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Research

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Insect-Symbiont Interactions

Antimicrobial Activity of Actinobacteria Isolated From the Guts of Subterranean Termites

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Abstract

Subterranean termites need to minimize potentially pathogenic and competitive fungi in their environment in order to maintain colony health. We examined the ability of Actinobacteria isolated from termite guts in suppressing microorganisms commonly encountered in a subterranean environment. Guts from two subterranean termite species, *Reticulitermes flavipes* (Kollar) and *Reticulitermes tibialis* Banks, were extracted and plated on selective chitin media. A total of 38 Actinobacteria isolates were selected for *in vitro* growth inhibition assays. Target microbes included three strains of *Serratia marcescens* Bizio, two mold fungi (*Trichoderma* sp. and *Metarhizium* sp.), a yeast fungus (*Candida albicans* (C.P. Robin) Berkhout), and four basidiomycete fungi (*Gloeophyllum trabeum* (Persoon) Murrill, *Tyromyces palustris* (Berkeley & M.A. Curtis) Murrill, *Irpex lacteus* (Fries) Fries, and *Trametes versicolor* (L.) Lloyd). Results showed both broad and narrow ranges of antimicrobial activity against the mold fungi, yeast fungus, and *S. marcescens* isolates by the Actinobacteria selected. This suggests that termite gut-associated Actinobacteria produce secondary antimicrobial compounds that may be important for pathogen inhibition in termites. Basidiomycete fungi were strongly inhibited by the selected Actinobacteria isolates, with *G. trabeum* and *T. versicolor* being most inhibited, followed by *I. lacteus* and *T. palustris*. The degree of inhibition was correlated with shifts in pH caused by the Actinobacteria. Nearly all Actinobacteria isolates raised pH of the growth medium to basic levels (i.e. pH ~8.0–9.5). We summarize antimicrobial activity of these termite gut-associated Actinobacteria and examine the implications of these pH shifts.

Key words: *Reticulitermes* spp., *Streptomyces* spp., pH, mold fungi, basidiomycetes

Subterranean termites live in an environment in which they are continually exposed to potentially pathogenic (e.g. mold and bacteria) and competitive (e.g. basidiomycete decay fungi) microorganisms (Kramm et al. 1982, Jayasimha and Henderson 2007). Healthy termite colonies and individuals, however, appear to be mostly devoid of microbial growth, suggesting inherent mechanisms of control. Various researchers have isolated and/or identified antimicrobial compounds from termites, including termicin and spinigerin (Lamberty et al. 2001), β-1,3 glucanase (Hamilton et al. 2011), and Gram negative binding proteins (Zeng et al. 2014). Rosengaus et al. (2004) identified antifungal activity produced in the sternal gland of the termite *Zootermopsis angusticollis*. In addition to endogenously derived antimicrobials, it is also possible that associated microorganisms may confer antimicrobial activity.

Associations with bacteria can play important roles in the ability of insects to adapt to novel environments and food sources (Maynard-Smith 1989, Kaltenpoth 2009). These roles include protection from pathogens, parasites, and predators (Brownlie and Johnson 2009). Bacterially mediated protection from pathogens has been observed in several systems, and the degree of specificity between the bacteria and insect host varies from loosely associated to strongly mutualistic linkages (Hölldobler and Wilson 1990, Currie et al. 1999, Kaltenpoth et al. 2005, Cardoza et al. 2006, Scott et al. 2008, Caldera and Currie 2012, Madden et al. 2013). Many of these interactions involve Actinobacteria, a phylum known to produce a diversity of antimicrobial secondary metabolites (Procópio et al. 2012). It is estimated that over 80% of medicinal antibiotics used today were derived from Actinobacteria, primarily members of *Streptomyces* (Procópio et al. 2012). Numerous studies to date have investigated antimicrobial activity of Actinobacteria isolated from a variety of insects (Poulsen et al. 2011, Seipke et al. 2011, Madden et al. 2013), including termites (Khucharoenphaisan et al. 2012, Matsui et al. 2012, Visser et al. 2012, Chouvenc et al. 2013) for the purpose of discovering novel antimicrobials. In one study,

Chouvenc et al. (2013) demonstrated increased termite survival caused by inhibition of the entomopathogen *Metarhizium anisopliae* by *Streptomyces* spp.

Although Actinobacteria have been shown to make up a small percentage of the total gut microbiota, they are consistently identified in culture-independent and culture-dependent studies of both higher and lower termite lineages (Watanabe et al. 2003, Fisher et al. 2007, Lefebvre et al. 2009, Berlanga et al. 2011, Boucias et al. 2013, Grieco et al. 2013, Arango et al. 2014). In this study, our primary goals were to isolate and characterize Actinobacteria associated with the gut of subterranean termites, and to evaluate antimicrobial activity of these isolates *in vitro*, with particular focus on inhibition of a variety of potentially invasive microorganisms.

Materials and Methods

Sample Collection and Termite Identification

Termite samples were collected from multiple sites within three locations in southern Wisconsin during the spring and summer of 2014 and 2015 (Table 1). Two subterranean termite species, *Reticulitermes flavipes* (Kollar) and *Reticulitermes tibialis* Banks, were identified morphologically and molecularly, as described in previous work (Arango 2015).

