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Microbial Resistance Mechanisms to the Antibiotic and Phytotoxin Fusaric Acid

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Abstract Fusaric acid (FA) produced by *Fusarium oxysporum* plays an important role in disease development in plants, including cotton. This non-specific toxin also has antibiotic effects on microorganisms. Thus, one expects a potential pool of diverse detoxification mechanisms of FA in nature. Bacteria and fungi from soils infested with *Fusarium* and from laboratory sources were evaluated for their ability to grow in the presence of FA and to alter the structure of FA into less toxic compounds. None of the bacterial strains were able to chemically modify FA. Highly FA-resistant strains were found only in Gram-negative bacteria, mainly in the genus of *Pseudomonas*. The FA resistance of the Gram-negative bacteria was positively correlated with the number of predicted genes for FA efflux pumps present in the genome. Phylogenetic analysis of predicted FA resistance proteins (FUSC, an inner membrane transporter component of the efflux pump) revealed that FUSC proteins having high sequence identities with the functionally characterized FA resistance protein FusC or Fdt might be the major contributors of FA resistance. In contrast, most fungi converted FA to less toxic compounds regardless of the level of FA resistance they exhibited. Five derivatives were detected, and the detoxification of FA involved either oxidative reactions on the butyl side chain or reductive reactions on the carboxylic acid group. The production of these metabolites from widely different phyla indicates that resistance to FA by altering its structure is highly conserved. A few FA resistant saprophytic or biocontrol strains of fungi were incapable of altering FA, indicating a possible involvement of efflux transporters. Deployment of both efflux and derivatization mechanisms may be a common feature of fungal FA resistance.

Keywords Fusaric acid · Resistance · Detoxification · Fungi · Bacteria · Antibiotic resistance · Soil microbiome · FUSC · FusC · Fdt · Efflux pump

Introduction

The fungal toxin fusaric acid (FA, Fig. 1; 5-butylpicolinic acid), which is produced by species of *Fusarium*, acts as a snon-specific toxin to plants and microbes. In plants, treatment with FA caused several negative effects to cell integrity (Gaumann 1958; Marre et al. 1993; Pavlovkin 1998; Jiao et al. 2013) and function (Kohler and Bentrup 1983; D'Alton and Etherton 1984; Marre et al. 1993; Samadi and Behboodi 2006). Other research suggests that FA can act as a virulence factor in Fusarium wilt of banana (Matsumoto et al. 1995; Dong et al. 2012), cotton (Liu et al. 2011), and tomato (Lopez-Diaz et al. 2017). *Fusarium oxysporum* f. sp. *vasinfectum* genotypes (Fov) from California and Australia produce greater than 1000 ppm of FA in culture filtrates (Liu et al. 2016a). These high FA producing genotypes and the sensitivity of cotton to FA (Gaumann 1957) are a substantial concern for cotton production. Mechanisms for resistance to Fov that target fusaric acid synthesis, transport, and metabolism have become a focus of new studies.

Soil-borne bacteria and fungi are potential sources of novel resistance to FA. Resistance to FA by efflux pumps containing FA resistance proteins (FUSC) and secondary metabolite transporters have been explored in *Klebsiella oxytoca*

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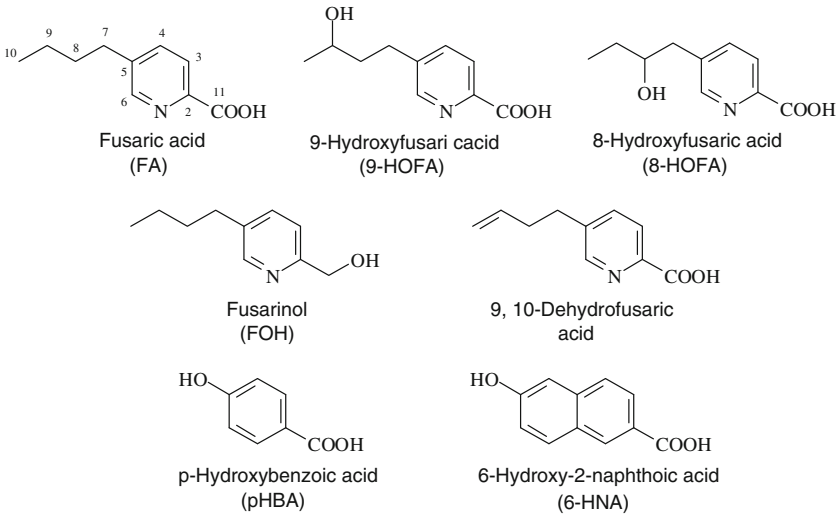

Fig. 1 Chemical structures of fusaric acid and its derivatives produced by fungal strains investigated in the present study. The structures of related aromatic carboxylic acids which are the substrates for the efflux pumps belonging to fusaric acid resistant protein family (FUSC) are also shown

(Toyoda et al. 1991), *Burkholderia cepacia* (Utsumi et al. 1991), *Stenotrophomonas maltophilia* (Hu et al. 2012) and two FA producing *Fusarium* spp. (Crutcher et al. 2015; Studt et al. 2016). Soil-borne fungi also may degrade or alter FA into less toxic forms (Fakhouri et al. 2003; Crutcher et al. 2014; Crutcher et al. 2017).

Several derivatives of FA have been identified and tested for phytotoxicity. The first derivatives detected in FA producing *Fusarium* cultures were 9-hydroxyfusaric acid (9-HOFA, Fig. 1), also known as fusarinolic acid (Braun 1960), and 9,10-dehydrofusaric acid (9,10-DHFA, Fig. 1) (Gaumann 1957). Phytotoxicity of 9-HOFA was significantly lower ($p < 0.05$) than that of FA (Stipanovic et al. 2011a). Other derivatives include fusaric acid methyl ester (Capasso et al. 1996), 9,10-dihydroxyfusaric acid methyl ester, 10-carboxyfusaric acid methyl ester (Burmeister et al. 1985), 3-butylpyridine (Vischetti and Esposito 1999), fusarinol (FOH, Fig. 1) (Crutcher et al. 2014), 8-hydroxyfusaric acid (8-HOFA, Fig. 1) (Crutcher et al. 2017) and several others (Stipanovic et al. 2011a; Liu et al. 2016b).

