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Research

Pine Engravers Carry Bacterial Communities Whose Members Reduce Concentrations of Host Monoterpenes With Variable Degrees of Redundancy, Specificity, and Capability

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Abstract

Bark beetles are eruptive forest insects that have the potential to cause landscape level mortality to conifer forests. The pine engraver, *Ips pini* (Say) (Coleoptera: Curculionidae), is the predominant pest of mature red pine (*Pinus resinosa* Aiton) plantations throughout the Great Lakes region of North America. Pine engraver attack elicits a localized response by host trees in which concentrations of terpenes rapidly exceed the tolerance levels of beetles and their fungal associates. We considered how bacterial associates degrade these toxins from the perspective of the symbiont communities of individual beetles. We demonstrate that 1) most pine engravers harbor bacterial communities that reduce monoterpene concentrations in vivo; 2) several individual bacterial isolates can reduce monoterpenes even at high concentrations; and 3) bacteria isolated from pine engravers are similar to those found in other bark beetles. Bacteria isolated from pine engravers decreased concentrations of (–)-α-pinene, myrcene, and 3-carene. Most beetles carried at least one bacterial isolate that reduced concentrations of at least one monoterpene. Different bacteria vary in the uppermost concentrations at which they can degrade monoterpenes. The community of bacteria associated with an individual beetle appears to have some manner of functional redundancy that could collectively increase the likelihood of successful host colonization.

Key words: bark beetle, microbial degradation, symbiosis, functional redundancy

Bark beetles (Curculionidae: Scolytinae) are subcortically feeding herbivores that can affect landscape processes, shape forest species compositions, and cause severe economic losses. For example, outbreaks of mountain pine beetle (*Dendroctonus ponderosae* Hopkins) (Coleoptera: Curculionidae), spruce beetle (*Dendroctonus rufipennis* Kirby) (Coleoptera: Curculionidae), and greater European spruce beetle (*Ips tyographus* L.) have caused extensive damage to various conifer species (Gregoire et al. 2015). Other species such as red turpentine beetle (*Dendroctonus valens* LeConte) (Coleoptera: Curculionidae) largely predispose trees to other agents in their native ranges but cause substantial damage in their introduced range (Yan et al. 2005). These outbreaks affect diverse ecosystem processes and cause substantial timber losses (Safranyik and Carroll 2006, Kurz et al. 2008, Campbell and Antos 2015, Pec et al. 2015).

Bark beetles complete most of their life history within their host plants, with the most economically important forest species primarily affecting conifers (Vega and Hofstetter 2015). Individuals of one sex select a host, tunnel through the bark into the phloem, and produce pheromones that attract both sexes (Blomquist et al. 2010). After mating, the female constructs a gallery along which she oviposits. The larvae eclose, feed on phloem and fungi, and pupate. Adults emerge from trees to search for new hosts.

Conifers possess formidable defenses, which bark beetles must overcome to successfully reproduce and develop. When entering a tree, beetles must contend with integrated physical and chemical components of defense, including resin ducts that exude large amounts of oleoresin (Franceschi et al. 2005). The chemical composition of oleoresin varies among conifer species but is dominated by terpenes (Keeling and Bohlmann 2006, Clark et al. 2014, Raffa 2014). Even at constitutive levels, the concentrations of monoterpenes in resin are often toxic to adult beetles (Raffa et al. 1995, Reid and Purcell 2011). Trees respond to attack by rapidly increasing monoterpene concentrations at the point of entry (Raffa et al. 1995, Keefover-Ring et al. 2016, Mason et al. 2017). High concentrations of monoterpenes are behaviorally repellant and toxicologically lethal to beetles, whereas diterpenes are more toxic to their fungal

associates (Kopper et al. 2005, Boone et al. 2013, Mason et al. 2015, Klepzig et al. 2015). Concentrations of monoterpenes present in local tissues during rapid-induced responses are lethal to most of the beetles (Raffa et al. 1995, Reid and Purcell 2011). In addition to defending against initial attackers, conifers can sometimes produce sufficiently high quantities of monoterpenes (Erbilgin et al. 2003, 2006) and antiaggregation compounds such as 4-allylanisole (Hayes and Strom 1994) to prevent entering beetles from eliciting the pheromone-mediated high attack densities needed to overcome tree defenses (Raffa et al. 2008).