Selective Isolation of Actinobacteria

Ten worker termites (larvae of the third or fourth instar) from each collection sample were briefly frozen and surface sterilized in 70% EtOH, prior to dissection. Extracted guts (whole) were placed directly into 1 ml of nutrient broth and ground with a pestle. A 1:10 dilution series was prepared, and 100 µl from each dilution was plated on selective chitin media (Hanshew et al. 2014). Isolation plates were incubated aerobically at 30 °C, during which colonies exhibiting characteristic Actinobacterial morphology (Bergey and Holt 1994) were selected and isolated on nutrient agar plates supplemented with 0.5% glucose.

DNA Extraction, Sequencing, and Analysis

Whole genomic DNA was extracted from Actinobacterial isolates using the Masterpure DNA kit according to manufacturer's specifications (Epicentre – Illumina Co., Madison, WI). Polymerase chain reaction (PCR) was performed using the universal bacterial primer set 27F (5'- AGA GTT TGA TCM TGG CTC AG -3') and 1496R (5'- CGG TTA CCT TGT TAC GAC TT -3'), corresponding to hypervariable regions V1-V9. PCR was performed in a standard 50 µl reaction consisting of 2 µl DNA template (20 ng/µl), 10 µl 5X Buffer (Promega, Fitchburg, WI), 1 µl 10 mM dNTPs, 3 µl 25 mM $MgCl_2$ (Promega), 1 µl combined 10 µM primers, 0.2 µl GoTaq (5U/µl) (Promega), and 32.8 µl H_2O. Thermocycler conditions began with an initial denaturation step at 95 °C for 5 min followed by 30 cycles each consisting of 45 s at 92 °C, 1 min at 58 °C for annealing, and 1 min at 72 °C for extension followed by a post-cycle extension at 72 °C for 5 min. PCR products were confirmed on 1.5% agarose electrophoresis gel prior to being cleaned using the Promega Wizard SV Gel and PCR clean up system (Promega). Big Dye sequencing reaction was then performed followed by a secondary clean-up step prior to submission to UW Biotech for analysis (University of Wisconsin-Madison). Resulting 16S rRNA gene sequences, along with a number of related publically available sequences (GenBank) and a few obtained from other termite/insect studies (C. Currie lab isolates), were aligned with Infernal (Nawrocki and Eddy 2013). Phylogenetic analysis was conducted using the maximum likelihood method implemented in RAxML using the GTRGAMMA substitution model with 100 bootstraps (RAxML 2006). Final trees were constructed in iTOL (Interactive Tree of Life, Letunic and Bork 2011).

In Vitro Inhibition Assays

Inhibition of Potential Antagonistic Microorganisms

Bacterial and fungal isolates were selected for antimicrobial inhibition assays based on their potential to become a contaminant and entomopathogen within a termite colony, as observed in laboratory experiments and published reports. These included two mold species, *Metarhizium* sp. and *Trichoderma* sp., and three different strains of *Serratia marcescens*, one of which was isolated from dead termites (RH02-FPL). The yeast *Candida albicans* (strain K1) was also included for activity comparison although it is not known to be associated with subterranean termites.

Actinobacteria isolates ($n = 34$) were inoculated along the center line of the six inner experimental wells in a 12-well plate (Fig. 1a). Each well contained 3 ml of YPM agar (2 g yeast extract, 2 g peptone, 4 g mannitol, 15 g agar, 1 liter H_2O). Actinobacteria were incubated at 28 °C for 5 d prior to the addition of the test pathogens. For mold pathogens, spore stocks of each mold were diluted 1:10, resulting in final concentrations of 9.1×10^6 and 1.2×10^5 cells per ml for *Trichoderma* sp. and *Metarhizium* sp., respectively. A total of 3 µl was inoculated in the center of the experimental well and the pathogen control well. For *S. marcescens* and *C. albicans*, 3 ml of broth containing the microorganism was inoculated into sterile 14-ml tubes with Luria-Bertani (LB) or yeast peptone dextrose (YPD) broth, respectively. Cultures were then incubated overnight at 28 °C in a shaker and then diluted 1:10, resulting in final concentrations of 2.3×10^{-8}, 2.8×10^{-8}, 4.5×10^{-8}, and 3.2×10^{-7} for *S. marcescens* 8055, *S. marcescens* RH02-FPL, *S. marcescens* 8059, and *C. albicans*, respectively. Diluted cultures were again used to inoculate 3 µl in the center of the experimental well and the pathogen control well.

Inhibition assay plates were maintained at 28 °C for 7 d and then results were recorded. Experimental wells were assigned a rating from 0–3 depending on the level of inhibition (0—no inhibition, 1—slight inhibition, 2—presence of a zone of inhibition, 3—complete inhibition; Fig. 1b).