In the case of Fusarium wilt of cotton, resistance to phytotoxins produced by the pathogen may provide disease control if incorporated into the host (Bell et al. 2003). FA detoxifying microorganisms are a potential source of such novel resistance. To identify new mechanisms of FA resistance, we screened soils from three *Fusarium*-infested cotton fields and isolated several microorganisms with high resistance to FA. Using HPLC, these microbes and those from available laboratory collections were evaluated for their ability to alter FA into potentially less phytotoxic derivatives.

Materials and Methods

Isolation of FA Resistant Microorganisms from Soil Soil collected from cotton fields infested with reniform nematode and *Fusarium* near Baton Rouge, LA and Weslaco, TX were obtained by A.F. Robinson (USDA ARS College Station, TX, retired) and maintained under greenhouse conditions with an alternating tomato-cotton rotation. Soil from Tallassee, AL was collected from a cotton disease evaluation field. One gram of soil was suspended in 10 ml of distilled water and dilutions of 10^{-2}, 10^{-3}, and 10^{-4} were made. Each dilution was spread on potato dextrose agar (PDA; Difco Laboratories, Detroit) plates for fungi and Luria-Bertani agar (LBA; Sigma-Aldrich, St. Louis) plates for bacteria, both containing 100 μg/ml FA. Individual colonies of bacteria or fungi were transferred to slants containing 500 μg/ml FA. Isolates that grew on the higher concentration of FA were retained for further analysis.

Identification of FA Resistant Isolates from Soil Eight bacterial isolates from soil capable of growing on slants containing 500 μg/ml FA were selected for further testing and identification. *Pseudomonas* spp. are common soil inhabitants (Fulthorpe et al. 2008) and provide biocontrol of *Fusarium* and detoxification of FA (Duffy and Defago 1997; Ruiz et al. 2015; Utsumi et al. 1991). Therefore, these bacterial isolates were screened for fluorescence on King's B and King's A media (King et al. 1954) and subjected to Gram stain. To further identify these isolates, the biochemical activities of the bacteria were determined with an API 20 E strip (bioMerieux, Inc., Durham).

Fungal isolates were evaluated microscopically for reproductive structures to identify the strains to genus and partial genes were sequenced to identify strains to species. *Aspergillus tubingensis* was previously sequenced and identified (Crutcher et al. 2014). For *Fusarium oxysporum*, the *Fusarium oxysporum* specific primer pairs, EF-1A and EF1885R, PHO48F and PHO2034, BT100F and BT1828R, were used to amplify and sequence the elongation factor gene, the phosphate permease gene and beta tubulin (BT) gene, respectively (Ortiz et al. 2017). Other primers used include the BT primers BT2a and BT2b (Glass and Donaldson 1995), the internal transcribed spacer region (ITS) primers

ITS4 and ITS5 (White et al. 1990) and calmodulin (CL) primers CL1 and CL2 (Serra and Peterson 2007). *Aspergillus flavus* and *Penicillium chrysogenum* were identified by ITS and BT, *Aspergillus terreus* by BT and CL, *Rhizopus microsporus* by ITS, and *Talaromyces pinophilus* by CL sequences.

Resistance Measurements for Bacterial and Fungal Isolates The FA resistant bacterial isolates from field soil, together with selected isolates from laboratory collections and a yeast isolate (Table 1), were evaluated for their resistance to FA by the agar dilution method (Masuda 1976). Bacterial suspensions were plated on tryptic soy agar (TSA; Sigma-Aldrich, St. Louis) containing 0, 50, 100, 200, 300, 400, or 500 μg/ml FA to determine the minimum inhibitory concentration (MIC) by calculating the lowest concentration of FA in which bacterial growth was inhibited. The yeast isolate that grew as single cells was evaluated in the same manner except *Schizophyllum* medium (glucose 20 g/l, potassium phosphate monobasic 0.46 g/l, potassium phosphate dibasic 1.28 g/l, magnesium sulfate 0.5 g/l, yeast extract 2 g/l and peptone 2 g/l, Bacto agar 20 g/l) was used. The tests were replicated three times.

The concentration of FA required to inhibit growth by 50% (IC_{50}) was measured for each fungal strain. Three mm diameter plugs from the edge of active colonies on PDA were transferred to the center of PDA plates containing 0, 50, 100, 200, 300, 400, or 500 μg/ml FA. At 24 to 96 h, depending on the growth rate of the specific fungus, the colony radius was measured in two perpendicular directions. Four replications were used for each FA concentration. The radial measurements were regressed against FA concentrations and the IC_{50} was calculated using the resulting regression equation. For all the fungal isolates, the R^2 value for growth inhibition by FA was more than 0.84 with the majority of the values above 0.95 (data not shown).

