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Multilocus Sequence Analysis highlights genetic diversity of *Acidovorax avenae* strains associated with sugarcane red stripe

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Abstract Pathogenic species of *Acidovorax* cause economically important diseases in monocotyledonous and dicotyledonous crops, including sugarcane, corn, rice, oats, millet, foxtail, watermelon and orchids. Sugarcane red stripe, caused by *Acidovorax avenae*, is present in the main production areas around the world. In Argentina, red stripe affects about 30% of stalks milled with important economic losses when severe infections occur. MLST was used to explore the genetic diversity of this bacterium associated with red stripe in Argentina, as well as their phylogenetic relationships. The MLST analysis included sequences from a total of 118 *Acidovorax*, 15 *A. avenae* strains isolated from Argentina sugarcane production areas, *A. citrulli* (93) from melon and watermelon, *A. avenae* (9) from rice, millet, corn, vasey grass and sorghum, and *A. oryzae* (1) from rice. MLST analysis revealed five novel sequence types (STs) for the sugarcane *A. avenae* strains, constituting a clonal complex with a common and close origin. When genetic relationships with other *Acidovorax* were explored, sugarcane strains were related to *A. avenae* from other hosts and more distantly to *A. citrulli*. Signals of frequent recombination in several lineages of *A. avenae* were detected and we observed that *A. oryzae* is closely related to *A. avenae* strains. This study provides valuable data in the field of epiphytological and evolutionary investigations of *A. avenae* strains causing sugarcane red stripe. Knowledge of the genetic diversity and host-strain specificity are important to select the genotypes with the best response to red stripe disease.

Key words Sugarcane, *Acidovorax*, genetic relationship, diversity, virulence, host strain

INTRODUCTION

Phytopathogenic *Acidovorax* species are currently not clearly classified. Three subspecies having different host ranges have been described for *A. avenae* (Willems and Gillis 2015): *A. avenae* subsp. *citrulli* infects Cucurbitaceae family members; *A. avenae* subsp. *cattleyae* infects only *Cattleya* and *Phalaenopsis* species; and *A. avenae* subsp. *avenae* infects Poaceae family members, including maize, rice, sorghum, corn, oats, barley, rye, various millets, vasey grass and sugarcane (Martin and Wismer 1989; Song *et al.* 2003). However, several authors adopted the reclassification up to species level proposed formerly by Schaad *et al.* (2008) as *A. avenae*, *A. cattleyae*, *A. citrulli* and *A. oryzae* sp. nov. (for rice isolates).

A. avenae, pathogenic for different monocotyledonous and dicotyledonous plants (Giordano *et al.* 2012), was reported as the causal agent of an infective outbreak of red stripe in sugarcane in Argentina by Fontana *et al.* (2013). In addition, the whole genome sequence of a virulent strain, *A. avenae* T10_60 for sugarcane, has been recently published (Fontana *et al.* 2016). The ability to accurately identify and differentiate *Acidovorax* pathogenic strains causing disease is of critical importance for epiphytological surveillance and for designing efficient crop-management procedures. The development of molecular typing methods based on sequence analysis such as

MLST (Multilocus Sequence Typing) has introduced valuable information for epiphytological investigation of these bacterial pathogens (Feng *et al.* 2009; Yan *et al.* 2013; Silva *et al.* 2016).

Here, we applied the MLST approach to explore the genetic diversity among *A. avenae* strains from sugarcane associated with red stripe disease and to understand phylogenetic relationships with other *Acidovorax* strains from different hosts and geographical origins. To investigate the relationship between the genetic diversity and severity levels of *A. avenae* strains, we also performed virulence tests.

MATERIALS AND METHODS

Bacterial strains used in this study

A. avenae strains used in this study, along with their host sugarcane genotype and cultivation region, are shown in Table 1. All strains were isolated from sugarcane infected with red stripe. Lysogeny broth (Bertani 2004) was used for bacterial replication, overnight at 30ºC, in a shaking incubator.