Table 1. Termite species and collection locations

Termite species	Collection location and GPS data	Habitat description
Reticulitermes flavipes	Janesville, WI N42°42.364 × W089°01.821	Small wooded area in the center of an urban area; south-central Wisconsin
	Muscoda, WI N43°12.157 × W090°25.372	Near home along the Wisconsin river; south-central Wisconsin
Reticulitermes tibialis	Hazel Green, WI N42°31.704 × W090°25.301	Under woody debris in the center of a north-facing, dry prairie; south-western Wisconsin

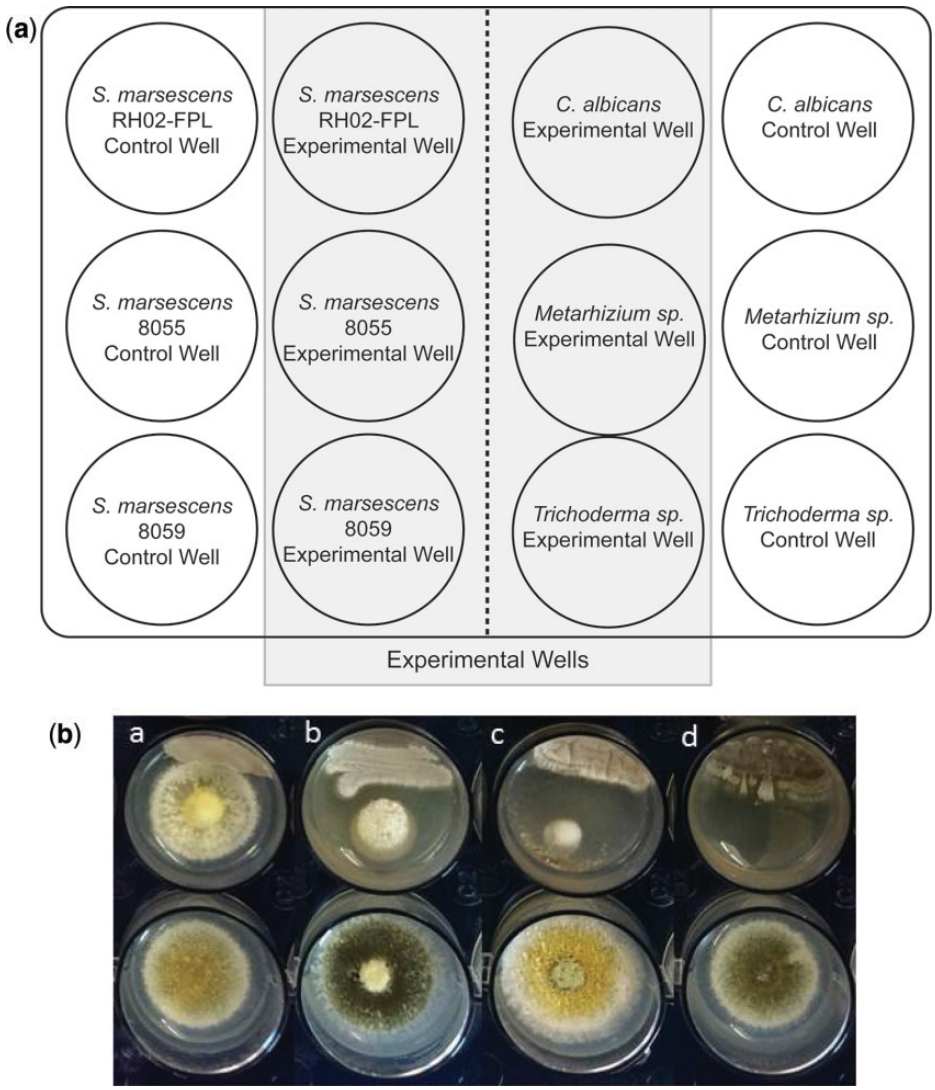

Fig. 1. (**a**) Experimental design for inhibition of potential entomopathogens; (**b**) experimental (top) wells showing no- to complete inhibition of mold fungi (ratings: 0–3) and controls (bottom). *Metarhizium* sp. used as an example for ratings 0 and 2, *Trichoderma* sp. used as an example for ratings 1 and 3.

Inhibition of Competitive Basidiomycete Fungi

Basidiomycete fungi were selected for inhibition based on their potential to serve as competitors for nutrients in dead, woody materials. This is supported by the observation that subterranean termites are rarely seen in wood with actively growing fungal hyphae. For these assays, two brown-rot fungi: *Gloeophyllum trabeum* MAD-617 and *Tyromyces palustris* 6137, and two white-rot fungi: *Irpex lacteus* MAD-517 and *Trametes versicolor* MAD-697, were obtained from the culture collection at the USDA-FS Forest Products Laboratory (Madison, Wisconsin). In vitro inhibition assays were performed on nutrient agar plates supplemented with glucose and yeast extract (nutrient agar + 0.5% glucose + 0.5% yeast extract). Actinobacteria used in part one were also used in basidiomycete inhibition assays with the addition of four additional isolates ($n = 38$ isolates). Actinobacteria were streaked on one side of square Petri plates and incubated for 2 d at 30 °C. A small, circular plug of freshly growing decay fungus (7 mm) was then inoculated ~4 cm from the edge of the bacterial streak and placed in an incubator at 28 °C (Supp. Fig. 1 [online only]). Three replicate plates of each Actinobacteria by fungus combination were assayed.