Table 1 Minimum inhibitory concentrations of fusaric acid to bacterial and yeast isolates and number of fusaric acid resistance FUSC proteins in the genome

Strain	Cell type	MIC (μg/ml)[a]	No. of FUSC[b]	Source[c]
Acidovorax avenae	G- bacillus	<50	1	DCG, Georgia
Agrobacterium radiobacter ATCC49644	G- bacillus	<50	1	PLPM, Australia
Burkholderia cepacia	G- bacillus	(500–800)	6	(Ouchi et al. 1989)
Burkholderia glumae 618	G- bacillus	200	2	PLPM, rice, Texas
Enterobacter cloacae ATCC13047	G- bacillus	100	2	RBM, spinal fluid
Erwinia amylovora 2029	G- bacillus	<50	0	DCG, pear
Klebsiella oxytoca ATCC8724	G- bacillus	200	2	ATCC
Pseudomonas aeruginosa CDRpa1	G- bacillus	300	7	Baton Rouge soil
Pseudomonas aeruginosa CDRpa2	G- bacillus	nd	7	Baton Rouge soil
Pseudomonas aeruginosa CDRpa3	G- bacillus	nd	7	Baton Rouge soil
Pseudomonas aeruginosa CDRpa4	G- bacillus	nd	7	Baton Rouge soil
Pseudomonas fluorescens CDRpf1	G- bacillus	400	4	Baton Rouge soil
Pseudomonas fluorescens CDRpf2	G- bacillus	300	4	Baton Rouge soil
Pseudomonas fluorescens CDRpf3	G- bacillus	200	4	Baton Rouge soil
Pseudomonas fluorescens CDRpf4	G- bacillus	200	4	Baton Rouge soil
Pseudomonas fluorescens Pf-5	G- bacillus	(1250)	4	(Ruiz et al. 2015)
Pseudomonas marginalis ATCC10844	G- bacillus	>500	2	DCG, plant derived foodstuff
Pseudomonas putida A514	G- bacillus	500	3	DCG, biocontrol, fruit tree
Pseudomonas syringae pv. *syringae B728a*	G- bacillus	400	2	DCG, bean, Wisconsin
Serratia marcescens ATCC13880	G- bacillus	100	3	RBM, pond water
Stenotrophomonas maltophilia	G- bacillus	(512)	2	(Hu et al. 2012)
Xanthomonas citri pv. *malvacearum*	G- bacillus	<50	1	PLPM, cotton, Texas
Bacillus cereus ATCC14579	G+ bacillus	<50	0	RBM
Bacillus subtillis ATCC6051	G+ bacillus	<50	0	RBM, Marburg, Germany
Corynebacterium flavescens ATCC10340	G+ bacillus	<50	0	RBM, dairy products, cheese
Curtobacterium flaccumfaciens	G+ bacillus	200	0	DCG, bean, Nebraska
Lysteria grayi ATCC19120	G+ bacillus	100	0	RBM, animal feces
Micrococcus luteus	G+ coccus	<50	0	RBM
Micrococcus lysodeikticus	G+ coccus	<50	0	RBM
Micrococcus roseus	G+ coccus	<50	0	RBM
Staphylococcus epidermidis ATCC14900	G+ coccus	<50	0	RBM, nose
Streptococcus mutans ATCC25175	G+ coccus	<50	0	RBM, carious dentine
Candida albicans	Yeast	100	0	RBM

[a] MIC concentrations in the parentheses are from cited references; nd, not determined

[b] Number of predicted FUSC genes in the genome

[c] Strains were obtained from soil collected in Baton Rouge, LA, American Type Culture Collection (ATCC), Department of Plant Pathology and Microbiology, Texas A&M University (PLPM), Dr. Dennis C. Gross, Department of Plant Pathology and Microbiology, Texas A&M University (DCG), or Dr. Rita B Moyes, Department of Biology, Texas A&M University (RBM)

FA Modification by Bacterial and Fungal Strains To determine if chemical conversion of FA or another unidentified method was the primary mechanism of resistance, cultures of all bacterial strains and fungal strains that grew in the presence of 50 μg/ml or more of FA were analyzed by HPLC. A single bacterial colony was inoculated into 2 ml of M9 medium (Sigma-Aldrich, St. Louis) containing FA at half of MIC concentration of the microorganism. Cultures were incubated at 27 °C and 200 rpm. After 72 h, cultures were collected and bacteria were spun down in a centrifuge at 10,000 rpm for 1 min. Culture filtrate samples (100 μl) were collected for HPLC analysis. *Pseudomonas* spp., unable to grow in M9, were grown in King's B medium and analyzed.

Fungal isolates were grown on PDA and two 3 mm plugs from the actively growing edge of the colony were used to inoculate 10 ml of PDB containing approximately the IC_{50} FA concentration of the fungal isolate. After 96 h, cultures were centrifuged and 100 μl of three biological replicates were collected for HPLC analysis.

HPLC Analyses of Culture Filtrates Culture filtrate was subjected to analysis using a computer-controlled Agilent Technologies HPLC instrument (Waldbronn, Germany) equipped with a model 1200 solvent degasser, 1200 quaternary pump, 1100 autosampler, 1100 diode array detector (DAD) and Rev.B.04.02 ChemStation-3D software. The analysis method used an Agilent Zorbax Eclipse XDB-C18 (5 μm, 4.6 mm × 150 mm) column and an isocratic mobile phase of 15% acetonitrile (ACN) and 85% H_2O (both containing 0.2% formic acid) run at 0.80 ml/min for 15 min. The chromatogram signal was monitored at 275 nm (bandwidth = 20 nm) referenced to 550 nm (bandwidth = 100 nm). Spectra were collected over 190–600 nm. The injection volume used was 5.0 μl. Using this method, FA appeared at 5.0 min. Pure FA (Stipanovic et al. 2011a), 9-HOFA (Stipanovic et al. 2011a), and FOH (Crutcher et al. 2014) were used as standards for identification and quantification. The quantification calibration curves for the three compounds were almost identical. Pure 8-HOFA (Crutcher et al. 2017) and 9,10-DHFA (Stipanovic et al. 2011b) were used as standards for identification via retention time and UV-Vis spectra. These two compounds and an unknown compound with a retention time of 3.2 min had UV-Vis spectra similar to that of FA (see Results section). Therefore, the three compounds were quantified using the FA quantification curve.