MLST analysis

PCR amplification and sequencing

The MLST scheme described by Feng *et al.* (2009) was used in this study. For the PCR, total genomic DNA from each *A. avenae* strain was extracted and purified according to the CTAB method described by Ausubel *et al.* (1992). The bacterial DNA was quantified with Qubit® (Invitrogen, Argentina), visualized by electrophoresis through 0.7% (w/v) agarose gel and stained with Gel Red (Genbiotech, Argentina). PCR amplifications were carried out in a final volume of 25 µL containing 1x Master Mix PCR (Promega, Italy), 0.8-1.0 µM of each primer and 10-20 ng of sample DNA. Reaction conditions included an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s for primer annealing, and an extension step at 72°C for 30 s. The final step was an extension period at 72°C for 5 min. ExoSap-IT Clean-up system (USB Co., Cleveland). was used for PCR products. Sequencing with forward and reverse primers was performed in a 3130xl Genetic Analyzer (INTA Castelar, Buenos Aires, Argentina).

MLST data analysis

Besides the 15 isolated strains from sugarcane, sequences of seven housekeeping genes downloaded from GenBank for strains of *A. citrulli* (93), *A. oryzae* (1) and *A. avenae* (9) from other hosts, were included in the MLST analysis. Sequences for the housekeeping gene are available under GenBank accession numbers MF623064 to MF623168 and EU928004 to EU928726 for sugarcane *Acidovorax* strains and other hosts, respectively. Allelic profiles for each strain were calculated using MLSTest software (Tomasini *et al.* 2013). A BURST analysis (Feil *et al.* 2004) using MLSTest to identify clonal complexes with a group definition of at least six shared alleles was performed (Tomasini *et al.* 2013). In addition, a Neighbor-joining (NJ) tree, with different node support measures, was built with MLSTest. Consensus trees summarizing the information of individual fragment trees (based on branch frequency into the NJ tree for each locus) were also assembled. Topological incongruence between locus trees and consensus networks were calculated by MLSTest to determine recombination into the *Acidovorax* species and the statistical significance was evaluated using the Templeton test (Tomasini *et al.* 2013).

Virulence assays

The virulence of sugarcane *A. avenae* strains representing the five ST determined by MLST analysis was evaluated on a susceptible sugarcane cultivar TucCP 77-42. *A. avenae* strains T10_61, S11_3, S22_3, SF17_4 and SF18_1 (ST5, ST1, ST4, ST2 and ST3, respectively) were used to inoculate young plants (less than 2 months). *A. avenae* T10_61 (Fontana *et al.* 2016), was also used as virulent positive control. Inoculum was prepared from a pure bacterial culture grown on Lysogeny broth on shaking incubator for 48 h at 30°C. Pathogenicity assay was carried out as previously described by Fontana *et al.* (2013). A total of 20 biological replicates (potted plants) were assessed for each treatment and the experiment was carried out once. A completely randomized experimental design was used.

Red stripe occurrence on leaves from seedlings was evaluated every day up to 10 days post-inoculation (dpi). The severity was evaluated once on 10 dpi as follows: 0 = no symptom; 1 = localized infection and less than three red stripes per leaf; 2 = advanced infection and more than three red stripes per leaf; 3 = severe infection with red stripe that reaches the apical bud; 4 = apical top rot and/or death of the apical top. This scale was developed by Fontana (2010) based on a similar scale described by Rott *et al.* (1994) with minor modifications and adapted to the red stripe disease characteristics. Data was used to calculate the mean severity for each plant. One-way analysis of variance (ANOVA) was performed for severity data analysis using the InfoStat software (Di Renzo *et al.* 2018).

Table 1. Sample description and strains used in this study.

