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ABSTRACT

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Lysobacter enzymogenes C3 is a bacterial biological control agent that exhibits antagonism against multiple fungal pathogens. Its antifungal activity was attributed in part to lytic enzymes. In this study, a heat-stable antifungal factor (HSAF), an antibiotic complex consisting of dihydromaltophilin and structurally related macrocyclic lactams, was found to be responsible for antagonism by C3 against fungi and oomycetes in culture. HSAF in purified form exhibited inhibitory activity against a wide range of fungal and oomycetes species in vitro, inhibiting spore germination, and disrupting hyphal polarity in sensitive fungi. When applied to tall fescue leaves as a partially-purified extract, HSAF at 25 μg/ml and higher inhibited germination of conidia of *Bipolaris sorokiniana* compared with the control. Although application of HSAF at 12.5 μg/ml did not reduce the incidence of conidial germination, it inhibited appressorium formation and suppressed Bipolaris leaf spot development. Two mutant strains of C3 (K19 and ΔNRPS) that were disrupted in different domains in the hybrid polyketide synthase-nonribosomal peptide synthetase gene for HSAF biosynthesis and had lost the ability to produce HSAF were compared with the wild-type strain for biological control efficacy against Bipolaris leaf spot on tall fescue and Fusarium head blight, caused by *Fusarium graminearum*, on wheat. Both mutant strains exhibited decreased capacity to reduce the incidence and severity of Bipolaris leaf spot compared with C3. In contrast, the mutant strains were as efficacious as the wild-type strain in reducing the severity of Fusarium head blight. Thus, HSAF appears to be a mechanism for biological control by strain C3 against some, but not all, plant pathogenic fungi.

Additional keywords: microbial fungicide, secondary metabolite.

The production of antibiotics, antimicrobial secondary metabolites, is an important mechanism of competition among microorganisms and plays an important role in many plant disease biological control systems (5,10). Antibiotics produced by biological control agents, in native or modified form, can also be the basis for modern fungicides. For example, pyrrolnitrin, an antibiotic involved in biocontrol by *Pseudomonas fluorescens* strain Pf5 (13), was chemically modified into two commercial fungicides, fenpiclonil and fludioxonil, which are used as seed treatments against diverse fungal pathogens (20).

While *Bacillus*, *Pseudomonas*, and *Streptomyces* are the most widely studied genera of bacteria in regards to the production of antibiotics that may be useful against plant diseases, the potential importance of other bacterial genera in this role is beginning to be recognized. *Lysobacter* is a bacterial genus known to produce a number of antibiotics (1,12,18,26,28,35). Myxin, first reported in *L. antibioticus* ATCC29479 (1,30), and xanthobaccins, produced by *Lysobacter* sp. SB-K88 (formerly reported as a strain of *Stenotrophomonas* sp. [11]) have antifungal activity and were implicated in biological control (27,31). Multiple, unidentified antifungal compounds were thought to be involved in antagonism by *L. enzymogenes* strain 3.1T8 against *Pythium aphanidermatum* (4). Transposon mutagenesis of a putative polyketide synthase (PKS) gene in 3.1T8 resulted in a loss of in vitro antagonism and reduced biocontrol activity (3). A heat-stable antifungal factor (HSAF) was isolated and characterized recently from C3, another biocontrol strain of *L. enzymogenes* (37). HSAF is a complex of at least three structurally-related compounds, the primary one being dihydromaltophilin, which was reported in a *Streptomyces* sp. (7) and is an analog of xanthobaccins. A hybrid PKS-nonribosomal peptide synthetase (NRPS) gene in C3 was shown to be responsible for biosynthesis of all of the HSAF compounds as mutants of C3 generated by disrupting specific domains in the PKS-NRPS gene no longer produced any of the HSAF associated compounds as identified by high-performance liquid chromatography (HPLC) and also lost in vitro antifungal activity (37). HSAF caused depolarized hyphal growth in *Aspergillus nidulans*, the effects being attributed to the disruption of sphingolipid biosynthesis in the fungus (25). Strain C3, which was reclassified from *Stenotrophomonas maltophilia* (33), is a biological control agent of multiple fungal and oomycetous pathogens (6,17, 21,22,38,39), with field efficacy demonstrated most extensively against *Bipolaris sorokiniana*, causing Bipolaris leaf spot in turfgrasses, and *Fusarium graminearum* (*Gibberella zeae* teleomorph), causing Fusarium head blight in wheat. Lytic enzymes produced by C3, particularly chitinases and β-1,3-glucanases, have important roles in biological control of *B. sorokiniana* and *P. ultimum* (29,40), while induced resistance also may be a mechanism for disease control by the strain (19). In these previous studies, none of the mechanisms alone could account for the total biological control capacity exhibited by C3. In one study (40), fractions from C3 culture fluid retained some antifungal activity after boiling despite chitinase activity being eliminated,

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and a low molecular weight fraction also exhibited antifungal activity. In another study (29), a mutant strain of C3 deficient in all β-1,3-glucanase activity was found to be unchanged in its ability to inhibit the oomycete *P. ultimum* on agar media compared with the wild type. These observations support secondary metabolites such as HSAF also being involved in antagonism of C3 against fungi and oomycetes.