Bioinformatics Analysis Sequences for functionally characterized fusaric acid resistant proteins FusC, Fdt-2 (Fdt), and FuaA (Toyoda et al. 1991; Utsumi et al. 1991; Hu et al. 2012) were used as query to BLAST probe the GenBank database (https://www.ncbi.nlm.nih.gov/genome) to identify FUSC proteins for the bacterial genomes listed in Table 1. Only those sequences retrieved at an E-value lower than 0.05 were taken into account. Other proteins such as transcription regulators, membrane fusion proteins (MFP), outer membrane factors (OMF), and membrane associated DUF1656 proteins residing in the same operon with the identified FUSC proteins were identified by conducting the Conserved Domain search with the retrieved respective sequences at NCBI (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Prediction of transmembrane regions for the FUSC and DUF1656 proteins was accomplished using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). A phylogenetic tree of predicted FUSC proteins was constructed using the Neighbor-Joining method implemented in the phylogenetic analysis program of MEGA 6.0 (Tamura et al. 2007). Kimura's two-parameter distance option and pair-wise deletion of gap option were used. Statistical support for the derived tree was obtained by running 1000 bootstrap replicates.

The relationship between the FA resistance and the number of FUSC proteins in the genome for Gram-negative bacteria was modeled using a linear regression approach (PROC REG, SAS version 9.4, SAS Institute Inc., Cary, NC). The log MIC value was treated as the outcome variable and the number of FUSC proteins as the regressor variable.

Results

Identification of FA Resistant Isolates FA resistant bacterial isolates from soil were screened for fluorescence on King's B medium, which enhances the production of the fluorescent secondary metabolite pyoverdine produced by *Pseudomonas* (King et al. 1954). All eight isolates fluoresced under UV light. To determine if any of these eight were *Pseudomonas aeruginosa*, each were plated on King's A media. *Pseudomonas aeruginosa* will fluoresce blue on this medium due to the production of pyocyanin. Four strains were positive for fluorescence, and therefore identified as *Pseudomonas aeruginosa*. The remaining four isolates were subjected to Gram stain and all were confirmed as small Gram-negative bacilli. The API 20 E strip identified these isolates as *Pseudomonas fluorescens*. The six fungal isolates were identified by microscopic observation of reproductive structures and sequencing of the ITS, beta-tubulin, calmodulin, elongation factor, or phosphate permease genes as *Aspergillus flavus*, *Aspergillus terreus*, *Fusarium oxysporum*, *Penicillium chrysogenum*, *Talaromyces pinophilus,* and *Rhizopus microsporus* (GenBank accession numbers: MF197729-MF197739).

FA Resistance of Bacterial Strains Highly resistant strains with MIC values greater than 300 μg/ml of FA, 9 in total, were found only in species of *Burkholderia, Pseudomonas*, and *Stenotrophomonas*, which are Gram-negative bacilli (Table 1). Only four out of 22 tested Gram-negative bacilli strains were unable to grow in 50 μg/ml of FA. In contrast, only two strains

with MIC values of 200 and 100 were found among the 5 tested species of Gram-positive bacilli, and other strains were unable to grow in the presence of 50 μg/ml of FA. None of five strains with cocci morphology grew at 50 μg/ml FA.

The capacity to modify FA by bacterial strains that were able to survive in 50 μg/ml FA was evaluated by HPLC analysis of filtrates from cultures containing FA at 50% of the MIC concentration. No change in the concentration of FA in the culture filtrate of any of the strains was observed and no FA derivatives were detected. These observations prompted the search for known fusaric acid resistant proteins in the bacterial species' genome that constitute a component of an efflux pump and belong to the FUSC family (Toyoda et al. 1991; Utsumi et al. 1991; Hu et al. 2012) (Table 1). Genes coding for FUSC proteins are present only in the Gram-negative bacteria with copy numbers ranging from 0 to 7 and are dispersed discretely in the chromosome(s). High copy numbers of FUSC generally corresponded with high FA resistance of the bacterium. Regression analysis of log MIC against copy number of FUSC in the corresponding genome among the tested Gram-negative bacteria showed the trend was significant ($P = 0.014$) with the regression equation, log MIC = 1.88 + 0.13 x No. of FUSC with R^2 of 0.36. None of the strains that were incapable of growing at 50 μg/ml of FA had more than one copy of FUSC.

Sequence comparisons of functionally characterized FUSC proteins, FusC in *Burkholderia cepacia*, Fdt in *Klebsiella oxytoca*, FuaA in *Stenotrophomonas maltophilia,* revealed identities of 21%, 24%, 41% between FusC and Fdt, FusC and FuaA, Fdt and FuaA, respectively. The FUSC proteins that had the highest sequence identity with FusC, Fdt, and FuaA were identified in each Gram-negative bacterial genome, labeled correspondingly with FusC, Fdt and FuaA designations, and evaluated for their phylogenetic relationships (Fig. 2). When two or three of the designated entries corresponded to the same FUSC protein, only the designation with highest identity was retained. The entry with the highest identity score among the three FusC, Fdt and FuaA designations is indicated in bold in Fig. 2. These FUSC proteins clustered either in clade III (FusC) or clade IV (Fdt and FuaA) and are further labeled in italic, except the three FUSC proteins (bold non-italic) from species that failed to grow in 50 μg/ml FA which clustered in clade V with other FUSC proteins. FUSC proteins that were annotated as aromatic carboxylic acid efflux pumps (AaeB) were included as