Strain	Sugarcane genotype	Cultivation region	Province	Date	Reference
T4_53	INTA NA 89-686	La Trinidad-south	Tucumán	2008	Fontana *et al.* 2013
T6_50	INTA NA 91-209	Cruz Alta-central	Tucumán	2008	Fontana *et al.* 2013
T8_45	TucCP 77-42	Las Piedritas-central	Tucumán	2008	Fontana *et al.* 2013
T10_61	INTA NA 89-686	Famaillá-central	Tucumán	2008	Fontana *et al.* 2013
S11_3	NA 85-1602	Colonia Santa Rosa	Salta	2008	Fontana *et al.* 2013
SF17_1; SF17_2; SF17_3; SF17_4; SF17_5; SF17_6 SF17_7	NA 85-1602	Tacuarendí	Santa Fe	2013	Fontana *et al.* 2018
SF18_1	NA 85-1602	Tacuarendí	Santa Fe	2014	Fontana *et al.* 2018
SF19_1_SF19_2 SF19_3; SF19_4	INTA 04-1604	Tacuarendí	Santa Fe	2014	Fontana *et al.* 2018
SF20_1; SF20_2 SF20_3; SF20_4	INTA CP 98-828	Villa Ocampo	Santa Fe	2014	Fontana *et al.* 2018
SF21_1; SF21_2 SF21_3; SF21_4 SF21_5	Unknown	Las Toscas	Santa Fe	2014	Fontana *et al.* 2018
S22_1; S22_2 S22_3:S22_4	NA 02-2320	Tabacal	Salta	2014	Fontana *et al.* 2018
M23_1; M23_2; M23_3; M23_4	Unknown	San Javier	Misiones	2014	Fontana *et al.* 2018

RESULTS

Clonal and host-associated origin for strains from sugarcane

Five Sequence Types (ST1 to ST5), not previously described, were defined among the 15 *A. avenae* strains from sugarcane analyzed in this study. Most of them were typed as ST1 or ST2 (each ST composed by six strains), whereas ST3, ST4 and ST5 were singletons (Figure 1). The BURST algorithm clustered such sequences in a single clonal complex meaning a common and close origin for all of them (Figure 2). In addition, the NJ-tree, to analyze the relationships with other *A. avenae* strains, showed that the sugarcane strains were clustered together and separated of other strains with a high bootstrap value and four loci supporting the split, suggesting a possible host-specificity (Figure 3). Topological incongruence between trees for each locus was not detected in these strains supporting the clonal behavior (data not shown). A Fisher exact test showed that no significant association was found between the strains analyzed and their geographic origin.

Recombination events in *A. avenae*

Sugarcane strains and *A. citrulli* conformed different clonal complexes, while other strains were not clustered together by a BURST analysis (i.e. singletons). In addition, the NJ analysis showed that such singletons were clustered in branches with low support (Figure 3) and with high and statistically significant topological incongruence. These results indicate frequent recombination among strains (Tomasini *et al.* 2014). Additional information about the recombination for *A. avenae* strains was obtained by building a consensus network (Figure 4). The network showed several square patterns indicating recombination. From the NJ-tree (Figure 3), and incongruence tests, it was possible to determine that of *A. oryzae* grouped with the *A. avenae* from rice.

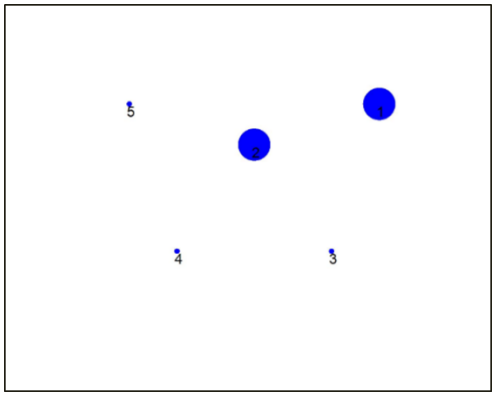

Figure 1. BURST graphic. Size of circle indicates number of strains contained in each ST for the *A. avenae* clonal complex**.**