Little is known as to the range of organisms that HSAF can inhibit, and the degree to which HSAF can affect plant infection by fungal pathogens. The role of HSAF in biological control of fungal pathogens by C3 also has not been established. The objectives of this study, therefore, were (i) to determine the spectrum of antimicrobial activity of HSAF and its effects on the morphology of fungal and oomycete pathogens; (ii) to determine the effects of HSAF on pre-penetration processes in *B. sorokiniana* on the phylloplane and the potential for the antibiotic to control foliar infection by this pathogen; and (iii) to ascertain whether HSAF might have a role in biological control of disease caused the fungi *B. sorokiniana* and *F. graminearum*.

MATERIALS AND METHODS

Microbial cultures. Strains of *L. enzymogenes* used in this study include C3, the wild-type (6); 5E4, a *clp* mutant of C3 inactive in antifungal antagonism and biological control (21); and HSAF-nonproducing mutants K19 and ΔNRPS disrupted in the ketosynthase domain of the PKS module and the condensation domain of the NRPS module, respectively, of the PKS-NRPS gene responsible for HSAF biosynthesis (37). Other microorganisms used in this study are listed in Table 1. Unless specified otherwise, all microorganisms were cultured routinely on one-tenth-strength tryptic soy agar (10% TSA; Sigma-Aldrich, St. Louis, MO) and cultures were incubated at 28°C. Strain C3 was grown in 10% tryptic soy broth (TSB; Sigma-Aldrich) at 28°C for 4 days with shaking at 180 rpm to produce broth cultures for extraction of HSAF. All *L. enzymogenes* strains were resistant to rifampicin via spontaneous mutation, and their populations in cultures, aqueous suspensions and on leaves were determined by dilution plating on 10% TSA amended with rifampicin and cycloheximide, both at 100 mg/liter.

TABLE 1. Antimicrobial spectrum of heat-stable antifungal factor (HSAF)

Organism[y]	Inhibition zone (mm)	
	Heated culture fluid	TLC[z]-purified HSAF
Fungi		
Aspergillus nidulans A28	18	16
Bipolaris sorokiniana	20	16
Candida albicans CHS1	15	9
Cryptococcus neoformans	22	17
Cercospora clavata	14	10
Fusarium graminearum PH1	19	13
Neurospora crassa	20	17
Pichia anomala	15	12
Rhizoctonia solani AG1-IA	25	17
Rhizopus stolonifer	20	16
Sclerotinia sclerotiorum	20	16
Saccharomyces cerevisiae JC482	14	0
Oomycetes		
Pythium ultimum var. *ultimum*	24	20
Phytophthora sojae	25	20
Bacteria		
Bacillus pumilus	15	0
Clavibacter michiganensis subsp. *nebraskensis*	12	0
Escherichia coli DH5α	0	0
Pectobacterium carotovorum subsp. *carotovorum*	0	0
Pseudomonas syringae pv. tomato DC3000	0	0
Xanthomonas campestris pv. *phaseoli*	0	0

[y] *P. syringae* pv. tomato was provided by J. R. Alfano; *S. cerevisiae* by M. B. Dickman; *P. sojae* by L. Giesler; *C. michiganensis* subsp. *nebraskensis, P. carotovorum* subsp. *carotovorum,* and *X. campestris* pv. *phaseoli* by A. K. Vidaver. All other microorganisms were from the culture collections of the authors.

[z] TLC = thin-layer chromatography.

Extraction and purification of HSAF. The antibiotic complex was investigated in this study in three forms: (i) fluid from a broth culture of C3 after removal of cells by centrifugation and filter sterilization; (ii) partially purified form extracted from culture fluid using ammonium sulphate and methanol (25); and (iii) purified form derived by thin-layer chromatography (TLC). These materials are referred to as culture fluid, extract, and TLC-purified HSAF, respectively. Prior to use in experiments, culture fluid and extract were heated at 70°C for 30 min to inactivate lytic enzymes. TLC-purified HSAF was produced from the extract using preparative TLC plates made with TLC-Silica gel 60 GF 254 (Sigma-Aldrich). The plates were developed in chloroform/methanol (3:1). Separated fractions were visualized under UV light (254 nm) and recovered by methanol/acetate acid (99:1). The sole fraction with antifungal activity (Rf value of 0.39) was considered to contain HSAF (24).

Assays for antifungal activity. A plate inhibition assay was used in determining antimicrobial activity of heated C3 culture fluid and TLC-purified HSAF across a range of plant pathogenic and saprotrophic microorganisms, some in the latter group also being potential human pathogens (Table 1). In tests with filamentous fungi and oomycetes, a 6-mm-diameter mycelial plug taken from the margin of an actively growing culture on 10% TSA was transferred to the center of a fresh plate of the medium. In assays involving yeast or bacteria, test plates were inoculated with the organism by spreading 40 µl of cell suspension (OD_{600} = 0.5) on the agar surface. To apply C3 culture fluid, wells (5 mm diameter) were cut into the agar at the perimeter of each plate and a 40-µl aliquot of culture fluid, or sterile 10% TSB used as the negative control, was added to each well. TLC-purified HSAF was tested by applying 20 µl of sample (dissolved in methanol) onto filter paper disks (5 mm), allowing the methanol to evaporate off, and then placing the disks on the agar surface at the periphery of plates. Filter paper disks loaded with the same volume of methanol were used as controls. Each HSAF sample was tested in two replicate wells or filter paper disks against each organism. Cultures were incubated at 28°C for 1 to 4 days, depending upon the organism, prior to the size of growth inhibition zones surrounding the wells and disks being measured.