Plates were rated for growth inhibition based on the amount of growth both towards (r_2) and away from (r_2) the Actinobacterial streak (~5 d). The zone of inhibition (d) between the fungus and the Actinobacteria was measured once the fungus stopped growing completely (~8–10 d). Percent inhibition of radial growth was then calculated [$100 \times (r_1\text{-}r_2)/r_1$] (Fokkema 1973). Since basidiomycete fungi are thought to be strongly influenced by pH, two measurements were taken with a micro-pH probe (MI-414-4P Micro Combination pH Needle Electrode; Microelectrodes Inc., Bedford, NH) between the fungus and the bacteria and on the opposite side of the plate from the bacterial streak. Results were then averaged for each plate after completion of the assay and measurements were used to determine if pH and inhibition were correlated.

Role of pH on Growth of *S. marcescens* and Mold and Basidiomycete Fungi

Inhibition of basidiomycete fungi was observed to be strongly associated with Actinobacteria-derived pH changes to the media (see below), so the effects of pH alone on growth of fungi and *S.*

marcescens were examined. Flasks of YPM agar were adjusted to pH 5, 6, 7, 8, 9, or 10 (±0.2) with acetic acid (CH_3COOH) or sodium hydroxide (NaOH) prior to autoclaving. One strain of the bacterium, *S. marcescens* RH02-FPL and three mold fungi, *Trichoderma* sp., *Metarhizium* sp., and *Rhizopus oryzae* were added to plates by inoculating 1 µl of a 1:10 dilution of a spore stock solution (cells/ml: 9.1×10^6 for *Trichoderma* sp., 1.2×10^5 for *Metarhizium* sp., 3.6×10^4 for *R. oryzae*, 2.8×10^{-8} for *S. marcescens*) to the center of the plate. All mold fungi were tested on 12-well plates with the exception of *R. oryzae*, which was done on separate Petri plates to avoid contamination. Basidiomycete fungi (*T. palustris*, *G. trabeum*, *I. lacteus*, and *T. versicolor*) were tested by adding a 7 mm, circular plug of freshly growing fungus to the center of the plate. Mold and basidiomycete fungi were incubated for 5 d at 30 and 26 °C, respectively. Measurements of growth (diameter) and notes of morphological changes were recorded daily.

Statistical Analysis

Two-factor nonparametric multivariate analysis of variance (MANOVA) was performed with the basidiomycete fungi (4 levels) and Actinobacteria isolates (including fungal control, 39 levels) as factors in a rank transformations of the three responses: percent inhibition, zone of inhibition, and pH. Individual, nonparametric two-factor analysis of variances (ANOVA) for each rank-transformed response was also examined, and mean comparisons were evaluated. Mean comparison adjustments were made using Tukey's method when comparing fungi and Dunnett's method when comparing Actinobacteria isolates to the controls. To examine the relationships between pH, growth inhibition (%) and zone of inhibition, the nonparametric Spearman's rho (ρ) statistic was also calculated across bacterial isolates for each of the basidiomycete fungi. All analyses were conducted in SAS® V9.4 (SAS Institute Inc. 2013).

Results

Genetic Analysis of Isolated Actinobacteria

Actinobacteria were readily isolated from termite guts, resulting in a total of 38 isolates selected for evaluation in the inhibition assays (18 from *R. flavipes* and 20 from *R. tibialis*). BLAST searches of the 16S rRNA gene region of sequenced cultures suggest that all isolates are members of *Streptomyces*. One isolate, JVTS A, although putatively an Actinobacteria morphologically, could not be amplified with the primers used in this study and was therefore left unclassified. Phylogenetic analysis showed clustering by collection location and by termite species (Fig. 2). BLAST results indicated genetic similarity of isolates from this study and a number of other insect associated *Streptomyces* spp., (e.g. dung beetle, bark beetle, wood wasp, ant), which were incorporated into the tree.

In Vitro Inhibition Assays

Inhibition of Potential Antagonistic Microorganisms

Bioassay results ranged from no inhibition to complete growth inhibition of the yeast, *C. albicans*, *S. marcescens* isolates, and the two mold fungi (Table 2). Certain Actinobacterial isolates showed some level of inhibition against all of the organisms tested, indicating broad antimicrobial activity, while others showed selective inhibition against either *S. marcescens* or mold fungi. Based on the Actinobacteria selected, it did not appear that there was any increase or decrease in inhibition of the *S. marcescens* isolate associated with termites (RH02-FPL), nor were the three strains of *S. marcescens* inhibited equally.

Inhibition of Competitive Basidiomycete Fungi

Nearly all termite-associated Actinobacteria exhibited antifungal activity against the basidiomycetes tested. Based on the nonparametric MANOVA, there were significant differences of inhibition of the fungal and bacterial isolates measured as the multivariate response of percent inhibition, zone of inhibition, and pH. There was no interaction between fungus and isolate (Wilk's Lambda *p*-values for fungus factor $P < 0.0001$, isolate factor $P < 0.0001$, or interaction $P = 0.78$). Individual analyses of the three responses showed similar outcomes. Growth inhibition varied by bacterial isolate and fungal species, with no significant interaction (interaction $p = 0.91$). Among the Actinobacteria isolates, 31 of 38 had significantly higher growth inhibitions than the controls based on a Dunnett's adjustment. There were strong differences in measurements of the zone of inhibition (d) between the fungus and the Actinobacteria, with the smallest zone in groups tested against *T. palustris* and the largest zones measured in assays against *T. versicolor* (Fig. 3).