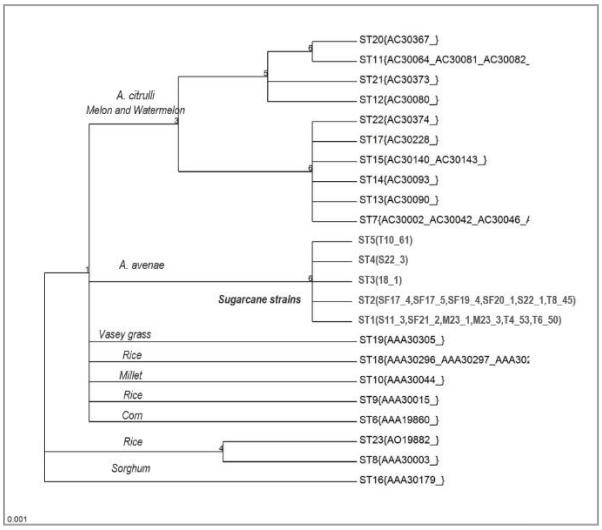

Figure 2. BURST analysis of sugarcane *A. avenae* strains showing clonal complexes.

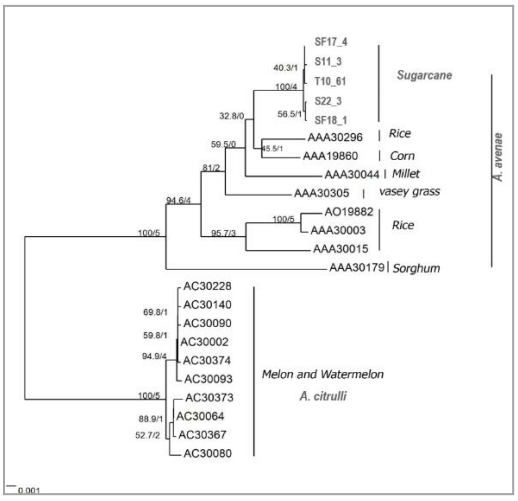

Figure 3. Neighbor-joining (NJ) tree for analyzed sugarcane strains and other *Acidovorax* strains. The tree was built based on nucleotide p-distance of seven concatenated loci. Support values (based on 1000 bootstrap replications) are shown at each branch.

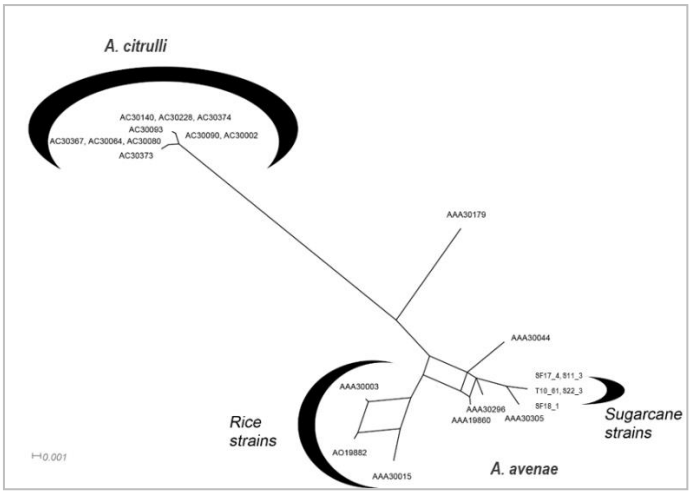

Figure 4. Consensus network of seven loci showing possible genetic exchange. Each split in the network is shown if at least two trees had such a split. Network regions with square patterns indicates probable recombination. Sugarcane *A. avenae* strains are circled on the right side.

Severity levels of *A. avenae* strains

Significant differences in the severity of symptoms were observed among strains from different STs (F=520.82; P <0.0001). Strains S22_3 and S11_3 were more virulent (mean severity ratings of 3.65 and 3.11, respectively) than strains SF17_4 and SF18_1 (mean severity ratings of 2.20 and 2.30, respectively), according to 0–4 rating scale (Figure 5). The strains S22_3 and S11_3 developed lesions on leaves considered as severe and generalized striations, affecting apical bud in some cases. Strains SF17_4 and SF18_1 exhibited an intermediate virulence developing typical red stripe lesions on leaves. The positive control, *A. avenae* T10_61, showed a lower level of symptom severity compared with the rest of the strains (mean severity ratings of 1.60). In all cases, first symptoms were observed after 48 hours of inoculation, but the severity was more evident for *A. avenae* S22_3 and S11_3 strains. Seedling death by apical bud rot (top rot) due to infection was not observed up to 10 dpi. *A. avenae* was successfully re-isolated from inoculated sugarcane leaves.