A spore germination assay was used for microscopic examination of the effects of HSAF on spore germination and hyphal development in fungi (*B. sorokiniana* isolate NE1 and *F. graminearum* isolate PH1) and the oomycete *P. ultimum* var. *ultimum* (here after referred to as *P. ultimum*) isolate P201. Conidia of the fungi were generated by growing the isolates on 10% TSA for 10 days. Sporangia of *P. ultimum*, which germinate only into hyphae (36), were generated by growing isolate P201 on plates of 10% TSA flooded with sterile distilled water for 3 days. Conidia or sporangia were harvested and suspended in sterile distilled water (100 ml for each plate). Mycelium was removed by filtering the suspensions through 4 layers of sterile cheese cloth. The conidia or sporangia were collected by centrifugation at 2,000 rpm for 1 min and resuspended in 10% TSB. Various volumes of TLC-purified HSAF dissolved in methanol were applied on slides and the methanol removed by evaporation at room temperature. Then, 5 µl of spore suspension was applied to the same location as the HSAF sample, and the antibiotic was allowed to dissolve in the liquid. Final concentrations of HSAF were 20 and 40 µg/ml. The slides were put in 15-cm-diameter petri dishes lined with moist filter paper and incubated at 28°C for 3 h for *P. ultimum*, or 12 h for *F. graminearum* and *B. sorokiniana*. Spore germination and hyphal growth were observed under a Zeiss compound microscope at magnification ×100 to ×400.

Evaluation of HSAF for Bipolaris leaf spot control. The effects of HSAF on epiphytic growth and infection of tall fescue by *B. sorokiniana* isolate NE1 was investigated in a growth chamber experiment under conditions previously described (39) using 45-day-old tall fescue (*Festuca arundinacea* Schreb. 'Kentucky-31') turf in 10-cm diameter pots. Partially purified HSAF extract was diluted to 12.5, 25, and 50 μg/ml with distilled water amended with the surfactant Induce (0.01%; Bayer Crop Science, Kansas City, MO), and then each dilution and a distilled water control was sprayed onto four pots of turf (20 ml per pot). One day after treatment application, turf foliage was inoculated with the pathogen *B. sorokiniana* by spraying suspensions of conidia (1×10^5 conidia/ml) in water amended with Induce. Misting was provided for the first 36 h after pathogen inoculation to stimulate pathogen germination and growth. Leaves were collected 12 h after pathogen inoculation and stained with lactophenol acid fuchsin for microscopic examination of conidial germination, hyphal growth, and appressorium formation on leaf surfaces. More than 130 germlings for each treatment were examined. Pots of inoculated turf were incubated for 8 days for disease development. Disease severity (percent infected leaf area) was visually assessed in at least 10 leaves per pot. The experiment was conducted twice, the data being pooled for analysis of variance (ANOVA) and means separation using PROC MIXED (SAS Institute Inc., Cary, NC).

Evaluation of mutants deficient in HSAF production. HSAF-nonproducing mutant strains K19 and ΔNRPS were compared with wild-type C3 for antagonism against *B. sorokiniana*, *P. ultimum*, the yeast *Saccharomyces cerevisiae*, and the gram-positive bacterium *Clavibacter michiganensis* subsp. *nebraskensis* in plate inhibition tests. In addition, the strains were evaluated for chitinase, β-1,3-glucanase, protease, lipases activities on media containing colloidal chitin, laminarin, skim milk, and Tween 80, respectively (21), and for biosurfactant activity (22). Colony morphology of each strain was examined on 10% TSA with the aid of a dissecting microscope.

Strains K19 and ΔNRPS were evaluated for biological control activity against *B. sorokiniana* in a series of five experiments. The first three experiments were conducted prior to the generation of strain ΔNRPS, and thus involved only strain K19 in comparison with wild-type strain C3 and the *clp* mutant strain 5E4. Strain ΔNRPS and wild-type C3 were tested in the last two experiments. The fourth experiment also involved mutant strain K19. Distilled water was used as the negative control in all experiments. Each bacterial strain was applied as a suspension of twice-washed cells (10^8 CFU/ml) from a 2-day-old culture on 1/10 TSA sprayed onto four pots of turf. The treated turf was incubated overnight in the growth chamber prior to inoculation with pathogen conidia. Foliage in two pots of tall fescue treated with a bacterial strain was sampled immediately after treatment, and 1 day and 7 days after treatment. The samples were assayed for populations of the applied strain by dilution plating (39). Inoculated turf was rated for severity of leaf spot at the end of 8 days incubation as described previously. In addition, the number of lesions on sampled leaves was counted and the length of leaves was measured to determined incidence of infection, i.e., number of lesions per 10 cm of leaf blade. Data from each experiment was analyzed separately by ANOVA, and means separation was performed using the Fisher's least significant difference (LSD) test.