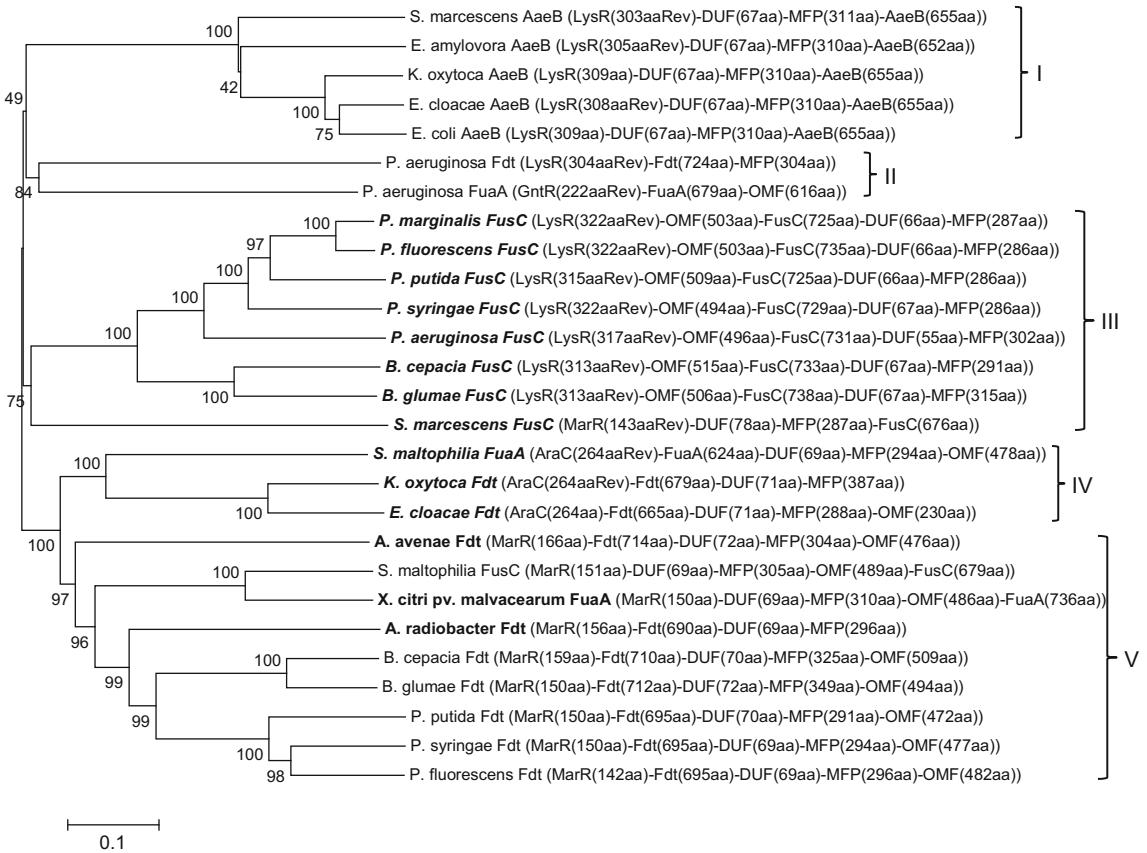

Fig. 2 Phylogenetic tree of predicted fusaric acid resistant proteins (FUSC) of Gram-negative bacteria. The tree was constructed using Neighbor-Joining method with Kimura's two-parameter distance option and pair-wise deletion of gap option. Statistical support for the derived tree was obtained by running 1000 bootstrap replicates. The composition of the operon containing FUSC protein was given in the entry's label and the size in amino acid sequence length are given in parentheses. When the transcription factor is reversely oriented relative to other members of the operon, it is indicated by 'Rev' following the size designation

references (Van Dyk et al. 2004) and were clustered in clade I. These proteins are effective efflux transporters of p-hydroxybenzoic acid (p-HBA, Fig. 1) and 6-hydroxy-2-naphthoic acid (6-HNA, Fig. 1). The remaining FUSC proteins were clustered in clade V, except the *Pseudomonas aeruginosa* Fdt and FuaA proteins which formed clade II.

Most of the operons containing FUSC proteins consisted of a transcription factor in the family of LysR, GntR, MarR, or AraC, an inner membrane efflux pump FUSC protein (652-735aa), a putative inner membrane efflux pump associated DUF1656 protein (DUF, 55-78aa), a membrane fusion protein (MFP, 286-387aa), and an outer membrane factor (OMF) containing double OEP domains (476-616aa) or a single OEP domain (230aa). Prediction of transmembrane (TM) regions by TMHMM Server v. 2.0 (Krogh et al. 2001) revealed 9 to 12 TMs with consensus of 10 TMs for FUSC proteins and 2 TMs for DUF1656 proteins. Absence of DUF, MFP, or OMF in an operon occurs occasionally with OMF being the most frequent. Members of clad I had a synteny structure of LysR (forward or reverse)-DUF-MFP-AaeB; clade IV, AraC (forward or reverse)-Fdt (or FuaA)-DUF-MFP-OMF; and clade III, LysR (Reverse)-OMF-FusC-DUF-MFP except *Serratia marcescens* which had MarR (reverse)-DUF-MFP-FusC. The majority of clade V members had a synteny structure of MarR-Fdt-DUF-MFP-OMF.

FA Resistance and Detoxification Products of Fungal Isolates Only two of the Ascomycetes, *Verticillium dahliae* and *Phymatotrichopsis omnivorum,* and both of the Oomycetes tested were unable to grow at 50 μg/ml of FA. The other isolates ranged in IC_{50} from 45 to 533 μg/ml, with *Aspergillus tubingensis* having the highest IC_{50} (Table 2). Ascomycetes had the greatest resistance to FA with IC_{50} values greater than 200 μg/ml, except for *Colletotrichum graminicola*, *Sclerotinia minor,* and *Trichoderma reesei*. The two Zygomycetes evaluated, *Mucor rouxii* and *Rhizopus microsporus*, grew in higher concentrations of FA than did the Basidiomycetes which had IC_{50} values of 100 μg/ml or less.