Bark beetles are associated with diverse microbial communities that facilitate their ability to overcome host nutritional and defensive barriers to survival and reproduction (Six and Klepzig 2004, Aylward et al. 2014). Fungal associates, such as yeasts and ophiostomatoids, range from obligate to facultative, and can provide nutrients and improve digestive efficiency (Ayres et al. 2000, Bentz and Six 2006) and detoxify phenolics and perhaps terpenes (DiGuistini et al. 2011, Hammerbacher et al. 2013, Wadke et al. 2016). Beetles also harbor opportunistic fungi that compete with larvae and their symbionts for phloem (Klepzig and Wilkens 1997, Cardoza et al. 2006).

The bacterial communities of bark beetles are less well studied than fungi and appear less diverse than in some other insects but include a relatively stable core of members (Morales-Jiménez et al. 2009, Aylward et al. 2014) that may assist beetles in contending with their subcortical environment. Bacterial symbionts of bark beetles appear to play roles in nitrogen acquisition (Morales-Jiménez et al. 2012), detoxification (Adams et al. 2011, Boone et al. 2013, Xu et al. 2015), stimulation of beneficial fungi (Adams et al. 2009, 2011; Therrien et al. 2015), inhibition of antagonistic fungi (Cardoza et al. 2006, Scott et al. 2009), and cellulose degradation (Delalibera et al. 2005). Unlike some other insects (Douglas 1998, Moran et al. 2008), bark beetles have no known obligate bacterial associates but rather a consortium of common facultative members such as *Serratia*, *Erwinia*, and *Rahnella*. Additionally, communities of bacteria associated with bark beetles may be enriched with genes that encode for enzymes associated with monoterpene degradation pathways (Adams et al. 2013). However, there is limited evidence that bacteria can reduce high concentrations of monoterpenes (Adams et al. 2013, Boone et al. 2013).

Bacterial communities associated with bark beetles are multifunctional, with multiple members performing various roles within the same species (Six 2013, Vega and Hofstetter 2015). It is unknown however, how much functional redundancy exists on an individual beetle basis. Most previous work on bacteria-bark beetle interactions has focused on how a specific bacterial associate may confer an advantage to the host beetle, but the frequencies of these associations are poorly known (Adams et al. 2010). Moreover, we have little quantification of functional communities from the beetle perspective, i.e., what proportion of beetles carry communities that have at least one member that can perform a particular function.

The pine engraver (*Ips pini* [Say] Coleoptera: Curculionidae) is transcontinentally distributed across North America (Wood 1982) and is the primary tree-killing pest of mature red pine, *P. resinosa* Aiton, in the Great Lakes region (Klepzig et al. 1991). It usually attacks weakened trees stressed by drought or root-feeding organisms. During colonization, pine engravers inoculate trees with the fungus *Ophiostoma ips* (Rumbold) Nannfeldt, which can facilitate some features of beetle development (Kopper et al. 2004) but also elicits tree defensive responses, especially local induction of monoterpenes (Raffa et al. 1995, Mason et al. 2017).

Our objectives were to 1) quantify the frequency and functional redundancy of pine engraver-associated bacterial communities that decrease concentrations of monoterpenes present in pines, in vitro, 2) assess the ability of individual bacteria to reduce concentrations of multiple monoterpenes, 3) determine the dosage of three monoterpenes needed to constrain the terpene-reducing activities of bacteria, and 4) compare bacteria associated with pine engravers to bacteria in other studies isolated from similar organisms.

Methods and Materials

Isolation of Bacteria

Pine engraver adults were collected from a red pine plantation established in 1964 by the Wisconsin Department of Natural Resources near Mazomanie, Dane County, Wisconsin. Insects were collected in June 2016 using multiple funnel traps (Lindgren 1983) baited with a 100 mg ipsdienol bubblecap lure (IP034; Chemtica Internacional) plus a lanierone bubblecap lure (IP043; Chemtica Internactional) suspended 1.3 m above-ground between red pine trees. Beetles were collected live to avoid cross-contamination (Aukema et al. 2005), by cutting open both ipsdienol and lanierone bubblecap lures and checking traps after 4 h (Pfammatter et al. 2016). Bacterial communities associated with 10 pine engraver adults were isolated by pulverizing live beetles to prevent contamination. To include both gut and surface bacterial communities in our analysis, beetles were not surface sterilized.