The mutant strains were compared with wild-type C3 in a series of four experiments for control of Fusarium head blight in wheat. Strain K19 was tested in all four experiments, while ΔNRPS was tested in one. Methods described previously (17) were used in evaluating the efficacy of the bacterial strains. Essentially, cell suspensions of C3 and the mutant strains, prepared as mentioned, and a sterile distilled water control were applied to flowering heads of 'Bobwhite' hard red spring wheat by spraying the heads to run-off. There were five or six replicate pots per treatment, each pot containing four plants with a total of five to seven flowering heads. Flowering heads were sampled immediately after bacterial treatment and just prior to pathogen inoculation for assay of the bacterial populations. Pots were placed in a moisture chamber overnight after bacterial treatment, and then the heads were sprayed with suspensions containing 5×10^5 conidia per ml of *F. graminearum*. The pots were incubated for another 48 h in the moisture chamber and then placed on a greenhouse bench. Sixteen to eighteen days after pathogen inoculation, the percent of diseased spikelets on each inoculated head was determined. Results for all heads in a pot were averaged prior to statistical analysis by ANOVA. Statistical analysis also was performed on arcsine-transformed data, but because the results were the same, only results using nontransformed data are reported. Each experiment was analyzed separately because treatments and numbers of replications differed among the experiments.

RESULTS

Antimicrobial activity of HSAF. Heated C3 culture fluid retained inhibitory activity against a wide range of fungi and oomycetes (Table 1). It was also inhibitory to gram-positive bacterial species but not gram-negative species. TLC-purified HSAF had a more restricted spectrum of activity compared to the heated culture fluid, lacking activity against *Saccharomyces cerevisiae* and all bacteria (Table 1).

The effects of HSAF on different stages of fungal growth were concentration dependent (Fig. 1). At higher concentration (40 μg/ml), TLC-purified HSAF inhibited spore germination in *B. sorokiniana*, *F. graminearum*, and *P. ultimum.* Although HSAF at the lower concentration (20 μg/ml) did not affect spore germination, it suppressed hyphal elongation and induced excessive branching in *B. sorokiniana* and *F. graminearum*. This same concentration slowed hyphal growth in *P. ultimum* compared with the control, but otherwise had no effect on hyphal morphology.

Effects of HSAF on infection of tall fescue by *B. sorokiniana.* HSAF extract applied at different concentrations to tall fescue leaves had different effects on germination and growth of *B. sorokiniana*. Although the frequency of germination at the lowest concentration (12.5 μg/ml) of HSAF extract was not affected compared with the distilled water control, the treatment caused excessive hyphal branching (Fig. 2B). There was also a significant reduction in appressorium formation accompanying the effects on hyphal branching. On control leaves, 72% of germlings developed at least one appressorium (Fig. 2A). However, only 22% of germlings on leaves treated with 12.5 μg/ml of HSAF extract developed appressoria. Higher concentrations of the extract (25 and 50 μg/ml) inhibited conidial germination or hyphal elongation (Fig. 2C).

All concentrations of HSAF extract applied to tall fescue turf reduced the severity of Bipolaris leaf spot compared with the distilled water control (Fig. 3). Levels of disease inhibition conferred by the extract were dosage dependant. The treatment factor was highly significant ($P = 0.0006$). Similar results were obtained in the two experiments in which HSAF extract was tested for disease control efficacy, the experiment × treatment interaction being insignificant.

Antagonism and biocontrol activity of HSAF-deficient mutants. Mutant strains K19 and ΔNRPS retained lytic enzyme and biosurfactant activities of wild-type C3, but they also exhibited altered colony morphology, being paler and more mucoid, compared to C3 (Table 2). The mutant strains lost antagonism activity against *P. ultimum*, while retaining inhibitory activity against *S. cerevisiae* and *C. michiganensis* subsp. *nebraskensis* (Table 2; Fig. 4).

Biocontrol activity against Bipolaris leaf spot was found to be associated with the ability of C3 to produce HSAF (Table 3). In

all experiments in which wild-type C3 and mutant strains were applied to tall fescue foliage, treatment with the wild-type strain resulted in significantly reduced levels of leaf spot incidence (lesions per centimeter of leaf) and disease severity (percent diseased leaf area) compared to treatment with the water control or the *clp*-disrupted mutant 5E4. In comparison to wild-type C3, HSAF-minus strains K19 or ΔNPRS exhibited complete loss of ability or decreased ability to reduce disease incidence and severity. There was no significant difference in leaf spot incidence or disease between treatment with either K19 or ΔNPRS and treatment with the water controls or 5E4 in any of the experiments. In the one experiment involving both K19 and ΔNPRS, there was no difference in biocontrol activity between the two mutant strains. All of the bacterial strains in each experiment colonized foliage to similar levels, with population levels of the mutant strains being within 1/2 $\log_{10}$ unit of the wild type (data not shown); from initial populations of 7.0 to 7.5 $\log_{10}$ CFU/g leaf, numbers of applied bacteria typically increased by 1 $\log_{10}$ unit over 8 days.

The ability of strain C3 to control Fusarium head blight, on the other hand, was not dependent on HSAF production (Table 4). In three out of the four experiments in which the HSAF-deficient mutant K19 was tested, K19 was equal to wild-type C3 in suppressing head blight development. In the remaining experiment, K19 exhibited an intermediate effect, with disease severity in the K19 treatment being statistically similar to that of C3 and the water control. In the one experiment in which ΔNPRS was evaluated, this mutant strain also was effective as wild-type C3 in controlling head blight. In all of the experiments, bacterial population levels on treated wheat heads were similar among the strains, with less than 1 $\log_{10}$ unit difference between strains being found in any given sampling (data not shown). Populations on wheat heads typically were around 10^7 CFU/g immediately after bacterial treatment, declining by approximately 1 $\log_{10}$ unit 1 day later when the heads were inoculated with the pathogen.