Five derivatives of FA were observed in culture filtrates of fungal isolates when exogenous FA was added to the growth media (Table 3 and Fig. 1). 8, 9-DHFA was produced only by the two strains of *Fusarium oxysporum* with and without the presence of exogenous FA. The concentration of 8, 9-DHFA was higher when FA was added than the negative control (data

Table 2 FA concentrations inhibiting mycelium growth by 50% (IC_{50}) for fungal species

Species	Phylum	IC_{50} (μg/ml)	Source[a]
Aspergillus flavus CDRaf1	Ascomycete	303	Weslaco
Aspergillus terreus CDRat2	Ascomycete	432	Weslaco
Aspergillus tubingensis CDRat1	Ascomycete	533	Baton Rouge
Colletotrichum graminicola	Ascomycete	56	CMK
Fusarium oxysporum CDR2047	Ascomycete	381	Tallassee
Fusarium oxysporum CA9	Ascomycete	400	(Kim et al. 2005)
Penicillium chrysogenum CDRpc1	Ascomycete	200	Baton Rouge
Penicillium roqueforti	Ascomycete	346	PLPM
Phymatotrichopsis omnivorum	Ascomycete	<50	CMK
Sclerotinia minor	Ascomycete	154	CMK
Talaromyces pinophilus CDRtp1	Ascomycete	365	Weslaco
Trichoderma harzianum YF	Ascomycete	238	CMK
Trichoderma reesei 6	Ascomycete	108	CMK
Trichoderma virens Gv29–8	Ascomycete	306	CMK
Verticillium dahliae V76	Ascomycete	<50	(Bolek et al. 2005)
Phanerochaete chrysosporium	Basidiomycete	45	PLPM
Rhizoctonia solani J1	Basidiomycete	74	CMK
Schizophyllum commune	Basidiomycete	75	PLPM
Ustilago maydis	Basidiomycete	102	CMK
Phytophthora nicotianae	Oomycete	<50	VA
Pythium ultimum	Oomycete	<50	CMK
Mucor rouxii	Zygomycete	269	PLPM
Rhizopus microsporus CDRrm1	Zygomycete	107	Weslaco

[a] Strains were obtained from Dr. Charles M. Kenerly, Department of Plant Pathology and Microbiology, Texas A&M University (CMK), Dr. Veronica Ancona, Texas A&M University-Kingsville Citrus Center (VA), the Department of Plant Pathology and Microbiology, Texas A&M University (PLPM), or from soil collected in Baton Rouge, LA, Tallassee, Al, and Weslaco, TX

Table 3 Concentrations of detoxification products of fusaric acid by fungal species as analyzed by HPLC

Species	FA added	9-HOFA (2.1 min)	8-HOFA (2.3 min)	Unknown (3.2 min)	FOH (3.9 min)	9,10-DHFA (4.2 min)	FA (4.9 min)
Aspergillus flavus	220	93.7 ± 2.6					130.7 ± 6.0
Aspergillus terreus	325	113.0 ± 9.8	109.6 ± 8.3				123.8 ± 11.5
Aspergillus tubingensis[a]	426	20.5 ± 1.0			175.8 ± 32.0		246.8 ± 25.3
Colletotrichum graminicola	52				53.0 ± 2.9		
Fusarium oxysporum	300	128.7 ± 5.7	40.0 ± 4.4			87.8 ± 1.9	48.6 ± 14.2
Fusarium oxysporum CA9[b]	325	193.6 ± 3.9	53.6 ± 3.1			137.1 ± 5.9	162.7 ± 13.3
Penicillium chrysogenum	180	30.2 ± 2.2	94.8 ± 3.1				57.4 ± 6.9
Penicillium roqueforti	297						272.9 ± 7.4
Sclerotinia minor	83			57.3 ± 2.4	26.0 ± 1.4		
Talaromyces pinophilus	280				103.2 ± 20.7		187.1 ± 30.1
Trichoderma harzianum	188						162.3 ± 3.2
Trichoderma reesei	84	10.6 ± 1.0			72.5 ± 2.7		
Trichoderma virens	220						230.6 ± 6.4
Phanerochaete chrysosporium	56				52.5 ± 1.6		
Rhizoctonia solani	80	26.6 ± 0.7	47.5 ± 0.7				
Schizophyllum commune[b]	70				23.8 ± 3.4		
Ustilago maydis	109				109.5 ± 1.8		
Mucor rouxii	220	46.0 ± 19.5	75.1 ± 20.6				90.4 ± 20.0
Rhizopus microsporus	100				103.8 ± 3.5		

Concentrations were expressed in μg/ml; times in the parentheses refer to the compounds elution times during HPLC analysis of the culture filtrate; FA: fusaric acid; 9-HOFA: 9-hydroxyfusaric acid, also known as fusarinolic acid; 8-HOFA, 8-hydroxyfusaric acid; FOH: fusarinol; 9, 10-DHFA: 9, 10-dehydrofusaric acid

[a] 8-HOFA was produced in cultures of *Aspergillus tubingensis* containing 200 μg/ml exogenous FA

[b] The sum of the FA derivative concentrations produced by *Fusarium oxysporum* CA9 was greater than the concentration of FA added; The sum of the FA derivative concentrations produced by *Schizophyllum commune* was less than the concentration of FA added

not shown). 9-HOFA and 8-HOFA were produced by a number of fungi in all the phyla represented. Three out of nine isolates produced 9-HOFA but not 8-HOFA. In contrast, FOH was produced by species that failed to produce both 9- and 8-HOFA, or 8-HOFA among species that were able to convert FA, except for *Aspergillus flavus*. *Sclerotinia minor* produced a novel peak at 3.2 min (Fig. 3). The UV-Vis spectrum of the unknown was similar to that of FA indicating that it was closely related to FA in structure (Fig. 3, inset).