Bacterial communities were isolated from each adult by homogenizing individual beetles in 1× phosphate-buffered saline (PBS) containing 0.1% Tween and plating triplicate samples on 10% tryptic soy agar (3 g of tryptic soy broth [TSB], 15 g agar, and 1-liter distilled water). Unique morphologies from each beetle were selected and subcultured until a single colony morphology was visible on each plate. Individual bacterial isolates were stored in an incubator at 24°C for 4 d before monoterpene exposure experiments.

Screening Isolates for Potential Monoterpene Degradation

Eighty individual bacterial isolates were screened independently for potential monoterpene degradation ability relative to 10 controls (no bacteria), following the approach of Adams et al. (2013) and Boone et al. (2013). Nonbacterial controls were used to establish a baseline for assessing relative monoterpene recovered. Bacterial isolates were inoculated into 2-ml 10% TSB in sterile test tubes and shaken for 4 d at 24°C, until all samples containing bacteria were turbid, while the controls exhibited no bacterial growth. All samples were amended with 5 µl of 95% (–)-α-pinene (Sigma-Aldrich) because it is the primary monoterpene of the host trees of pine engravers in the Great Lakes region (Raffa and Smalley 1995, Mason et al. 2017) and is repellant and toxic to beetles at high concentrations (Raffa et al. 2005). Samples were shaken for 36 h. Bacterial growth was terminated by freezing samples overnight in a –30°C freezer. After thawing samples, 1 ml of *n*-hexane was added to each sample and shaken for 4 h. One hundred microliters of the *n*-hexane-monoterpene phase was transferred to 2-ml autosampler vials with 400-µl glass inserts and open top caps with polytetrafluoroethylene (PTFE)/silicone septum. The *n*-hexane-monoterpene phase was diluted with 100-µl *n*-hexane containing 5 µl/ml *m*-xylene as an internal standard.

Concentrations of monoterpenes were analyzed by gas chromatography (GC) using a Cyclodex-B enantioselective capillary column (30 m × 0.25 mm I.D., film thickness 0.25 µm; Agilent Technologies) and helium as the carrier gas at 1 ml/m. Two microliters of each sample was injected (260° injector temperature) into a Hewlett Packard 5890 GC in the split mode (30:1 split ratio) and quantitated with a

flame ionization detector (250°C detector temperature). Runs were 27.2 min long with a ramp of 3°C/min to 200°C (Keefover-Ring et al. 2016). Peak areas were integrated, and isolates were compared to nonbacterial controls by plotting (Howe 2017). We selected 22 isolates that showed promising biological activity, including at least one isolate/beetle (Supp Fig. 1 [Online only]).

Frequencies and Functional Redundancy of Bacteria That Decrease Concentrations of Monoterpenes

The 22 bacterial isolates and 12 nonbacterial controls were grown in triplicate in 2 ml of TSB in 4-ml screw thread vials with PTFE septa for 4 d. Isolates and four of the controls were assigned to each of the three in vitro monoterpene treatments: 95% pure (–)-α-pinene, 90% pure myrcene, or 95% pure (1*S*)-(+)-3-carene. Both enantiomers of α-pinene were recovered, but (+)-α-pinene was omitted because there was no difference between the two compounds (linear model of (–)-α-pinene vs (+)-α-pinene; $R^2 = 0.99$; Supp Fig. 2). Five microliters of the respective treatment monoterpene was added to all samples including the nonbacterial controls and shaken for 36 h. All samples were placed in a –30°C freezer to terminate bacterial growth. Remaining monoterpene was extracted with 1-ml of *n*-hexane, and 100 µl of *n*-hexane/monoterpene mixture was diluted with 100-µl of *n*-hexane containing 2.5 µl/ml *m*-xylene. Samples were analyzed by GC using the same method as previously described.