DISCUSSION

The primary compound having HSAF activity has a chemical structure and molecular weight identical to dihydromaltophilin (37), a polyketide antibiotic containing both a macrocyclic lactam structural unit and a tetramic acid residue (7). This would place HSAF in a family of antibiotics that includes maltophilin produced by *Stenotrophomonas maltophilia* (16) and *Streptomyces* sp. (7), ikarugamycin from *Streptomyces phaeochromogenes* (15), xanthobaccins from *Lysobacter* sp. SB-K88 (11), alteramide A from *Alteromonas* sp. (32), and discodermide from the marine sponge *Discodermia dissoluta* (8). The sensitivity of a wide range of filamentous fungi and oomycetes and lack of sensitivity in bacteria and some yeasts to HSAF in this study is consistent with

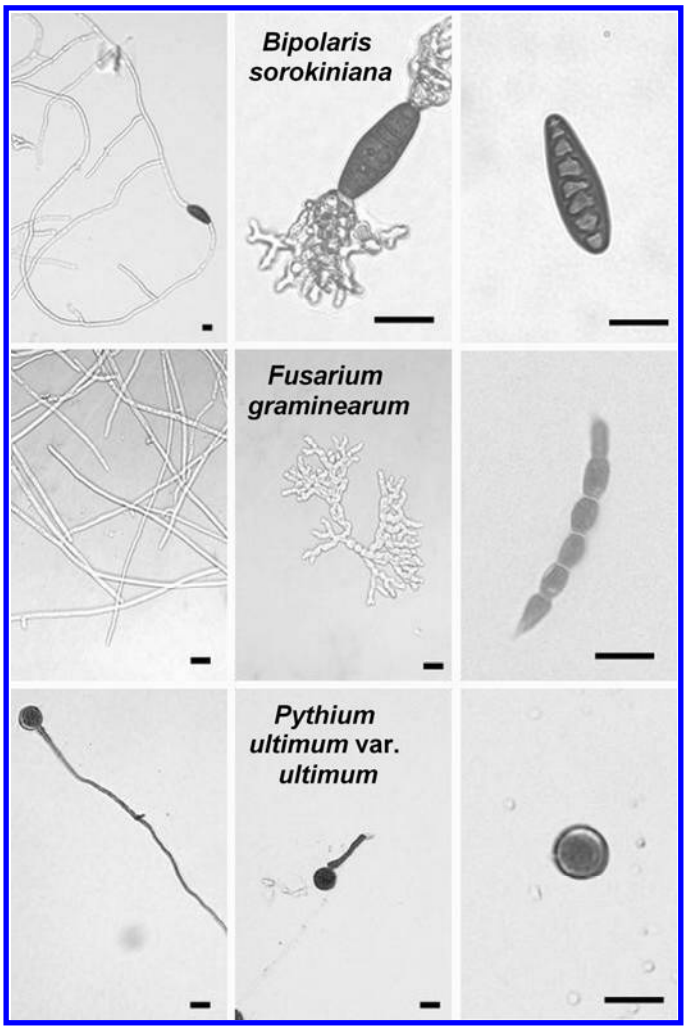

Fig. 1. Effects of thin-layer chromatography-purified heat-stable antifungal factor(HSAF) on spore germination and hyphal growth in *Bipolaris sorokiniana*, *Fusarium graminearum*, and *Pythium ultimum* var. *ultimum*. HSAF concentrations were 0, 20, and 40 µg/ml in the left, center, and right panels, respectively. Bars indicate 30 µm.

