes 1 and 2) and moderate levels of recombination were detected between strains of different phylogroups (Fig. 7A, box 4). Interestingly, almost no gene conversion was detected between turfgrass strains and maize strains (Fig. 7A, boxes 5 and 6). These observations suggest that very limited genetic exchange occurred between the turfgrass

Fig. 3. Geographical distribution and genetic correlation of the turfgrass *A. avenae* strains. **A**, Geographical distribution of the turfgrass *A. avenae* strains by phylogroups in the United States. **B**, Matrix of geographical distance and genetic distance of the mapped strains, generated by Mantel's test.

isolates and maize isolates; however, significant amounts of genetic exchange occurred within the turfgrass-specific *A. avenae* populations, both within and across the two phylogroups.

We also determined the level of recombination in the T3SS pathogenicity island in turfgrass and maize strains (Fig. 7B). Similar to the results from the whole-genome analysis, limited gene conversion was detected between turfgrass and maize isolates in the T3SS pathogenicity island (Fig. 7B, boxes 5 and 6), whereas higher levels of gene conversion were detected among turfgrass isolates (Fig. 7B, boxes 1, 2, and 4). Interestingly, across-group recombination (group 1-group 2, group 1-Sa2, and group 2-Sa2) occurred at a much higher level on the T3SS pathogenicity island (Fig. 7B, box 4) than that of the whole genome (Fig. 7A, box 4). Alignment of the gene conversion fragments to the T3SS pathogenicity island revealed that most of the cross-group gene conversion (Fig. 6A, magenta lines) occurred in the region of the identified host-specific genes (16,000 to 27,000 bp) (Fig. 6B, ORFs highlighted in green). Together, our results suggest that recombination between the two turfgrass phylogroups has played an important role for the evolution of host specificity in turfgrass *A. avenae*.

Three recombination events can explain the observed T3SS phylogeny.

Our analyses suggest that recombination is a likely cause for the high sequence conservation of T3SS genes among turfgrass-infecting *A. avenae* strains. To infer a likely time frame for when the recombination occurred, we generated a nucleotide alignment of the host-specific T3SS genes (Acav_0525 to Acav_0536). The inferred phylogeny (Fig. 8A) is consistent with the whole-genome phylogeny (Fig. 8B) within both turfgrass phylogroups with two exceptions: URI9, a strain that is listed as a group 1 turfgrass strain in the whole-genome phylogeny (Fig. 8B), is now grouped with group 2 turfgrass strains in the T3SS host-specific phylogeny (Fig. 8A), and Sa2, a strain that is listed as a single clade separate from the group 1 and group 2 turfgrass strains (Fig. 8B), is now grouped with group 1 strains in the T3SS host-specific phylogeny (Fig. 8A). Hence, we can explain the difference between the host-specific T3SS tree and the whole-genome phylogeny with only three recombination events: one recombination occurred between the ancestor of group 1 turfgrass and the ancestor group 2 turfgrass strains, the second recombination event occurred between URI9 and the ancestor of INDB2 and SF12, and the third recombination event occurred between Sa2 and the ancestor of QHB1, MDB1, and NCT3 (Fig. 8B).

As these recombination events may have occurred a long time ago, the low genetic variation of the Acav_0525-Acav_0536 region is likely due to strong purifying selection acting to preserve sequence identity. To test this hypothesis, the ratios of non-synonymous to synonymous substitutions (dN/dS) of genes located in the T3SS pathogenicity island were measured in group 1 and group 2 turfgrass *A. avenae* strains (KL3, INV, URI9, QHB1, NCT3, COLB1, SF12, INDB2, QH1, MOR, SH7, and MD5). Of all 33 genes in the T3SS pathogenicity island, 17 genes were detected to be under purifying selection (Table 4, dN/dS < 1, $P < 0.01$). However, genes under purifying selection are concentrated at the first 10-kb of the T3SS pathogenicity island and mostly encode for the T3SS structure and regulation (Table 4, Acav_0510-Acav_0519). Within the 11 genes in the host-specific region (Acav_0525-Acav_0536), only three genes (Acav_0525, Acav_0527, and Acav_0531) were affected by purifying selection with statistical significance (Table 4). However, when inspecting the data closely, it is obvious that the high P values are not due to dN/dS values that are indistinguishable from 1, because there are dN/dS values that are 0 (the lowest possible value) but still not significantly different from 1. Hence, low significance is due to the fact that genetic variation in this part

of the genome is too low (Table 4, piDn and piDs values) to sample enough substitutions to allow for accurate dN/dS measures. This indicates that dN/dS is not an appropriate measure to gauge selection strength across the host-specific T3SS region.

Relative sequence diversity as a measure of selection.