Dose–Response Interactions of Bacterial Isolates with Monoterpenes

Based on the results of the previous experiment, five bacterial isolates were selected for an in vitro dose–response experiment. Bacterial isolates were each subjected to five doses (1, 2.5, 5, 10, and 20 µl) of each monoterpene individually with three technical replicates per isolate/monoterpene/dose combination (three technical replicates by three monoterpenes by five doses) and 45 nonbacterial controls (three replicates by three monoterpenes by five doses). These doses were selected to span the range of monoterpene concentrations of host constitutive and induced tissue (Raffa and Smalley 1995, Mason et al. 2017). The 20-µl doses of represent a 1% (v/v) monoterpene concentration which corresponds to the induced concentrations of (–)-α-pinene biosynthesized in both jack and red pines. Bacteria were grown for 4 d, frozen, and extracted. Monoterpene concentrations were analyzed by GC using the same method as previously described.

Bacterial Sequencing

The 22 bacterial isolates used in the first experiment were sequenced. DNA was extracted using a Masterpure DNA kit (Epicenter Cat. No. MC85200) and 16s RNA universal bacterial primers 27F—5′-AGA-GTT-TGA-TCM-TGG-CTC-AG-3′ and 1492R—5′-CGG-TTA-CCT-TGT-TAC-GAC-TT-3′ (Promega) were used for polymerase chain reaction (95°C for 5 min; 30 × [92°C for 45 s; 58°C for 1 min; 72°C 1 min]; 72°C for 5 min) using GoTaq Green Master Mix (Promega). PCR products were run on an agarose gel with a 100 basepair ladder as a standard for 1 h at 100 V. Since the samples only had one band of DNA, samples were submitted without doing PCR clean up. Samples were submitted to the University of Wisconsin Biotech Center for sequencing.

Sequences were aligned using the ClustalOmega algorithm in GenomeCompiler. Pairwise comparisons were calculated for each isolate used in this study (Supp. Fig 3 [Online only]). Comparison sequences (Supp. Table 1 [Online only]) were obtained from NCBI Blast using the search words '*Dendroctonus*' and '*Ips pini*,' and type sequences were adapted from Mason et al. (2015). Multiple sequences were aligned using ClustalOmega multiple alignment tool, trimmed at the 5′ and 3′ ends, and then realigned. This alignment was used to construct a molecular phylogeny based on maximum likelihood, using a GTR+I+G model and 1,000 bootstraps (Kumar et al. 2016). Branches that were represented in <50% of bootstrap replicates are collapsed. High-quality sequences were deposited in GenBank under accession numbers (MG926577:MG926584).

Statistical Analysis

All statistical analyses were performed in R Studio (version 1.0.136). Signal peaks from the GC were integrated and standardized based on the *m*-xylene internal standard (peak area/internal standard peak area). Proportional change versus the nonbacterial controls was calculated based on the pooled (all monoterpenes together) median nonbacterial controls [(standardized peak area – median nonbacterial control)/median nonbacterial control] for investigating frequency and functional redundancies of bacterial communities, while dose-response controls were calculated for each monoterpene-dose combination independently. Controls were pooled in the first case to accommodate sample size. Samples were relativized based on nonbacterial controls because the sample size was much lower than the experimental sample sizes, which precludes us from using most direct parametric tests. Median control was used because of one pooled control outlier, which was omitted for analysis (chi-squared tests for outliers [$\chi^2 = 6.27$, $P = 0.012$]). Conclusions were not changed by using medians instead of means.

Models of monoterpene reduction relative to the control were calculated using independent one-sample Student *t*-tests for each compound with a null hypothesis of $\mu = 0$, representing no change relative to the nonbacterial controls. The 22 samples for each *t*-test were sufficient for a one-sample *t*-test to behave appropriately and no assumptions were violated. Pearson's correlation tests were used to assess if change in monoterpene concentrations were correlated among the three monoterpenes. Dose–response curves were generated using the ggplot2 package in R. Multiple linear regression was used to fit a model of the relationship between monoterpene added and relative monoterpene recovered. Assumptions of heteroscedascity, linearity, and normality for multiple linear regression were examined by plotting and were met for each analysis. The effects of dose, treatment, and their interaction on relative monoterepene recovered were analyzed by analysis of variance (ANOVA), and assumptions of heteroscedascity, linearity, and normality were met for an ANOVA.