All Actinobacteria isolated in this study produced alkaline conditions in the growth media within the testing period, with pH measurements ranging from 8.0–9.5 in the absence of fungal growth. Control plates with the basidiomycete fungus alone indicated the optimal pH of the fungus is acidic (*I. lacteus*—6.5, *T. versicolor*—5.8, *G. trabeum*—7.0) to very acidic (*T. palustris*—2.5). Results from *T. palustris* are consistent with previous research showing this fungus lowers pH of its growth substrate by producing oxalic acid early in the decay process (Shimada et al. 1994, Arango et al. 2009). Thus, *T. palustris* was found to be the least inhibited by Actinobacteria induced shifts in pH. Although individual Actinobacteria isolates showed a range of activity across the various pH levels, results are suggestive of increasing fungal inhibition with increasing pH levels (Supp. Figs. 2 and 3 [online only]).

There were strong, positive associations ($P < 0.01$) between growth inhibition (%) and zone of inhibition (d), as well as between growth inhibition (%) and plate pH, and zone of inhibition and plate pH (Table 3). When controlling for pH in statistical analysis, the associations between growth inhibition and zone of inhibition are reduced, but still significant, with the exception of *T. palustris*, where a greater range of pH values were observed. This suggests that although pH has a significant inhibitory effect, there are likely additional factors contributing to growth inhibition (e.g. secondary metabolites), but to a lesser degree.

Role of pH on Growth of *S. marcescens* and Mold and Basidiomycete Fungi

The effect of pH alone on fungi and bacteria showed a variety of growth and morphological changes. Mold fungi were relatively tolerant of shifts in pH, and grew at all levels tested (Fig. 4). The rate of growth and the production of pigmentation, however, was reduced at the higher pH levels (8, 9, 10) in *Trichoderma* sp. and *R. oryzae*. This may translate to delayed sporulation at higher pH ranges. Growth of the bacterium *S. marcescens* (isolate RH02-FPL) was not inhibited at the higher pH levels, but rather was delayed at pH 5.

Basidiomycete fungi were much less tolerant to changes in pH than mold fungi, with most isolates showing complete growth inhibition at pH 9 and 10 over the 5 d assay (Fig. 5). The white-rot fungus, *T. versicolor*, was the most tolerant to pH shifts, and was the only basidiomycete that grew at pH 9. All basidiomycete fungi were able to grow on the pH 8 plates, but initiation of growth was slower than at the lower pH levels (i.e. pH 5-7).