Alternatively, we can determine relative sequence diversity as a measure of purifying selection. Sequence diversity can be low either because selection acts to reduce diversity (every mutation has a lower fitness than any new mutation) or because

Fig. 4. Virulence of the turfgrass and maize *Acidovorax avenae* strains on maize and turfgrass. **A**, Population of different *A. avenae* strains inoculated on maize (*Zea mays* 'Peach Cream') and creeping bentgrass (*Agrostis stolonifera* 'PennA4'). Inoculation of maize was performed by cutting the tip of the second expanded leaf off across the veins, using an overnight culture adjusted to 10⁸ CFU ml⁻¹. Inoculation of turfgrass was performed by cutting the root of the surface-sterilized grass and maintaining the root of the grass in a bacterial suspension of 10⁸ CFU ml⁻¹. The inoculated maize and turfgrass plants were maintained at 26 and 22°C (day and night, respectively) and 80% humidity and at 32°C, 80% humidity, respectively. The internal pathogen population was sampled by plating at 72 h postinoculation. Strains are color coded by their phylogroups (green: maize, red: turfgrass group 1, blue: turfgrass group 2). Statistical analysis was performed using analysis of variance with $\alpha = 0.05$. The level of significance was calculated using LSD ($\alpha = 0.05$). **B**, Survival rate of creeping bentgrass 'PennA4' after inoculation of turfgrass and maize-pathogenic *A. avenae* strains. The experiment was repeated two times and data from one experiment is presented in this figure.

the rest of the genome is under positive selection and our region of interest is evolving neutrally (or under less positive selection pressure). Either way the selection on the host maintains the sequence identity in the host-specific T3SS region. To determine relative sequence diversity, we measured the Shannon entropy for each nucleotide across the T3SS pathogenicity island in the turfgrass strains. The host-specific region of the T3SS (16,000 to 25,000 bp) displayed a significantly lower nucleotide diversity compared with the rest of the genome (98% of all 500-bp windows across the genome have higher nucleotide diversity than the turfgrass-specific region [Fig. 9B]). When compared with the diversity of the whole genome, both the T3SS pathogenicity island and the T3SS host-specific region display diversity values below the genome average, with the value in T3SS host-specific region (22% of the genome average) much lower than the entire T3SS (55% of the genome average) (Fig. 9A). This observation strongly suggests purifying selection as the main driver of evolution for the T3SS pathogenicity island and especially for the T3SS host-specific region. In addition, one region, which aligns with *hpaP* (Acav_0517) within the T3SS, showed a significantly higher level of nucleotide diversity (195% of the genome average [Fig. 9B]). This suggests that the T3SS pathogenicity island is also affected by diversifying selection in addition to the strong

purifying selection of the host-specific region. Compared with the turfgrass strains, no significant purifying selection was observed in either the T3SS host-specific region or the T3SS pathogenicity island in maize strains (103 and 93% of the genome average, respectively [Supplementary Fig. S4]).

DISCUSSION

In our study, we sequenced a set of 18 *A. avenae* strains, 14 of which were isolated from a recent BED outbreak on creeping bentgrass (turfgrass). We found that not only do the 14 strains not belong to the same clonal strain but that they form two major phylogroups within the *A. avenae* species (if not including the single strain Sa2, which is separately grouped as a single clade). It is unusual that a novel disease is caused by a diverse range of strains, as most disease outbreaks are caused by a highly virulent clonal plant pathogen (e.g., *Xylella fastidiosa* strains associated with olive quick decline syndrome in Italy and some of the phylotype II strains of *Ralstonia solanacearum* causing diseases on banana and potato [Ailloud et al. 2015; Marcelletti and Scortichini 2016]). Although BED in creeping bentgrass has not been described before, the strains causing the disease have not recently emerged but probably

Fig. 5. Phylogeny of genes that group turfgrass-infecting *Acidovorax avenae* strains into a monophyletic group. The phylogeny of a selection of four genes in the type III secretion system pathogenicity island are displayed. The phylogeny was inferred using nucleotide sequence alignment of the individual genes by maximum likelihood with a general time reversible model and was color-coded by hosts and phylogroups (green: maize, red: turfgrass group 1, blue: turfgrass group 2, black: Sa2, yellow: outgroups). A 50% cutoff was used to create the bootstrap consensus tree, and bootstrap support values greater than 60% are indicated at each node.

diverged millions of years ago, as our phylogenetic analyses and host-specificity assays show. Instead, it is more likely that the hot summers between 2009 and 2012 provided an optimal environment for *A. avenae* to thrive on creeping bentgrass, which probably resulted in more severe symptoms than usual. Alternatively, *A. avenae* strains could be adapting to creeping bentgrass and slowly increasing in virulence. However, this hypothesis seems less likely since such an adaptation would have to happen in parallel across very different strains and within the exact same time frame.

Although the BED outbreak between 2009 and 2012 displayed low virulence phenotypes, a severe outbreak may still occur as observed for the kiwifruit pathogen *P. syringae* pv. *actinidiae*, in which a highly pathogenic *P. syringae* pv. *actinidiae* clone emerged from a highly diverse set of related low-virulence *P. syringae* pv. *actinidiae* strains and spread worldwide within a few years (McCann et al. 2013). Hence, it is possible (and the high diversity and recent population expansion may even make it likely) that, from this population of strains, a highly virulent strain will emerge that can cause a devastating future outbreak.

We also showed that the high genetic diversity we observe between turfgrass strains was not correlated with their geographic origin. This suggests that dispersal rates of *A. avenae* are much higher than genetic diversification rates. At this point, it is unclear whether high dispersal rates are an effect of human activity or whether *A. avenae* is easily dispersed by wind, rain, seeds, or animals.

In laboratory experiments, we have shown that both *A. avenae* isolates from turfgrass and maize are host specific despite the distant relationship between some of the strains. This begs the question what determines host specificity in *A. avenae*. Two common mechanisms can explain bacteria gaining pathogenicity of a specific host, i.e., acquiring host-specific virulence genes through horizontal gene transfer or adaptation to generate host-specific alleles (McCann and Guttman 2008).