Results

Comparison of Monoterpene Recovered Between Bacterial Samples and Controls

Monoterpene concentrations recovered from isolates of the bacterial community in pine engraver were on average 14% lower ($t = -6.22$, $P < 0.001$) than from nonbacterial controls (Fig. 1). The bacterial samples amended with 3-carene were 15% lower than nonbacterial controls, while (–)-α-pinene and myrcene were 14% and 12% lower, respectively (Table 1). Thirty-three bacterial samples fell outside of the 99% confidence interval for pooled nonbacterial controls. There was no relationship between monoterpene recovered on an isolate basis [(–)-α-pinene: myrcene, $t = 1.7$, $P = 0.104$; (–)-α-pinene: 3-carene, $t = 0.04$, $P = 0.972$; myrcene: 3-carene, $t = 0.93$, $P = 0.363$].

Beetle Bacterial Communities Have Varying Abilities to Decrease Monoterpene Concentrations

Bacterial communities showed varying abilities to decrease monoterpenes (Fig. 2). Beetles 1, 4, 7, 9, and 10 all had at least four isolates

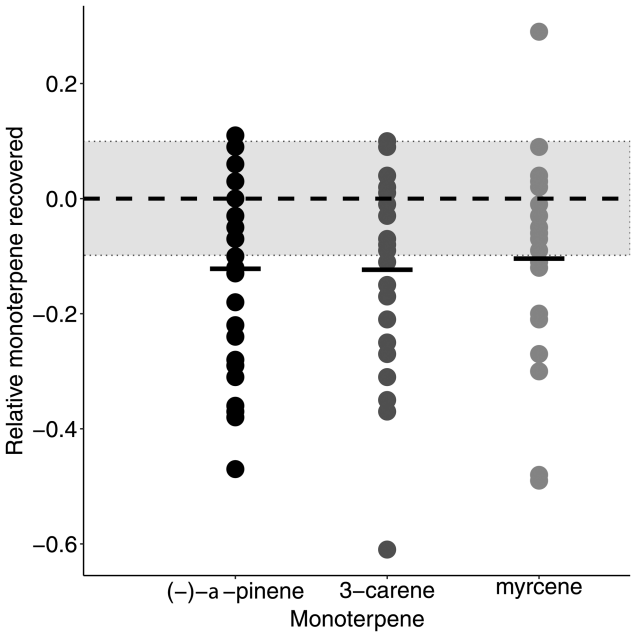

Fig. 1. Monoterpene recovered relative to pooled nonbacterial controls for 22 different bacteria isolated from pine engravers. Mean reduction is represented by solid black line for each monoterpene. The gray box represents the 99% confidence interval for pooled nonbacterial controls.

Table 1. Mean estimates of percent reduction in monoterpene concentration for 22 samples relative to the median control for each monoterpene

Monoterpene	Mean estimate	T-statistic	*P*-value
(−)-α-pinene	−0.14	−3.76	**0.001**
3-carene	−0.15	−3.91	**<0.001**
Myrcene	−0.12	−3.02	**0.006**

Significance assessed by independent Student *t*-tests (H_0: $\mu = 0$) with 21 degrees of freedom. Bold denotes significance at the $\alpha = 0.01$ level.

that decreased monoterpenes by more than the 99% confidence interval for nonbacterial controls. Nine out of 10 beetles carried at least one bacterium that reduced monoterpene concentrations by more than the 99% confidence interval for pooled nonbacterial controls (Fig. 2). Three beetles carried communities of bacteria that reduced only one monoterpene, and five beetles carried bacteria that reduced all three monoterpenes (Fig. 2). Most beetles (8/10) carried bacteria that reduced 3-carene by at least 10%, while seven and five reduced myrcene and (−)-α-pinene by at least 10%, respectively. There was also one positive outlier (from beetle 8) that represented a 20% increase in monoterpene concentration relative to the controls, but this result was likely due to a pipetting error.