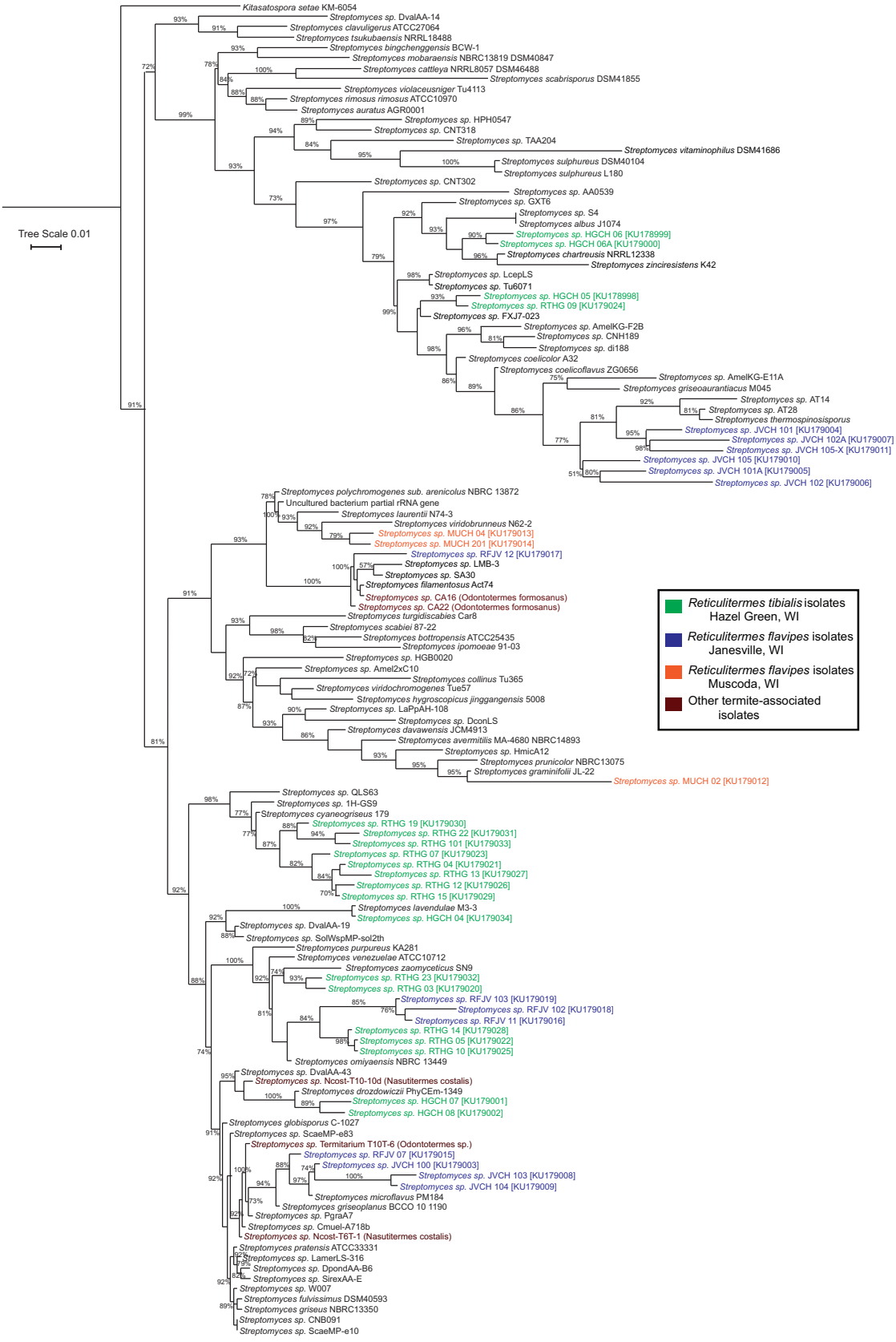

Fig. 2. Phylogenetic tree based on partial 16S rRNA sequences of Actinobacteria isolated from subterranean termite guts compared to other publically available *Streptomyces* and bacterial strains. Isolates used in this study are shown in green (Hazel Green, WI isolates; R.t.), blue (Janesville, WI isolates; R.f.), and orange (Muscoda, WI isolates; R.f.) and assigned accession numbers from the NCBI database are given in brackets. Other termite-associated strains are labeled with the termite species in parentheses in brown. Bootstrap values are based on 100 replicates. Percent support is given for all nodes $\geq$ 70%.

Table 2. Inhibition of potential entomopathogenic microorganisms arranged first by termite species and second by decreasing levels of microbial inhibition

	Isolate code	Mold fungi		*Yeast*	*Serratia marcescens*		
		Metarhizium sp.	*Trichoderma* sp.	*Candida albicans*	Strain 1 (8055)	Strain 2 (RH02-FPL)	Strain 3 (8059)
Reticulitermes tibialis isolates	HGCH 05	++	+++	++	++	++	++
	RTHG 13	++	+	++	+++	+++	++
	RTHG 22	++	++	++	++	++	+++
	RTHG 15	++	++	++	++	++	++
	RTHG09	++	+++	++	++	–	++
	RTHG19	++	++	+	++	++	++
	RTHG03	+	–	+++	++	++	++
	HGCH 04	++	+	++	++	++	–
	RTHG04	++	+	++	++	–	++
	HGCH 06A	++	+	++	–	+	–
	HGCH 06	++	++	+	–	–	–
	HGCH 07	–	–	–	++	++	–
	RTHG10	–	–	–	–	++	++
	RTHG23	–	–	–	–	++	–
	HGCH 08	–	–	+	–	–	–
	RTHG05	–	–	–	–	–	–
Reticulitermes flavipes isolates	JVCH 100	+++	++	+++	++	++	++
	JVCH 101	++	++	++	++	++	++
	JVCH 101A	+++	+++	+++	++	–	–
	JVCH 102	++	++	+	++	++	–
	JVCH 102A	+	–	++	++	++	++
	JVCH 103	+	++	+++	++	–	–
	JVCH 104	+++	–	+++	+	–	–
	JVCH 105	++	++	++	–	–	–
	JVCH 105X	++	++	–	++	–	–
	JVTS A	++	++	+	–	–	–
	MUCH 02	++	+	+	–	–	–
	MUCH 04	++	+	–	–	–	–
	MUCH 201	++	–	–	–	–	–
	RFJV102	+	–	+	–	–	–
	RFJV11	–	+	–	–	–	–
	RFJV12	–	–	–	–	–	–
	RFJV07	–	–	–	–	–	–
	RFJV103	–	–	–	–	–	–

No inhibition, 0 (–); slight inhibition, 1 (+); zone of inhibition present, 2 (++); complete inhibition, 3 (+++).

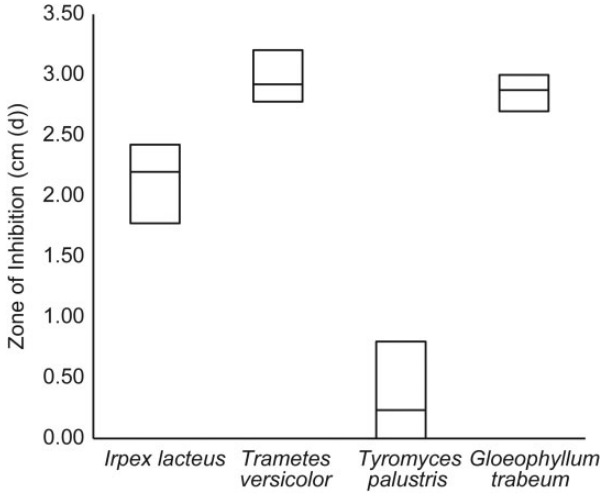

Fig. 3. Median (cm) zone of inhibition (d) values for all Actinobacteria isolates by basidiomycete fungus. Boxes represent the 25% and 75% quartiles.