We tested these hypotheses with extensive whole-genome sequence analyses and phylogenetic analyses. We first performed BLAST to search for putative virulence factors that are only present in turfgrass strains but not in maize strains. However, this effort did not identify any genes that may explain the host specificity in turfgrass. Although putative host-specificity or virulence genes including the T3 effectors vary significantly in their presence among strains (Supplementary Fig. S5), we could not find virulence factors that are present exclusively in turfgrass strains. However, as many of the genomes are draft genomes, we cannot exclude the possibility that some virulence genes are missing due to variations in sequencing depth that can cause erroneous assemblies. Next, we identified a cluster of genes in the T3SS that placed all turfgrass-infecting strains into a single monophyletic group. These turfgrass-infecting strains are from diverse phylogenetic backgrounds, including strains from two distinct phylogroups (group 1 and group 2) and a separately grouped strain (Sa2). The fact that the identified genes are highly conserved in diverse strains infecting the same host and that they are involved in virulence raises the possibility that this region is responsible for determining turfgrass-specific growth of *A. avenae*.

The 10-kb turfgrass-specific region we discovered in this study represents a significant portion of the T3SS pathogenicity island, accounting for about one third of the entire pathogenicity island. Genes in this region encode both putative T3 effectors, structural components of the secretion apparatus, and proteins with unknown functions. The T3SS in *A. avenae* has been poorly studied, and the functions of most genes in the T3SS pathogenicity island remain unvalidated or uncharacterized. Among the genes that are conserved in the T3SS pathogenicity island, four genes (*lrp*, Acav_0526, Acav_0527, and Acav_0528)

were predicted to encode T3 secreted proteins (Table 3), which suggests that they are putative T3 effectors or harpin proteins. Although the functions of these genes are unknown, homolog search revealed that one of these four genes, *lrp* (Acav_0525), encodes a putative GalA like T3 effector (GalA1), which was previously shown to manipulate the host ubiquitin-proteasome system (Remigi et al. 2011). T3 effectors and harpins are at the front line of suppressing host immunity and promoting pathogenicity (Büttner and He 2009). Some have even been shown to be important determinants of host specificity (Baltrus et al. 2012). It is possible that the homologs in turfgrass-infecting *A. avenae* may grant the ability to evade host surveillance.

In addition to the T3 secreted proteins, we also identified three genes encoding components of the inner ring of the secretion apparatus (*hrcN*, *hrcL*, and *hrcJ*). *hrcN* encodes an ATPase located on the cytoplasm side of the inner membrane and HrcL and HrcJ are proteins associated with HrcN. Previous reports showed that HrcN and other proteins located on the inner ring of the secretion apparatus are involved in the translocation of T3 effectors (Büttner and He 2009). Although the exact fitness advantage of maintaining a conserved inner membrane ring is not known, it is possible that the turfgrass *A. avenae* utilizes the conserved sequences on the inner membrane to mediate the translocation of host-specific T3 effectors and thus confer host specificity. The remaining putative host-specific genes do not share homology with proteins of known function. Future experiments are needed to determine their role in pathogenicity and host specificity.

Sequence variations of T3 effector proteins causing variations in pathogenicity have been documented in previous studies (Stevens et al. 1998; Yang et al. 2005). For example, seven different natural allelic variants were identified in the T3 effector HopX in

Table 3. Genes that cluster all turfgrass-infecting *Acidovorax avenae* strains into a monophyletic group^a

Genomes	Gene name	Predicted gene function
Acav_0216		Hypothetical protein
Acav_0303		Hypothetical protein
Acav_0483		Chemotaxis methyl-accepting receptor
Acav_0509		Chorismate mutase
Acav_0525^b	<i>lrp</i>	Putative GalA like T3 effector (with leucine-rich repeat)
Acav_0526^{bc}		Hypothetical protein
Acav_0527^c		Hypothetical protein
Acav_0528^c		Hypothetical protein
Acav_0529		Hypothetical protein
Acav_0530	<i>hrcN</i>	T3S apparatus ATPase
Acav_0531	<i>hrcL</i>	T3S apparatus protein, HrpE/YscL
Acav_0533	<i>hrcJ</i>	YscJ/HrcJ family T3S apparatus lipoprotein
Acav_0534	<i>hrpB2</i>	T3S protein HrpB2
Acav_0535	<i>hrpB1</i>	T3S protein HrpB1/HrpK
Acav_0536		Hypothetical protein
Acav_0715		Single subunit mitochondrial RNA polymerase
Acav_0952		S-adenosylmethionine synthetase
Acav_1526		(2Fe-2S)-binding domain-containing protein
Acav_1813		Alcohol dehydrogenase
Acav_2773	<i>iscS</i>	Cysteine desulfurase
Acav_2826		Hypothetical protein
Acav_2827	<i>estB</i>	Phospholipase
Acav_2829		Class I peptide chain release factor
Acav_2839		Fe ³⁺ -transporting ATPase
Acav_2853	<i>clsA</i>	Cardiolipin synthetase
Acav_3448		Flavin-containing monooxygenase-like protein

^a Bold indicates genes in the type III secretion (T3S) system pathogenicity island.