Functional Redundancy of Bacteria Decreasing Monoterpene Concentrations

The bacterial communities carried by pine engravers have varying numbers of isolates that decrease concentrations of each monoterpene (Fig. 3). Half of the beetles sampled carried bacteria that reduced 3-carene by at least 30%, myrcene by 20%, and (−)-α-pinene by 10%. Most beetles (3-carene, 80%; myrcene, 60%) only carried one isolate that reduced 3-carene and myrcene by more than the 99% confidence interval for pooled nonbacterial controls. Half of

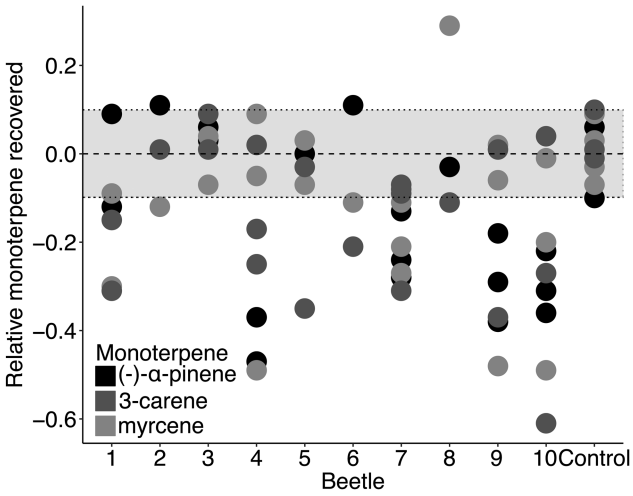

Fig. 2. Concentration of monoterpene relative to control for 22 bacterial isolates sorted by source beetle. Monoterpene recovered relative to control represents percent change relative to median nonbacterial control for each monoterpene. Gray box represents 99% confidence interval for pooled nonbacterial controls.

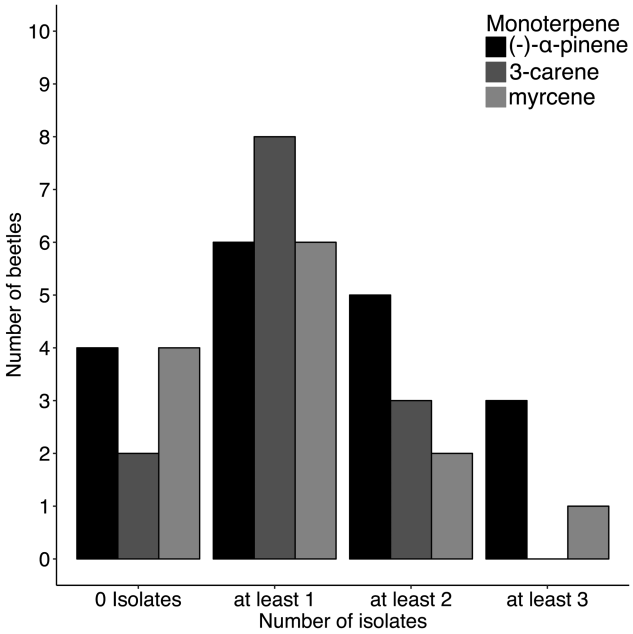

Fig. 3. Number of beetles with a bacterium that reduced monoterpenes by more than the 99% confidence interval for nonbacterial controls versus the number of isolates.

the pine engravers carried two isolates that reduced (−)-α-pinene and 30% of beetles carried three isolates that reduced (−)-α-pinene.

Bacteria isolated from the same beetles were at most 80% similar to each other (Supp Fig. 3), indicating these isolates represent distinct species. Sequence similarities of 97% are accepted for assigning species level (Schloss and Handelsman 2005).