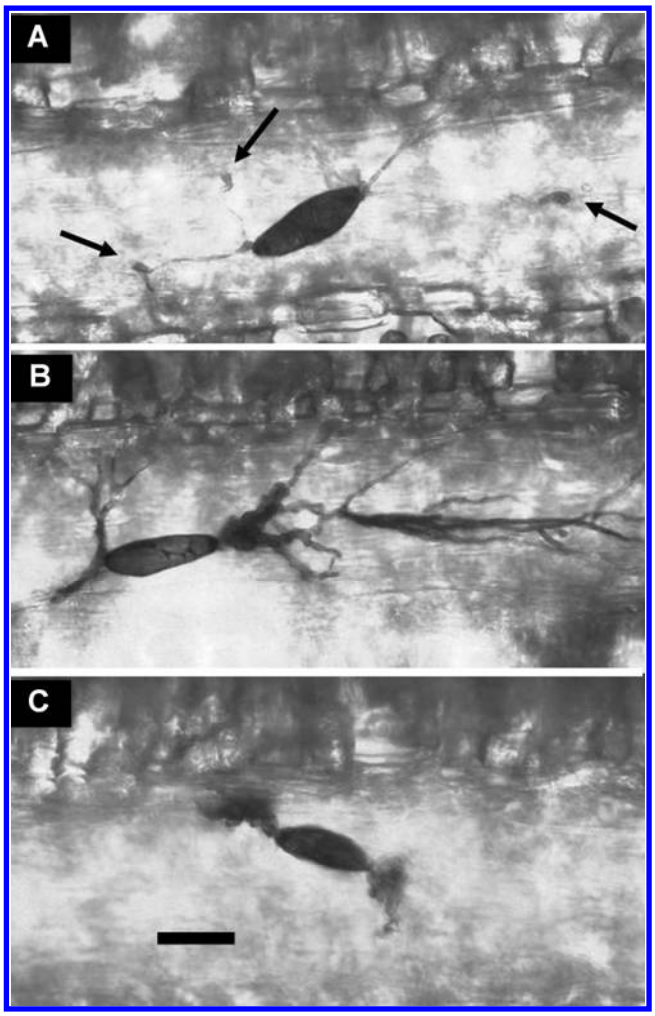

Fig. 2. *Bipolaris sorokiniana* hyphal growth and appressorium formation on the surface of tall fescue leaves treated with **A,** distilled water, **B,** 12.5 µg/ml heat-stable antifungal factor (HSAF) extract, and **C,** 50 µg/ml HSAF extract. Arrows in panel A point to appressoria. Specimens were stained with lactophenol acid fuchsin 12 h after inoculation of leaves with pathogen conidia. Bar indicates 30 µm.

the spectrum of activity reported for maltophilin and xanthobaccins (16,27). The multiple unidentified antifungal compounds presumed to be produced by of *L. enzymogenes* 3.1T8 (4) might also include an antibiotic in this group, as the partial sequence for a PKS gene in 3.1T8 (3) was used to find the homologous gene for HSAF biosynthesis in strain C3 (37).

HSAF appears to be the primary factor responsible for antagonism by C3 against filamentous fungi and oomycetes in plate tests. This is consistent with the finding that loss of β1,3-glucanase activity in C3 did not affect antagonism against *P. ultimum* (29). HSAF, however, is not the sole heat-stable antimicrobial component produced in vitro by C3 because unknown heat-stable factors with antimicrobial activity against *S. cerevisiae* and bacteria also were found in the culture fluid. Whether a single compound is responsible for activity against bacteria and *S. cerevisiae* has not been determined. Myxin, a phenazine antibiotic produced by strains of *L. antibioticus*, is a candidate for such a compound as it is heat stable and has activity against bacteria and *S. cerevisiae* (30); it has not, however, been reported to be produced by *L. enzymogenes*.

HSAF causes inhibition of hyphal elongation and disruption of hyphal polarity in filamentous plant pathogenic true fungi, which is consistent with observations of HSAF effects on the model saprophytic fungus *Aspergillus nidulans* (25). While similar morphological effects were observed with the related antibiotic xanthobaccin A on the oomycete *Aphanomyces cochliodes* (14), we did not find HSAF to alter branching morphology in the oomycete *P. ultimum*. This would suggest that HSAF and xanthobaccins have different effects on oomycetes or that *P. ultimum* is unique in its response to HSAF.

HSAF also exhibited antifungal activity when applied to tall fescue foliage. Because of the difficulty in preparing sufficient amounts of TLC- or HPLC-purified material for the experiments, partially-purified extract was used instead. It can be assumed that the observed effects using the extract were due solely to HSAF because HPLC analyses of the extract and TLC-purified HSAF revealed a similar compliment of peaks associated with HSAF activity (24,37). These peaks were responsible for all activity against filamentous fungi as the peaks and antifungal activity were absent from extracts prepared from HSAF-deficient mutants (37). On the leaf surface, HSAF at lower concentrations disrupted appressorium formation, and thus, while being fungistatic rather than fungicidal, it is nevertheless effective in suppressing foliar

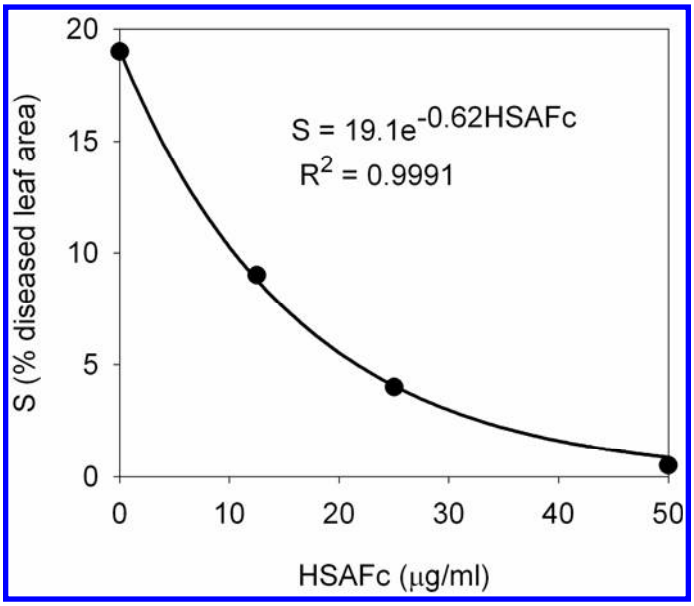

Fig. 3. Suppression of Bipolaris leaf spot on tall fescue by various concentrations of heat-stable antifungal factor extract (HSAFc). Disease severity (S) was measured 8 days after inoculation of turf with conidia of *Bipolaris sorokiniana.* Results are means from two experiments, each with four replicates.