^b Putative T3S protein, predicted by Effective ELD (based on eukaryotic-like domains). Only predictions with high confidence was displayed.

^c Putative T3S protein, predicted by Effective T3 (based on the N-terminal signal peptide).

P. syringae (Stevens et al. 1998). The wild-type allele of HopX induces the hypersensitive response in bean, while all other variants do not. In another study, host-adapted polymorphisms were found in multiple virulence-related genes (such as the T3 effector) in banana pathogenic and nonpathogenic *Ralstonia solanacearum* strains. The authors suggest that these polymorphisms may explain differences in host range (Ailloud et al. 2015). Although these examples establish the correlation between host-specific alleles and the corresponding phenotypes, the distribution of these host-specific alleles in natural pathogen populations, as well as the evolutionary mechanisms responsible for the formation of these alleles, are not well understood. In this study, we provide evidence that the conserved host-specific sequences could be shared in distantly related phylogroups of pathogens. We also showed that the host-specific alleles were formed by recombination and were preserved by purifying selection.

The evolutionary mechanisms that cause the observed allelic diversity are not very well understood; however, as we show in this study, recombination generally plays a major role

(Awadalla 2003; Baltrus et al. 2017; Menardo et al. 2016; Mhedbi-Hajri et al. 2013; Yan et al. 2008). Strains causing a disease outbreak often show signs of extensive recombination with related strains (McCann et al. 2013; Monteil et al. 2013). For instance, the *P. syringae* pv. *actinidiae* strain causing the 2008 kiwifruit outbreak displays significant levels of homologous recombination, about 8.3 and 21.4% of the NZ V-13 genome (one of the outbreak strains) arose from recombination with strains from Korea and Japan, respectively (McCann et al. 2013). In our study, we show that turfgrass-pathogenic *A. avenae* strains are also significantly affected by recombination; on average, 7.54% of the genome was affected by recombination when comparing any two turfgrass-pathogenic *A. avenae* strains (Fig. 7). Generally, it is difficult to determine the number of recombination events needed for divergent bacterial strains to infect a common host. Naively, one would assume that attaining host specificity is a long and complex multistep recombination process. In contrast, in our study, we demonstrate that as few as three recombination events may have been sufficient to enable divergent *A. avenae* to

Fig. 6. Genetic variance and gene conversion fragments mapped to the type III secretion system (T3SS) pathogenicity island in turfgrass- and maize-infesting strains. **A**, Gene conversion fragments identified in the T3SS pathogenicity island. The gene conversion fragments between any two strains were identified by first aligning the nucleotide sequences of the T3SS pathogenicity islands and, then, using the T3SS nucleotide alignment as input in GENCONV to identify gene conversion fragments. The identified fragments were mapped back to the T3SS pathogenicity island and were color-coded by the identity of the two strains. **B**, Open reading frames (ORFs) in the T3SS pathogenicity island. Gene names and their corresponding identification code (Acav_0510-Acav_0543) are labeled above the ORFs. The green-colored ORFs are genes that place turfgrass-infesting strains into a monophyletic group. **C**, The alignment of the nucleotide sequences of the T3SS pathogenicity island in 15 *Acidovorax avenae* strains. Consensus sequence and polymorphisms are indicated by green color and black vertical lines. Strains were color-coded by their hosts and phylogroups (green: maize, red: turfgrass group 1, blue: turfgrass group 2, black: Sa2).

infect the same host. We'd like to address that the number of recombination events is estimated based on the phylogeny of a concatenated nucleotide sequence of the host-specific genes. It is possible that more recombination events occurred between closely related strains. However, due to the extremely low nucleotide diversity in the host-specific T3SS region, it is impossible for us to further quantify these events at the level of each individual gene.

Apart from the recombination event that allowed host specificity, we observe high recombination rates between *A. avenae* turfgrass strains but very limited recombination between turfgrass and maize strains. This suggests that most of the recombination events between the turfgrass-infecting strains occurred after the host shift of one of the *A. avenae* turfgrass-infecting clades, emphasizing the importance of frequent physical contact and sharing the same ecological niche for high

Fig. 7. Recombination occurred at the genome level and in the type III secretion system (T3SS) pathogenicity island. **A.** Percentage of the genome involved in gene conversions. **B.** Percentage of the T3SS pathogenicity island involved in gene conversions. The gene conversion fragments were identified by GENE-CONV, using the nucleotide alignment of the whole-genome sequences or the T3SS pathogenicity island sequences. Percentage was calculated by dividing the size of all gene conversion fragments with the size of the aligned sequences. Boxes numbered 1 to 6 represent all possible combinations of different phylogroups. The simulated *P* values of all gene conversion fragments are between 0 and 0.019 (for fragments detected in the genome, section A) and between 0 and 0.05 (for fragments detected in the T3SS pathogenicity island, section B).

recombination rates. Hence, our data shows that host specificity can lead to differentiation (and eventually speciation) for bacterial strains that infect different hosts, but it can also lead to the opposite effect (convergent evolution) when two divergent clades infect a common host. We also show that purifying selection across the turfgrass-specific T3SS region is much stronger in turfgrass-infecting strains than in maize-infecting strains. The fact that the turfgrass-specific region is not conserved in maize supports suggestions that this region has not been affected by recombination with distantly related bacteria, because nucleotide diversity is identical to the mean diversity across the genome.