Penicillium roqueforti, Trichoderma harzianum, and *Trichoderma virens* failed to metabolize FA, while *Colletotrichum graminicola*, *Sclerotinia minor, Phanerochaete chrysosporium*, *Rhizopus microsporus*, *Rhizoctonia solani, Schizophyllum commune, Trichoderma reesei*, and *Ustilago maydis* were able to completely convert FA to derivatives. *Talaromyces pinophilus* also completely converted 200 μg/ml of FA to FOH (data not shown). For all isolates, the total concentration of FA conversion products

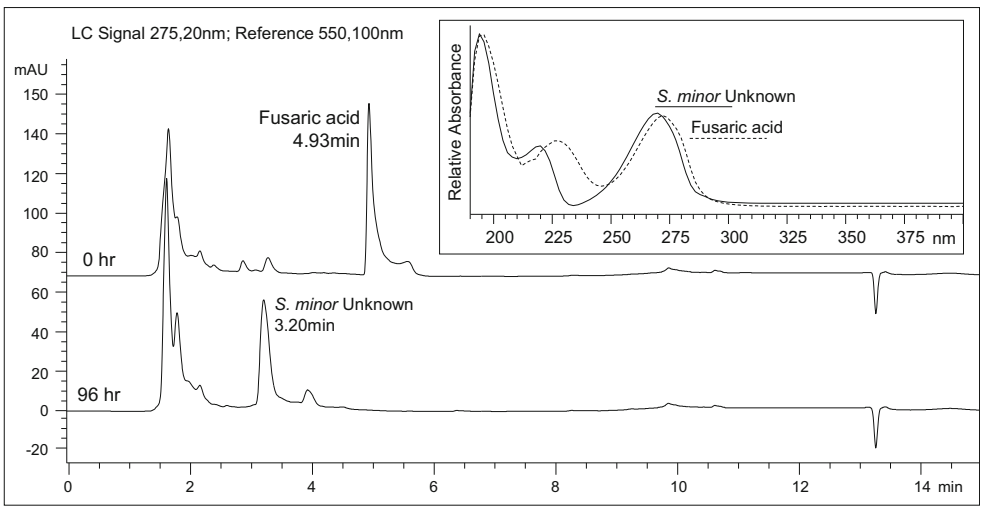

Fig. 3 HPLC chromatograms of cultures of *Sclerotinia minor* supplemented with fusaric acid. Culture filtrates taken at 0 h (top) and 96 h (bottom) after adding fusaric acid were analyzed and show the formation of an unknown compound at 3.2 min with the associated disappearance of fusaric acid peak over the time. Inset shows the UV-Vis spectrum of fusaric acid (dashed line) and the unknown (solid line)

along with unconverted FA equaled the original concentration of FA added, except for *Fusarium oxysporum* CA9 and *Schizophyllum commune.* For these two isolates, a 68% increase and a 66% decrease of total FA related compounds were found in the culture filtrates compared to original added FA concentration, respectively.

Discussion

Eight bacterial isolates obtained from the soil screen fluoresced on King's A and King's B agar, and were identified as *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* by their chemical reactions on the API 20E strip. These *Pseudomonas* spp. isolated from soil and the laboratory isolates of *Pseudomonas* spp. were the only bacteria capable of growth when exposed to greater than 500 μg/ml FA when cultured as a lawn (data not shown). Under these conditions, Gram-negative bacilli grew on higher concentrations than did Gram-positive bacilli. To more thoroughly examine FA sensitivity, MIC values were used to measure the ability of the bacteria to grow on increasing amounts of FA as a discrete colony. Under these conditions, *Pseudomonas marginalis* had the highest MIC at greater than 500 μg/ml of FA with pseudomonads overall having the greatest resistance (Table 1). These findings are supported by the observed resistance of *Pseudomonas* spp. to FA and their effective use as biocontrol agents against a variety of *Fusaria* (Duffy and Defago 1997; Bolwerk et al. 2003; Quecine et al. 2016). Both *Bacillus cereus* and *Bacillus subtilis* were incapable of growth at 50 μg/ml FA supporting previous reports of FA sensitivity in *Bacillus* spp., and negating their effectiveness as biocontrol agents against species of *Fusarium* (Bacon et al. 2004; Bacon et al. 2006). All of the Gram-positive cocci tested in this assay were incapable of growing in FA.

None of the bacterial isolates converted FA into its derivatives and no decrease in the amount of FA in the medium was observed during a growth period of 72 h in the presence of FA (data not shown). We hypothesize that the primary mechanism for high resistance to FA in Gram-negative bacteria is the transport of the compound by an efflux pump. This theory is supported by previous research with *Klebsiella oxytoca, Burkholderia cepacia,* and *Stenotrophomonas maltophilia* revealing that DNA sequences responsible for resistance encode efflux pumps consisting of a FUSC family inner membrane transporter, a MFP, and an OMF (Toyoda et al. 1991; Utsumi et al. 1991; Hu et al. 2012). The production of siderophores also may provide resistance to FA in some bacterial species (Ruiz et al. 2015).

Both the number of FUSC proteins in the genome and sequence identities of the FUSC proteins may contribute to the effectiveness of the strains' FA resistance. Regression analysis of log MIC values against the number of FUSC proteins in the genome showed a significant positive correlation between them ($P = 0.014$, $R^2 = 0.36$). FUSC proteins of the FA resistant strains having the highest amino acid sequence identities with the functionally characterized FusC, Fdt, and FuaA clustered in clades III and IV: the FusC type (clade III) and the Fdt/FuaA type (clade IV). This indicates that there are two major types of FUSC FA resistance proteins and they are probably the major contributors of FA resistance.

FUSC proteins also include the aromatic carboxylic acid efflux pump proteins (AaeB) clustered in clade I. The substrates for these proteins, pHBA and 6-HNA, like FA, contain aromatic carboxylic acid moieties (Fig. 1). These compounds are much less toxic than FA to *E. coli* with MIC values of 13,800 μg/ml for pHBA and 3760 μg/ml for 6-HNA (Van Dyk et al. 2004) compared to 50 μg/ml for FA (Utsumi et al. 1988). Whether Aae pumps confer resistance to FA remains to be elucidated. The OMFs are missing from these Aae operons, but might reside outside the operon (Van Dyk et al. 2004).