Discussion

Genetic similarity of Actinobacteria isolates originating from the same location, and between isolates from the two termite species, suggests an environmental or colony component to gut bacterial populations, which is consistent with previous studies of Wisconsin termite populations (Arango et al. 2014). Clustering observed in phylogenetic analysis with other insect-associated Actinobacteria, however, may suggest a more permanent association between Actinobacteria and the termite gut. Book et al. (2016) identified a number of *Streptomyces* clades dominated by host-associated strains. Genetic similarity of Actinobacteria isolated in this study with bacteria from these host-associated lineages may be supportive of a more stable association. Further research, however, is required to fully understand this complex relationship.

Despite these sources of genetic variation, all of the isolates tested raised the pH of the growth medium to alkaline conditions during the inhibition assays. This characteristic has not previously been attributed to Actinobacteria associated with the termite gut, and has the potential to help clarify the function of these bacteria within the termite system. Shifts in pH alone can influence microbial

Table 3. A nonparametric Spearman's rho (ρ) statistic was calculated using individual plates between each pair of variables and for each bacterial isolate (growth inhibition (%), zone of inhibition, plate pH)

	Spearman correlation coefficient Prob > |r| under H_0: $\tilde{n} = 0$		Spearman partial correlation coefficient Prob > |r| under H_0: partial $\tilde{n} = 0$	
Fungus	Growth inhibition[a] × Zone of inhibition[b]	Growth inhibition × pH	Zone of inhibition × pH	Growth inhibition × Zone of inhibition (Adjusted for pH)
G. trabeum	0.36 **	0.41**	0.51**	0.30**
T. palustris	0.36**	0.44**	0.67**	0.07
I. lacteus	0.60**	0.38**	0.54**	0.49**
T. versicolor	0.72**	0.46**	0.48**	0.51**

[a] Growth inhibition (r-values).
[b] Zone of inhibition (d values) as measured in Supp. Fig. 1 (online only).
**P*-value < 0.01.

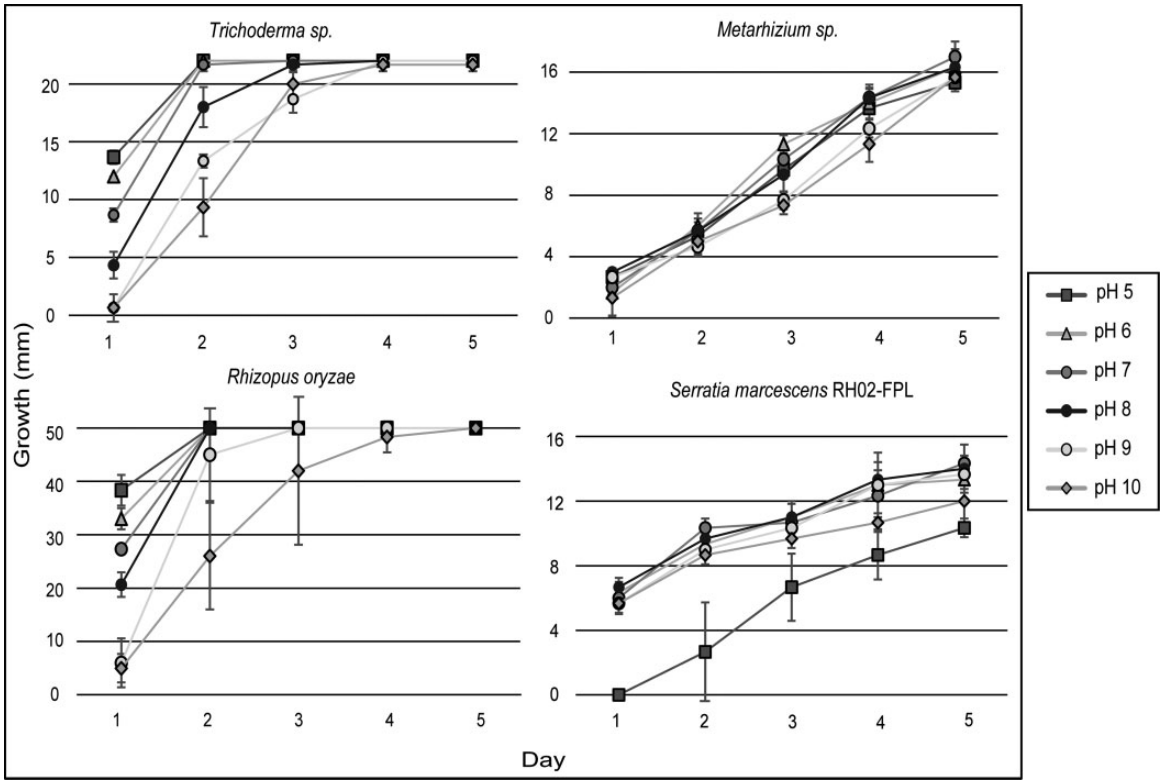

Fig. 4. Growth of *S. marcescens* and mold fungi on YPM plates with pH ranging from 5–10.

growth. In vitro assays against potentially competitive basidiomycete fungi indicate that Actinobacteria-induced pH shifts significantly reduced growth inhibition, possibly independent of or in conjunction with any Actinobacteria produced antifungals. These results agree with findings from other studies examining fungal growth and pH. For example, Ejechi and Obuekwe (1996) found that the brown-rot basidiomycete fungus *Gloeophyllum* was particularly sensitive to changes in pH and grow optimally at pH 6.5 or less, compared to the mold fungi, *Trichoderma viride* and *Penicillium* sp., which grew best under conditions approaching neutral pH. Another potential explanation for how high pH reduces basidiomycete growth could be inhibition of iron chelation by siderophores (Slade et al. 1986, Jellison et al. 1990, Eisendle et al. 2004).