The sequence conservation we observed in the turfgrass-specific T3SS region is much higher than that of the rest of the genome. Our analyses show that this is likely due to strong purifying selection acting on this region of the genome. Previous studies have shown that purifying selection plays an important role in maintaining essential functions of the T3 secretion apparatus (Guttman et al. 2006; McCann and Guttman 2008). Results from this study provide evidence that purifying selection also helps in maintaining sequence conservation of host-specific alleles.

Here, we provide an example of two divergent pathogen lineages converging onto the same host by acquiring or maintaining, or both, a conserved set of virulence genes. However, this is not the only way that different lineages of pathogens adapt to the same host. The divergent pathogens may also be able to acquire different assemblages of virulence factors when adapting to the same host. For example, hazelnut decline in Greece and Italy was caused by two entirely distinct lineages of *P. syringae* pv. *avellanae* (Pav) (O'Brien et al. 2012). These two lineages of *P. syringae* pv. *avellanae* contain divergent T3 effector repertoires, which implies that the two lineages may have developed distinct mechanisms to infect hazelnut trees (O'Brien et al. 2012). The reason for choosing different adaptive paths may be due to geography. The two pathogen lineages in Greece and Italy are geographically separated by the Ionian sea (allopatric speciation), whereas the two phylogroups of the turfgrass pathogens in this study are located in the same geographical region without any significant geographical barriers (sympatric speciation). It is conceivable that geographical barriers will play less of a role for pathogen evolution as globalization advances. Human activities and transportation of plant materials will provide ample opportunity for pathogens to share host-specific virulence factors on a global scale.

In conclusion, our study provides a comprehensive analysis of the bacterium *A. avenae* that has led to a recent outbreak

of BED in turfgrass. We have shown that a diverse set of strains is responsible for causing the disease and have identified a set of genes that is likely to be responsible for conferring host specificity to turfgrass. Hence, here we provide the basis for developing tools to monitor and control the disease and, hopefully, prevent a more severe outbreak in the future.

Table 4. The ratios of nonsynonymous to synonymous substitutions (dN/dS) of genes located in the type III secretion system pathogenicity island in turfgrass *Acidovorax avenae* isolates

GeneID ^a	piDn	piDs	dN/dS	P value ^b
Acav_0510 ⁺	2.736	11.486	0.099	0.001
Acav_0511 ⁺	2.431	7.778	0.114	0.001
Acav_0512 ⁺	0	3.514	0	0.001
Acav_0513 ⁺	4.375	14.806	0.095	0.001
Acav_0514 ⁺	0	3.556	0	0.001
Acav_0515 ⁺	0.972	6.75	0.047	0.001
Acav_0516	9.069	5.125	0.769	0.26
Acav_0517 ⁺	4.792	5.167	0.334	0.005
Acav_0518 ⁺	1.556	16.5	0.038	0.001
Acav_0519 ⁺	1.708	14.056	0.058	0.001
Acav_0520	7.806	2.139	1.347	0.809
Acav_0521 ⁺	3.417	10.972	0.113	0.001
Acav_0523	1.472	0.444	1.647	0.965
Acav_0524	9.34	7.91	0.439	0.827
Acav_0525⁺	5.076	4.174	0.738	0.005
Acav_0526	0	1.181	0	0.237
Acav_0527⁺	1.333	1.847	0.19	0.001
Acav_0528	1.361	1.528	0.521	0.016
Acav_0529	0.778	0.931	0.275	0.242
Acav_0530	0.528	8.139	0.031	0.15
Acav_0531⁺	0.153	1.764	0.061	0.001
Acav_0532 ⁺	0.611	1.903	0.331	0.002
Acav_0533	1.028	0.486	1.102	0.089
Acav_0534	0.5	0	No_dS	0.715
Acav_0535	1.097	0.431	0.489	1
Acav_0536	0	0.444	0	0.356
Acav_0537	0	0.306	0	0.278
Acav_0538	0	0.306	0	0.047
Acav_0539	1.111	1.861	0.163	0.047
Acav_0540 ⁺	0.278	2.472	0.034	0.003
Acav_0541 ⁺	0.278	0.556	0.148	0.001
Acav_0542	3.472	10.194	0.134	0.131
Acav_0543 ⁺	2.5	16.306	0.05	0.001

^a Genes highlighted in bold are the turfgrass-specific genes. Genes noted with a plus (+) suggest significant negative selection ($P < 0.01$).

^b P values represent statistical significance for negative selection and are calculated by Fisher's exact test.

Fig. 8. Inference of the number of recombination events needed for the formation of the conserved type III secretion system (T3SS) gene cluster. **A**, Phylogeny of the concatenated nucleotide sequence alignment of Acav_0525 to Acav_0536. **B**, Whole-genome phylogeny indicating the only three recombination events necessary to explain difference between the host-specific T3SS tree and the whole-genome phylogeny.

MATERIALS AND METHODS

Bacterial strains.

Acidovorax avenae isolates from creeping bentgrass were obtained from turfgrass samples with BED symptoms sent to the Michigan State University turfgrass diagnostic laboratory between 2009 and 2012. All other *Acidovorax* isolates were obtained from R. Walcott at the University of Georgia, except for isolates AA_30003 to AA30305 in Table 1, in which nucleotide sequences for individual genes were obtained from an *Acidovorax* multilocus sequence typing (MLST) database on the PAMDB website. Bacteria were cultured in lysogeny broth (LB) medium and stored at -80°C in 15% of glycerol.