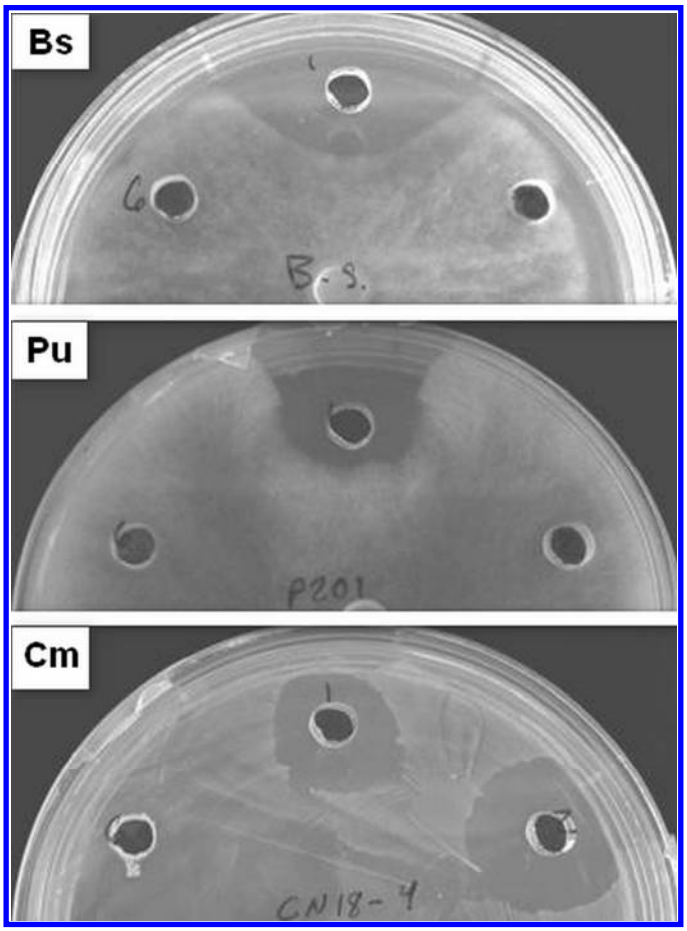

Fig. 4. Comparison of cell-free fluid from 10% tryptic soy broth cultures of *Lysobacter enzymogenes* C3 and heat-stable antifungal factor-deficient mutant strain K19 for inhibition of growth by *Bipolaris sorokiniana* (Bs), *Pythium ultimum* var. *ultimum* (Pu), and *Clavibacter michiganensis* pv. *nebraskensis* (Cm) on 10% tryptic soy agar. In each panel, wells contained (left to right) sterile broth, C3 culture fluid, or K19 culture fluid.

TABLE 2. Phenotype of wild-type *Lysobacter enzymogenes* C3 and heat-stable antifungal factor (HSAF)-nonproducing mutant strains K19 and ΔNRPS

		Lytic activity[x]					In vitro growth inhibition[y]			
Strain	Colony morphology	Chi	Glu	Pro	Lip	Biosurfactant activity[z]	Bs	Pu	Sc	Cm
C3	Pale yellow, convex	+	+	+	+	+	+	+	+	+
K19	Cream-colored, mucoid	+	+	+	+	+	–	–	+	+
ΔNRPS	Cream-colored, mucoid	+	+	+	+	+	–	–	+	+

[x] Chitinase (Chi), β-1,3-glucanase (Glu), protease (Pro), and lipase (Lip) activities were determined on media containing colloidal chitin, laminarin, skim milk, and Tween 80, respectively.

[y] Plate inhibition assays were conducted with bacterial cells on 10% tryptic soy agar against *Bipolaris sorokiniana* (Bs), *Pythium ultimum* var. *ultimum* (Pu), *Saccharomyces cerevisiae* (Sc), and *Clavibacter michiganensis* subsp. *nebraskensis* (Cm).

[z] Biosurfactant activity was based on spreading of culture fluid droplets on polystyrene compared with sterile 10% trypticase soy broth.

TABLE 3. Comparison of heat-stable antifungal factor (HSAF)-deficient mutant strains K19 and ΔNRPS with wild-type *Lysobacter enzymogenes* C3, *clp* mutant 5E4, and water control in five experiments for effects on incidence and severity of Bipolaris leaf spot on tall fescue

	Incidence (lesions/cm leaf)[x]					Severity (% diseased leaf area)[x]				
Treatment	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5
C3	10.2 B[y]	3.0 B	4.1 B	5.9 B	2.7 B	8.9 B	2.0 B	1.8 B	8.5 B	3.2 C
K19	11.6 B	4.1 AB	5.2 AB	–	5.1 A	19.8 AB	7.0 A	6.2 A	–	8.4 B
ΔNRPS	–[z]	–	–	9.9 A	4.6 A	–	–	–	18.5 AB	9.1 B
5E4	15.5 A	5.5 A	6.9 A	–	–	27.2 A	7.6 A	6.5 A	–	–
Water	15.2 A	5.4 A	6.1 A	9.8 A	5.7 A	29.4 A	8.6 A	7.2 A	22.0 A	20.5 A
P	0.005	0.054	0.036	0.031	0.002	0.053	0.034	0.019	0.020	<0.001
$LSD_{0.05}$	3.0	2.0	1.8	3.1	1.2	15.3	4.5	4.0	13.5	4.6

[x] N = 4 in all experiments.
[y] Values in a column followed by the same letter are not significantly different according to Fisher's least significant difference (LSD) test (α = 0.05).
[z] – = not tested.