The FUSC efflux pumps, like the related ABC, MFS, and RND superfamilies tripartite efflux pumps of Gram-negative bacteria (Daury et al. 2016; Hu et al. 2012; Saier and Paulsen 2001), are also composed of an inner membrane transporter (FUSC), a periplasmic adapter protein MFP, and an outer membrane channel protein OMF (Fig. 2). The almost universal presence of a small (less than 78aa) putative MFP associated DUF1656 family protein consisting of 2 transmembrane segments suggests that this protein may be vital for the efflux system. Only two other tetrapartite efflux systems have been identified in Gram-negative bacteria (Delmar et al. 2014). The Gram-positive bacteria lack FUSC proteins, yet two of the ten tested strains were able to resist moderate concentrations of FA (MIC less than 200 μg/ml). Either a different efflux protein or a formidable membrane barrier may be responsible for this resistance (Bansal-Mutalik and Nikaido 2011).

Fungi also displayed a wide range of resistance to FA. Only a few plant pathogens such as the oomycetes *Phytophthora nicotianae* and *Pythium ultimum*, and ascomycetes *Verticillium dahliae* and *Phymatotrichopsis omnivorum* were highly sensitive to FA and unable to grow at 50 μg/ml FA. The oomycete *Phytophthora infestans* was previously found to be sensitive to FA (Son et al. 2008). The plant pathogens *Ustilago maydis*, *Sclerotinia minor*, *Rhizopus microsporus*, *Rhizoctonia solani*, and *Colletotrichum graminicola* were also sensitive to FA with IC_{50} values less than 160 μg/ml FA. Like the tested bacterial strains, no fungal isolates, even the FA producing *Fusarium* isolate had complete resistance to FA. The strains with the highest IC_{50} values were species of *Fusarium* or saprophytic/beneficial microorganisms that are ubiquitously found in soil environments.

Unlike the bacteria and the yeast screened for this work, various derivatizations of FA by filamentous fungi were observed for both the soil and laboratory isolates. Four of the five detected FA derivatives were previously characterized as the less toxic compounds 9-HOFA (Stipanovic et al. 2011a), 8-

HOFA (Crutcher et al. 2017), 9,10-DHFA (Stipanovic et al. 2011b), and FOH (Crutcher et al. 2014). The remaining FA derivative produced by *Sclerotinia minor* appears to be a novel compound. Future work will include identifying this compound and testing its phytotoxicity. Thus, these fungi are able to resist FA through modifying FA into less toxic compounds. Only a few fungi, namely *Penicillium roqueforti*, *Trichoderma harzianum*, and *Trichoderma virens,* were incapable of modifying FA yet had relatively high resistance against FA. Like for Gram-negative bacteria, efflux by transporters may be the primary mechanism for the FA resistance in these fungal isolates. In FA producing *Fusarium* spp., both the FA efflux by a MFS transporter and derivatization of FA into less toxic 9-HOFA and 9,10-HDFA are responsible for FA resistance (Crutcher et al. 2015; Studt et al. 2016). Deployment of both efflux and derivatization mechanisms may be a common feature of the FA resistance of fungal isolates and requires further elucidation.

9-HOFA, 8-HOFA, and 9,10-DHFA are oxidized FA derivatives, while FOH is a reduced FA derivative. In most cases, when the reductive pathway product FOH dominated the derivatization products of FA by an isolate, none or minor amounts of oxidized FA derivatives were produced. Likewise, when oxidative FA derivatives constituted the majority of the derivatized products, no reductive derivative of FA was observed (Table 3). Thus, the oxidative pathway and the reductive pathway seem to be alternative biological adaptations. Both the reductive and oxidative pathway FA derivatives, FOH, 9-HOFA, and 8-HOFA, were produced by all the phyla of filamentous fungi tested: Ascomycota, Basidiomycota, and Zygomycota. The presence of these enzymatic reactions in such evolutionarily distant fungi indicates that these mechanisms of detoxification are preserved or convergent and may serve other functions.

Overall, there were substantial differences in resistance among fungal isolates; however, there was no correlation between the level of resistance to FA and the ability to alter FA. Even though their IC_{50}s were lower than 100 μg/ml FA, *Colletotrichum graminicola*, *Phanerochaete chrysosporium*, and *Schizophyllum commune* reduced FA to FOH and *Rhizoctonia solani* oxidized FA to 9- or 8-HOFA. Previously, only microorganisms with high resistance to FA were evaluated for detoxification of FA (Utsumi et al. 1991; Fakhouri et al. 2003; Crutcher et al. 2014). The observation of a variety of chemical modifications of FA by strains with low FA resistance, including the production of the unknown compound by *Sclerotinia minor*, indicates that other resistance mechanisms may exist. The loss of FA from cultures of *Schizophyllum commune* without an increase of an apparent derivative suggests that FA was completely catabolized. In contrast, the concentration of FA derivatives plus unconverted FA exceeded the initial concentration of FA added to cultures of *Fusarium oxysporum* CA9, due to continuing synthesis of FA by the isolate.

A cytochrome P450 was implicated in the production of 9-HOFA and 9,10-DHFA, but this gene was not found within the FA biosynthetic cluster of *Fusarium fujikuroi* (Studt et al. 2016). Two enzymes, a carboxylic acid reductase and an alcohol oxidoreductase would be required to reduce FA to FOH (Li and Rosazza 2000). *Trichoderma reesei* 6 converted 86% of added FA to FOH. The availability of a complete genome sequence for this isolate (http://genome.jgi.doe.gov/Trire2/Trire2.home.html) would be useful for discovery of genes that encode for these enzymes.

Potential dissemination of *Fusarium oxysporum* f. sp. *vasinfectum* strains from Australia and California (Davis et al. 1996; Davis et al. 2006) that produce large quantities of FA are a threat to U.S. cotton production. The discovery of resistance mechanisms that detoxify FA could provide methods for the production of transgenic cotton cultivars with resistance to Fusarium wilt or be used to improve efficacy of biocontrol agents. The results presented here provide numerous targets for gene discovery in several fungal species, including those with genome sequences available.

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