DNA isolation.

Bacterial strains were cultured in 5 ml of LB broth at 28°C . The bacterial cells were collected from a culture in exponential growth phase (optical density = 0.3). Total DNA was isolated by using a Qiagen DNeasy kit (Qiagen, Valencia, CA, U.S.A.), following its protocol for bacterial DNA isolation. The quality and quantity of the purified DNA was checked by a Nanodrop 2000C (Nanodrop Products, Wilmington, DE, U.S.A.).

MLSA.

MLSA was performed as previously described by Feng et al. (2009). Briefly, a set of seven loci (*gmc*, *ugpB*, *pilT*, *lepA*, *trpB*, *gltA*, and *phaC*) were polymerase chain reaction (PCR)-amplified from bacterial isolates originating in this study. The PCR products were purified using Exonuclease I (Fermentas Life Sciences, Vilnius, Lithuania) and FastAP Thermosensitive alkaline phosphatase (Fermentas Life Sciences), according to the manufacturer's protocol. DNA was sequenced at the Michigan State University Research Technology Support Facility, using an ABI 3730 automated sequencer. Sanger sequencing reads were assembled using the CodonCode Aligner (v. 2.0.6) (CodonCode Corporation, Centerville, MA, U.S.A.) to obtain 2 $\times$ coverage sequences. All sequences were aligned using ClustalW in MEGA5 (Tamura et al. 2011). The sequences of seven genes are concatenated and

are used in the ML analysis and Bayesian method of phylogenetic inference. The ML tree was generated using the general time reversible (GTR) model in Geneious 9.1.6 (Kearse et al. 2012).

Bayesian method of phylogenetic inference (Mau et al. 1999; Rannala and Yang 1996) was performed using MrBayes 3.3.2 (Ronquist and Huelsenbeck 2003). We assumed a GTR model with estimated parameter values for base frequencies, proportion of invariant sites, and for the shape of the gamma distribution with four categories. Ten million generations of two simultaneous Markov chain Monte Carlo chains were performed and trees were sampled every 500 generations. Four chains (three heated and one cold) were run, with heated chains at temperature of 0.2. Chain length, subsample frequency, and burn-in length are set at 1,100,000, 200, and 100,000, respectively. *A. citrulli* 00-1 was used as the outgroup.

Genome sequencing and assembly.

The quality and quantity of the DNA were measured using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, U.S.A.). The genomes of 15 *Acidovorax* strains (Cat98-1, KL3, INV, QHB1, MDB1, NCT3, Sa2, AA78-5, AA99-2, COLB1, SF12, INDB2, QH1, MOR, SH7) were sequenced using Illumina MiSeq technology at the Michigan State University Research Technology Support Facility. Genomic DNA was prepared using the Illumina TruSeq DNA library prep kit LT. Sequencing was performed on an Illumina MiSeq in a pair-end 250-bp read with a v2 reagent kit. The mean insert size of the 15 *Acidovorax* sequencing libraries ranged from 498 to 638 bp, with an average of 540 bp.

The Illumina sequencing files were first checked by FastQC to determine the sequencing quality. Trimmomatic v0.32 (Bolger et al. 2014) was used to trim off low-quality reads that do not meet the preset threshold. Reads that passed the filter were checked by FastQC again to confirm the improvement of quality from the original reads. The trimmed reads were then assembled by SPAdes de novo genome assembler v3.5.0 (Bankevich et al. 2012) into scaffolds with multiple K-mer lengths: 21, 33, 43, 53, 65, and 77. The de novo assembled genomes were analyzed by QUAST (Gurevich et al. 2013), using ATCC19860 as reference

Fig. 9. Nucleotide diversity analysis across all 14 turfgrass-infecting *Acidovorax avenae* strains. **A**, Histogram of the mean per nucleotide diversity (Shannon entropy) calculated for nonoverlapping 500-bp long sequence windows across a REALPHY alignment for all 14 turfgrass-infecting *A. avenae* strains. Red arrows indicate the mean diversity for the type III secretion system (T3SS), the T3SS turfgrass-specific region, and for *hpaP*. **B**, The mean per nucleotide diversity across the T3SS region for nonoverlapping 500-bp sequence windows.

genome. The assembled genomes (scaffolds) were ordered by MUMmer through the wrapper program ABACUS v 1.3.1 (Assefa et al. 2009). The pseudomolecules of all 15 genomes were generated and were used as input for gene annotation and prediction by Prokka v.1.11 (Seemann 2014).

Three *A. avenae* strains (MD5, URI9, and AA81-1) were sequenced using PacBio sequencing technology at the Yale Center for Genome Analysis. The library was prepared using the PacBio template preparation kit 1.0. Sequencing was performed on a PacBio RS II, using P6C4 reagent kit. One SMRT cell was used for each bacterial genome. The numbers of reads as well as the mean sequence lengths are 150,292 reads at 6,571 bp (MD5), 150,292 reads at 6210 bp (URI9), and 150,292 reads at 8,253 bp (AA81-1). The genome coverage for the three strains are 86×, 84×, and 111× (for MD5, URI9, and AA81-1, respectively). The genome sequences were assembled using the HGAP protocol of PacBio SMRT analysis software (Chin et al. 2013). The comparison of MD5, URI9, and AA81-1 was performed by MUMmer 3, using the default nucmer setting for fairly similar sequences (Kurtz et al. 2004).