Figure 5. Severity differences of red stripe symptoms on sugarcane cultivar TucCP 77-42 used for the virulence assays. According to 0–4 rating scale: A) 0= no symptoms; B) 1 = localized infection and less than three red stripes per leaves; C) 2 = advanced infection and more than three red stripe per leaves; D) 3 = severe infection with red stripe that reaches the apical bud; E) 4 = apical top rot and/or death of the apical top.

DISCUSSION

In Argentina, in the last 15 years red stripe of sugarcane has become the most serious plant disease causing industrial losses of 30%. To manage the disease, resistant cultivars play a crucial role and several clones have been developed. Thus, knowledge on the genetic diversity of the causal agent, *A. avenae,* is an important factor to be considered for improving an accurate diagnosis and/or for the selection of tolerant sugarcane cultivars. In this study, *A. avenae* strains isolated from different sugarcane genotypes cultivated in the main crop areas were selected to explore their genetic diversity applying a MLST analysis.

The MLST defined five ST among the 15 sugarcane strains analyzed. These strains clustered together suggesting host specificity with a relatively recent origin and clonal behavior. Such host specificity in different clades of *A. avenae* was also observed for other groups (Yan *et al.* 2017). It has already been demonstrated that association of *A. avenae* is stronger with the host than with the geographical origin (Feng *et al.* 2009; Yan *et al.* 2013). In our study, *A. avenae* strains from sugarcane, were clustered separately from *A. citrulli* from watermelon and melon strains, and closer to *A. avenae* from Poaceae origin (millet, rice, corn, vasey grass and sorghum).

As in Feng *et al.* (2009), we found two clonal complexes grouping the *A. citrulli* with a clear separation from the other *A. avenae* strains and *Acidovorax* spp. In addition, Yan *et al.* 2013 reported the occurrence of 73 STs distributed into three clonal groups among 118 strains of *A. citrulli* from Chinese watermelon. Even though a new taxon for the *A. avenae* from rice, *A. oryzae*, was proposed by Schaad *et al.* (2008), we observed that *A. oryzae* is closely related to other *A. avenae* strains from rice. We also detected phylogenetic incongruence in *A. avenae* suggesting frequent recombination in some clades. Recombination between different lineages has been described for virulence genes in some *A. avenae* that share the same host (Zeng *et al.* 2017). This is relevant because new highly virulent strains may originate in such clade, where recombination is frequent (Feil *et al.* 1999). Recombination in other plant pathogens has also been reported. Timilsina *et al.* (2015) found evidence of multiple recombination events between *Xanthomonas euvesicatoria* and *X. perforans,* which indicates that there have been shifts in the species composition of bacterial spot pathogen populations due to the global spread of dominant genotypes and that recombination between species has generated genetic diversity in these populations.

It is important to highlight that despite their close relationships by MLST, sugarcane strains showed virulence differences in the virulence assays. However, this is not contradictory because virulence factors are codified by genes that mutate faster than housekeeping genes (Moxon *et al.* 1994). Consequently, there is much more relevant genetic diversity that is hidden to the MLST resolution power.

When virulence tests were performed on the red stripe susceptible sugarcane genotype TucCP 77-42, results showed, in agreement with those reported by Fontana *et al.* (2013), that red stripe symptoms developed earlier in Tucumán sugarcane variety (TucCP 77-42) inoculated with a pathogenic strain from another province.

Our study provides an invaluable platform for epiphytological and evolutionary investigations of novel clones of *A. avenae* strains. The knowledge of genetic diversity and host-strain specificity has great value in the selection of genotypes with the best response to the red stripe disease.