TABLE 4. Comparison of heat-stable antifungal factor (HSAF)-deficient mutant strains K19 and ΔNRPS with wild-type *Lysobacter enzymogenes* C3 and water control in four experiments for effects on the severity of Fusarium head blight in wheat

	Disease severity (% diseased spikelets)[x]			
Treatment	Expt 1	Expt 2	Expt 3	Expt 4
C3	8.7 B[y]	10.6 B	35.6 B	5.1 B
K19	22.8 B	24.1 AB	29.9 B	3.0 B
ΔNRPS	–[z]	–	–	11.2 B
Water	57.5 A	36.2 A	71.3 A	27.0 A
P	0.010	0.033	<0.001	<0.001
$LSD_{0.05}$	27.0	18.5	18.4	9.6

[x] N = 6 in experiments 1 and 2; N = 5 in experiments 3 and 4.
[y] Values in a column followed by the same letter are not significantly different according to Fisher's least significant difference (LSD) test (α = 0.05).
[z] – = not tested.

infection by a fungal pathogen. Maintenance of hyphal polarity in *Colletotrichum gloeosporioides* was reported to be related to appressorium formation (9), and thus it is plausible that the target of HSAF regulates both biological processes. Natural product-derived fungicides with activity against specific fungal infection stages have been suggested as future tools for fungal disease control (34), and appressorium formation has been suggested as such a target (2). Dihydromaltophilin was effective in suppressing grape downy mildew caused by *Plasmopara viticola* when applied to grape foliage (7). Similarly, xanthobaccin A applied to sugar beet seed suppressed damping-off disease caused by *Pythium* spp. (27). The effects of these antibiotics on diseases caused by true fungi, however, had not been reported. With its unique mode of action (25), and its effectiveness in appressorium formation on the phylloplane and suppressing disease development shown in this study, HSAF may be a model for a new generation of fungicides.

In a study on *Lysobacter* sp. SB-K88 (27), one piece of evidence offered for xanthobaccins being involved in biological control by SB-K88 was the observation that spontaneous mutants exhibited reduced xanthobaccin production also were reduced in biocontrol activity. The mutants, however, were not checked for pleotrophic effects. In our experience with C3, such spontaneous mutants can be readily found, but loss of antibiotic production in these mutants is associated with loss of lytic enzyme activity as well, most likely because the mutations occur in regulatory genes, such as *clp*, which globally controls expression of antagonism traits in C3 (22). Through mutant strains K19 and ΔNRPS, which were disrupted specifically in the PKS-NRPS gene, we provide more definitive evidence that production of HSAF is a mechanism in the biological control of some fungal pathogens.

While we have not attempted to detect HSAF on the foliage of C3-treated plants, results with the HSAF-deficient mutants K19 and ΔNRPS suggest that the bacterium produces HSAF on the phylloplane of tall fescue in sufficient quantity to affect infection by *B. sorokiniana*. While HSAF appears to be a key mechanism in reducing the severity (symptomatic leaf area) of Bipolaris leaf spot, the antibiotic has a lesser role in suppressing leaf spot incidence (lesions per leaf length), as evidenced by the HSAF-deficient mutant K19 exhibiting only partially diminished ability to reduce infection incidence. The number of lesions on a leaf is dictated primarily by the success rate of prepenetration processes, i.e., spore germination and appressorium formation, whereas symptomatic leaf area is also determined by the degree to which the pathogen grows through the mesophyll and produces toxins (23). We surmise that *B. sorokiniana* is particularly sensitive during the prepenetration phase to any one of the multiple mechanisms of antagonism elaborated by C3. Thus, lytic enzymes excreted by C3 (29,40) or host resistance activated by C3 (19) may be sufficient to inhibit infection in the absence of HSAF. Inhibition of fungal metabolism and growth within leaf tissues, on the other hand, likely requires the full complement of mechanisms possible in C3, and thus, loss of HSAF production represents loss of an important component of a biocontrol system. This explanation is also consistent with the previous finding that loss in C3 of β-1-3-glucanase activity specifically also resulted in greatly reduced capacity of C3 to suppress the severity of disease caused by *B. sorokiniana* (29).

HSAF is not an important mechanism of biological control in all fungal disease situations as shown by the HSAF-minus mutants being unchanged from the wild-type C3 in their ability to control Fusarium head blight. These results, however, were not associated with low sensitivity of *F. graminearum* to HSAF, as purified HSAF was very active against the fungus in vitro. It is possible that HSAF was not produced by C3 on the surface of wheat heads and that biological control of *F. graminearum* by C3 was due instead, to induced resistance, as surmised in a previous study (17).

In conclusion, we demonstrated HSAF has the potential to arrest fungal growth and infection stages on the phylloplane and, consequently, suppress disease development. We also showed the importance of HSAF as a mechanism of biological control of certain fungal pathogens by C3. These findings, coupled with the previous discoveries of the unique mode of action of HSAF (25) and the genes for HSAF biosynthesis (37), point to the potentials of using C3 as a model for understanding the biosynthesis and activity of an entire family of antibiotics, and ultimately using them as the basis for developing new fungicides and identifying new strains of biocontrol agents.

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