Whole-genome alignment, SNP analyses, and phylogeny.

Fifteen assembled draft genomes (Cat98-1, KL3, INV, QHB1, MDB1, NCT3, Sa2, AA78-5, AA99-2, COLB1, SF12, INDB2, QH1, MOR, SH7) and five complete genomes (AA81-1, URI9, MD5, ATCC19860, AAC00-1) were used as input for a reference sequence alignment-based phylogeny (REALPHY) analysis (Bertels et al. 2014). REALPHY produced a merged alignment from five individual reference sequence alignments (Cat98-1, KL3, ATCC19860, QH1, SF12), using default parameters. From this alignment, a phylogeny was constructed with PhyML using a GTR substitution model as well as 100 bootstrap replicates (Guindon et al. 2005). The topology of the merged tree is the same as the consensus tree of the five individual reference trees (Felsenstein 1989). The merged tree was rooted using Cat98-1 and AAC00-1 as outgroups. The merged alignment of the 20 *Acidovorax* genomes generated from REALPHY was used as input file in Geneious 9.1.6 for determining the number of SNPs. Watterson's theta (Watterson 1975) was calculated using the Eglib package (De Mita and Siol 2012).

Mapping turfgrass-pathogenic *A. avenae* by phylogroup and Mantel's test.

The turfgrass-pathogenic *A. avenae* strains were mapped to the map of United States and color-coded by their phylogroups as determined by MLSA. Strains Sa2 and URI1 are separately listed because Sa2 was grouped as a separate clade in the genome-wide SNP alignment phylogeny. The pairwise genetic distance from the seven-gene MLST sequence alignment was calculated using the 'ape' package (Paradis et al. 2004) in R with the TN93 evolutionary model (Tamura and Nei 1993). The pairwise geographical distance was calculated based on the GPS coordinates of the isolation origin, using the 'geosphere' package in R. Mantel's test was performed using the 'ape' package in R with 10,000 permutations.

Population test and virulence assay.

The population test of *A. avenae* strains on maize was performed as follows. Two-week-old maize seedlings (*Zea mays* 'Peach Cream') grown from seed in Promix potting soil (Quakertown, PA, U.S.A.) were used in the inoculation assay. *Acidovorax* overnight cultures were adjusted to a cell concentration of 10^8 CFU ml⁻¹. Inoculation was performed by dipping sterile scissors into a bacterial suspension and cutting the tip of the second expanded leaf off, across the veins. The inoculated plants were kept in a temperature- and humidity-controlled growth chamber (26 and 22°C day and night temperatures, 80% humidity). The plants were surface-sterilized and pathogen population was sampled by plating at 72 h postinoculation. Statistical analysis

was performed using the one-way analysis of variance (ANOVA) model in the 'stats' package in R. Letters above the bars denote the level of significance ($\alpha = 0.05$). For virulence phenotype observation, the inoculated maize leaves were collected 7 days after inoculation and virulence phenotype was documented.

The population test of *A. avenae* strains on turfgrass was performed as follows. Five-week-old creeping bentgrass (*Agrostis stolonifera* 'Penn A4') grown in horticultural grade perlite was used in the population assay. Prior to inoculation, whole plants were disinfested in 10% sodium hypochlorite for 1 min, followed immediately by a rinse in sterile distilled water for 1 min. Top growth from individual plants was trimmed with a sterile razor blade so that each plant measured 20 mm from the crown. Lower roots were sliced with a sterile razor blade so that all roots measured 10 mm in length from the longest root tip to the crown. One milliliter of overnight culture of *A. avenae* strains (10^8 CFU ml⁻¹) was added into each well of a 48-well flat-bottomed, polystyrene Costar cell culture plate (Corning Inc., Corning, NY, U.S.A.). Plates were covered with pierceable adhesive sealing foil and were punctured with a sterile pipette tip in the center of the well to accommodate plant insertion. The prepared creeping bentgrass was placed into each well of a 48-well plate and was incubated at 32°C with 80% humidity. Populations of *A. avenae* internally present in each plant were sampled at 72 h post-inoculation, by plating. Statistical analysis was performed using the one-way ANOVA model in the 'stats' package in R. Plants for survival rate testing were kept at 32°C and survival rates were documented at 4, 7, 11, 13, 15, and 17 days postinoculation. For virulence phenotype observation, six-week-old creeping bentgrass (*Agrostis stolonifera* 'Penn A4') grown in Promix potting soil in foam cups was first cut by sterile scissors and was then inversely dipped in bacterial suspension (0.5× phosphate buffered saline containing bacteria at the concentration 10^8 CFU/ml). Inoculated plants were maintained at 32°C with 80% humidity. The virulence phenotype was documented at 72 h postinoculation.

Genome-wide identification of host-specific genes in turfgrass *A. avenae*.