Inhibition assays against mold fungi and *S. marcescens* showed a broad range of antimicrobial activity, which are unlikely to have been caused solely by shifts in pH, but rather production of antimicrobial secondary metabolites. Elevated pH levels were, however, shown to reduce growth rate in certain test organisms. At the higher pH ranges, growth was delayed in mold fungi, specifically both strains of *Trichoderma* sp. and *R. oryzae*, but, by day five measurements of growth were nearly equal across all pH ranges. *Metarhizium* sp., a known entomopathogen, was able to tolerate all test pH conditions equally. The one *S. marcescens* strain examined showed a slight delay in growth at the lower pH range (i.e. pH 5), but no inhibition at the higher pH levels. Slowing mold growth or inhibiting sporulation by Actinobacteria-induced alterations in pH could potentially contribute to colony protection, by allowing time for colony maintenance and

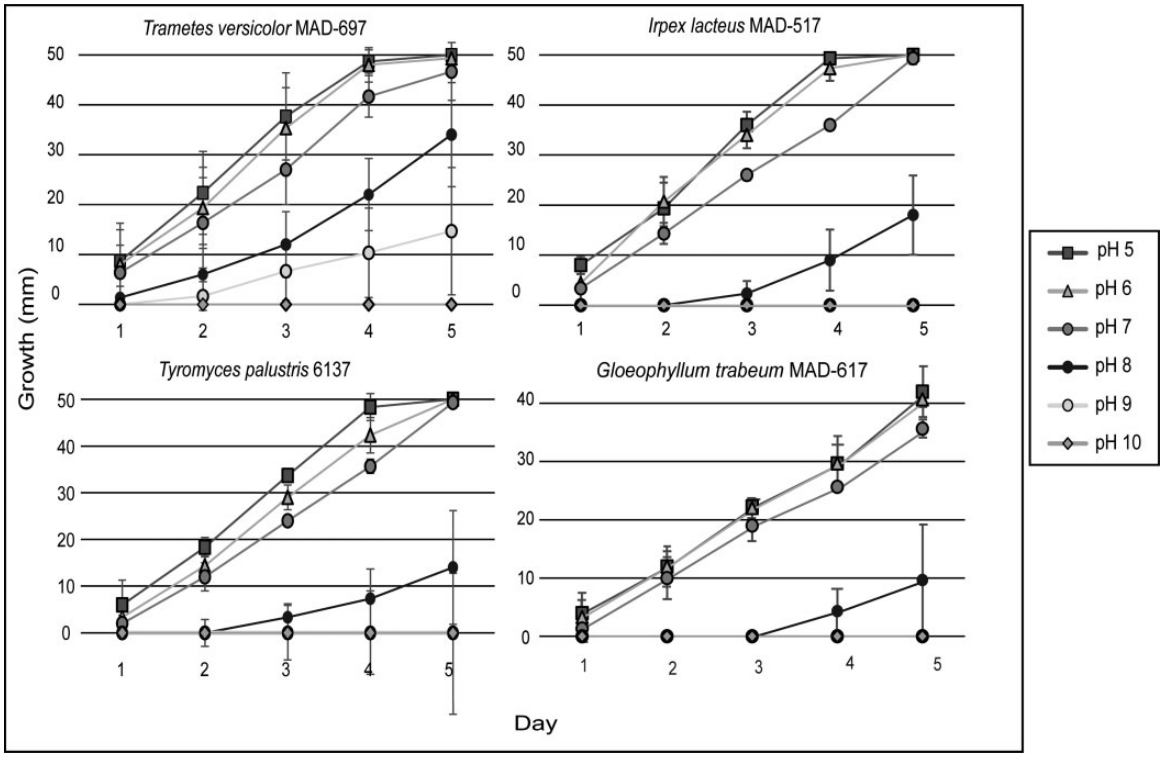

Fig. 5. Growth of basidiomycete fungi on YPM plates with pH ranging from 5–10.

grooming behaviors by workers, effectively controlling a pathogen before it can infect the colony.

In summary, Actinobacteria, specifically *Streptomyces* spp., were easily isolated from *Reticulitermes* spp. guts, and expressed a diversity of antimicrobial characteristics against potentially invasive microorganisms *in vitro*. Alteration of pH by these Actinobacteria is a novel observation that may eventually help elucidate relationships between subterranean termites and Actinobacteria. Future research will focus more closely on the implications of Actinobacteria-produced shifts in pH, specifically their role in the termite gut and implications for termite survival and colony-wide microbial control.

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