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L'analyse des séquences multi locus met en évidence la diversité génétique des souches d'*Acidovorax avenae* associées aux rayures rouges de la canne à sucre

Résumé. Les espèces pathogènes d'*Acidovorax* sont responsables de maladies importantes sur le plan économique dans les cultures monocotylédones et dicotylédones, notamment la canne à sucre, le maïs, le riz, l'avoine, le millet, la sétaire, la pastèque et l'orchidée. Les rayures rouges de la canne à sucre, causée par *Acidovorax avena*, est présente dans les principales zones de production du monde. En Argentine, les rayures rouges touchent environ 30% des tiges usinables, entraînant d'importantes pertes économiques en cas d'infection grave. Le MLST a été utilisé pour explorer la diversité génétique de cette bactérie associée aux rayures rouges en Argentine, ainsi que leurs relations phylo-génétiques. L'analyse MLST comprenait des séquences provenant d'un total de 118 souches *Acidovorax*, 15 souches d'*A. avenae* isolées des zones de production de canne à sucre de l'Argentine, *A. citrulli* (93) du melon et de pastèque, *A. avenae* (9) du riz, le mil, le maïs, l'herbe de vasey et le sorgho et *A. oryzae* (1) obtenue du riz. L'analyse MLST a révélé cinq nouveaux types de séquence (ST) pour les souches de canne à sucre d'*A. avenae*, constituant un complexe clonal d'origine commune et proche. Lorsque les relations génétiques avec d'autres *Acidovorax* ont été explorées, les souches provenant de la canne à sucre étaient apparentées à *A. avenae* provenant d'autres hôtes et plus lointainement à *A. citrulli*. Des signaux de recombinaison fréquente dans plusieurs lignées d'*A. avenae* ont été détectés et nous avons observé qu'*A. oryzae* est étroitement apparenté aux souches d'*A. avenae*. Cette étude fournit des données précieuses dans le domaine des études épiphytologiques et évolutives des souches d'*A. avenae* provoquant les rayures rouges de la canne à sucre. La connaissance de la diversité génétique et de la spécificité souche-hôte est importante pour sélectionner les génotypes présentant la meilleure résistance à la maladie des rayures rouges.

Mots-clés: Canne à sucre, *Acidovorax*, relation génétique, diversité, virulence, souche-hôte

El análisis de secuencias multilocus evidencia la diversidad genética de cepas *de Acidovorax avenae* asociadas con la estría roja en caña de azúcar

Resumen. Las especies fitopatógenas de *Acidovorax* causan enfermedades en cultivos tanto monocotiledóneos y dicotiledóneos, que incluyen caña de azúcar, maíz, arroz, avena, mijo, sandía y orquídeas. La estría roja de caña de azúcar, causada por *Acidovorax avenae*, está presente en las principales áreas de producción del mundo. En Argentina, esta enfermedad llegó a afectar hasta un 30% de tallos molibles en infecciones severas con importantes pérdidas económicas. Para explorar la diversidad genética de esta bacteria, así como sus relaciones filogenéticas, se aplicó un análisis MLST. El MLST incluyó un total de 118 secuencias de cepas de *Acidovorax*, 15 *A. avenae* aisladas de diferentes áreas de producción de caña de azúcar de Argentina, *A. citrulli* (93) de melón y sandía, *A. avenae* (9) de arroz, mijo, maíz, pasto vasey y sorgo y *A. oryzae* (1) de arroz. El análisis de MLST reveló cinco nuevos tipos de secuencia (ST) para las cepas de caña de azúcar *A. avenae*, que constituyen un complejo clonal con un origen común y cercano. Cuando se investigó la relación genética con otras *Acidovorax*, las cepas de caña de azúcar se mostraron cercanas con *A. avenae* de otros huéspedes, pero más distante de A. *citrulli*. Se evidenciaron señales de recombinación frecuente en varios linajes de *A. avenae* y observamos que *A. oryzae* está estrechamente relacionada con las cepas de *A. avenae*. Este estudio proporciona datos valiosos en el campo de las investigaciones epifitológicas y evolutivas de las cepas de *A. avenae* que causan estría roja en caña de azúcar. El conocimiento de la diversidad genética y la especificidad cepa-hospedante son importantes para seleccionar genotipos con la mejor respuesta frente a los biotipos más virulentos y predominantes en la región.

Palabras clave: Caña de azúcar, *Acidovorax*, relación genética, diversidad, virulencia, cepa-hospedante