The core genome (genes that are shared across all strains) was determined for six turfgrass group 1 (KL3, INV, QHB1, MDB1, NCT3, URI9), seven turfgrass group 2 (SH7, QH1, MOR, COLB1, INDB2, SF12, MD5), and four maize (AA78-5, AA99-2, AA81-1, ATCC19860) *A. avenae* strains. The protein sequences were used for core and pan genomes analyses, using Roary (Page et al. 2015) with a BLASTp identity cutoff of 80% for the comparison among *Acidovorax* strains. Homologs with at least 80% protein sequence identity that are present in at least 13 of the 20 *Acidovorax* strains were used to identify genes with sequence conservation in turfgrass-pathogenic *A. avenae* strains (group 1 and group 2 strains and Sa2). Nucleotide sequences were aligned using MUSCLE v3.8.31 (Edgar 2004) and built gene trees using 'FastTree' (Price et al. 2009) with the neighbor joining method (Saitou and Nei 1987). Gene trees were analyzed in the 'ape' package to identify monophyletic groups that cluster strains adapted to the turf host. The monophyletic gene trees were further filtered by visualizing tree topologies in R using the 'ggtree' package (Yu et al. 2016), with tree tip labels colored according to their host range and phylogroups. Tree topologies that cluster turfgrass-pathogenic *A. avenae* strains (group 1 and group 2 strains and Sa2) apart from the maize-pathogenic strains were selected as gene candidates for *A. avenae* turfgrass host-specific genes. The procedure was implemented in R (3.1.3).

Recombination test.

The genome alignments of 20 *Acidovorax* strains generated by REALPHY was used to test for recombination in a Phi test as implemented in SplitsTree (Huson and Bryant 2006). After confirming the existence of recombination, the alignment

file was used as input file in GENCONV, with default settings (Sawyer 1989), to predict the locations of gene conversion fragments. The multiple-comparison corrected *P* values representing the possibility of gene conversion events were calculated with 10,000 random permutations. Regions with *P* < 0.05 were identified as significant gene conversion fragments. The number of gene conversion fragments between two strains as well as the total size of these fragments were documented and displayed in heat maps generated by Plotly. Gene conversion fragments in the T3SS pathogenicity island were identified by the same method, using the DNA alignment of the T3SS pathogenicity island as input file. The visualization of DNA alignment of the T3SS pathogenicity island was performed in Geneious 9.1.6 (Kearse et al. 2012).

Selection (dN/dS), Tajima's D, and nucleotide diversity analyses.

The T3SS pathogenicity island sequences of 15 *Acidovorax* strains were extracted and aligned using MUSCLE (Edgar 2004). dN/dS ratios (ω) were calculated using EggLib (De Mita and Siol 2012) for each gene located within the T3SS pathogenicity island. Whether the deviation from a dN/dS value of one was significant was determined for each gene's dN/dS value using Fisher's exact test.

To calculate the per nucleotide diversity, we generated a REALPHY alignment with URI9 as reference for all 13 turfgrass-infecting strains (without Sa2). For each nucleotide site, we calculated the Shannon entropy as follows.

$$D_j = \sum_{i \text{ in ATGC}} p_{ij} \log_2 p_{ij},$$

where D_j is nucleotide diversity for position j in REALPHY alignment and p_{ij} is the proportion of nucleotide i in alignment at position j .

We calculated the mean nucleotide diversity over non-overlapping 500-bp windows across the whole alignment.

Presence and absence of putative virulence factors.

The presence and absence of the genes with putative virulence functions in turfgrass-pathogenic *A. avenae* subsp. *avenae* were determined by applying BLAST (Altschul et al. 1997) to search for homologs of virulence-related genes in *Acidovorax citruli* AAC00-1 (NC_008752) and *Acidovorax avenae* ATCC19860 (NC_015138). Virulent related genes in AAC00-1 used to search with are T2SS genes *gspI* (Aave_4150-Aave_4160), *gsp2* (Aave_0916-Aave_0926), endoglucanase gene (Aave_2102), and flagella genes (Aave_4383-Aave_4403). Virulent related genes in ATCC19860 used to search with are T4SS (Acav_0967-0971), T6SS (Acav_1504-1521), VgrG proteins (Acav_0298, Acav_0662, Acav_2399, Acav_3111, Acav_3369, Acav_3676, Acav_3724, Acav_1905), and T3SS (Acav_0510-0543). Putative T3 effectors in turfgrass-pathogenic *A. avenae* subsp. *avenae* were identified by performing BLAST of turfgrass-pathogenic *A. avenae* subsp. *avenae* using the amino acid sequences of effectors identified in *Acidovorax citruli* and closely related species (genera *Xanthomonas*, *Pseudomonas*, and *Ralstonia*). BLAST was performed using Geneious 9.1.6. The amino acid sequences of Acav_0510-Acav_0543 of the turfgrass pathogen URI9 were searched in the EffectiveDB using default parameters for the T3 secretion prediction (Eichinger et al. 2016). Default prediction settings were used (Effective T3: minimal score = 0.9999, 2.0.1 model; Effective ELD [based on eukaryotic-like domains] minimal score = 4). Only predictions with high confidences were included in the results.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

FastQC: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>
 GENCONV program: <http://www.math.wustl.edu/~sawyer/geneconv>
 'geosphere' package in R: <https://cran.r-project.org/web/packages/geosphere/geosphere.pdf>
 MUMmer 3: <http://mummer.sourceforge.net>
 PAMDB Acidovorax MLST database at (http://genome.ppps.vt.edu/cgi-bin/MLST/search_alleles.pl)
 Plotly website: <https://plot.ly>
 REALPHY online tool: <http://realphy.unibas.ch>