Bacterial Response to Monoterpene Doses

Various bacterial samples exhibited different responses to increases in monoterpene dose (Fig. 4). Across all isolates, there was a 19% decrease in monoterpenes recovered relative to nonbacterial controls. For all five doses applied, there was a decrease in monoterpene recovered relative to the nonbacterial controls; 1.0 µl was decreased

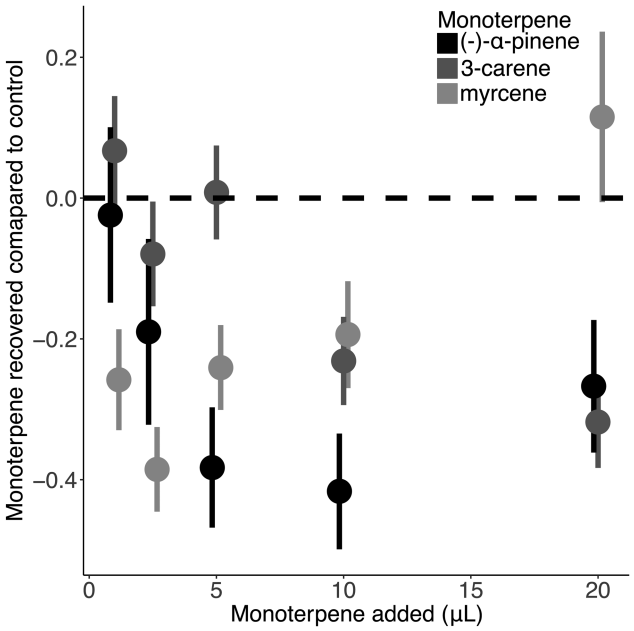

Fig. 4. Response of bacteria to five doses of monoterpenes. Points represent mean monoterpene recovered/(dose by bacteria) relative to median nonbacterial controls. Error bars represent standard error. Points are jittered for clarity.

by 7%, 2.5 µl by 22%, 5 µl by 21%, 10 µl by 28%, and 20 µl by 16%. There was not a significant effect of dose on monoterpene recovered relative to nonbacterial controls (F = 0.27, P = 0.60), and the effect of monoterpene added was moderately significant (F = 3.43, P = 0.03). There was however, a strong dose by monoterpene interaction present (F = 14.43, P < 0.001).

Composition of Bacteria From Pine Engravers That Reduce Monoterpene Concentrations

Bacteria used in this study are similar to bacteria previously isolated from pine engravers and *Dendroctonus* spp. beetles (Fig. 5). Within and among each beetle community, there were no bacteria more than 80% similar to each other (Supp Fig. 3). Isolates were not identified to genus level because of shortness of sequence reads and region of 16S-rRNA targeted. We used a phylogenetic approach to compare isolates with isolates from pine engravers and *Dendroctonus* spp. Many of the sequences isolated in this study are closely related to unidentified bacterium previously isolated from pine engravers and are similar to *Erwinia* spp., *Serratia* spp., and *Enterobacter* spp.

Discussion

These results indicate that most pine engravers carry bacteria that reduce concentrations of monoterpenes present in their host plants, in vivo. Communities of bacteria associated with individual beetles vary in their abilities to decrease monoterpene concentrations, responses to different monoterpenes, and functional redundancies. These communities contend with (–)-α-pinene by having multiple members that moderately decrease concentrations, while they contend with 3-carene and myrcene by having one member that greatly decreases concentrations. This appears to correspond with patterns in host tree chemistry. Throughout the Great Lakes region, pine engravers attack red pine, jack pine (*Pinus banksiana*), and white pine (*Pinus strobus*), all of which have defenses that are comprised of about 70% (–)-α-pinene and only 1–3% 3-carene and myrcene (Erbilgin et al. 2001, Aukema et al. 2010, Mason et al. 2017).

Bacteria isolated from pine engravers appear to have different tolerances to increasing doses of monoterpenes, similar to variation observed among symbionts of *Dendroctonus* species. A 1% (v/v) concentration (equivalent to our 20-µl dose) of 3-carene and (–)-α-pinene prevented growth of bacterial associates of mountain pine beetle and inhibited growth of bacterial associates of red turpentine beetle, while 1% myrcene stimulated growth of bacterial associated mountain pine beetle and did not affect associates of red turpentine beetle (Adams et al. 2011). The 20-µl dose of (–)-α-pinene also approximates the induced concentrations biosynthesized in both jack and red pines, which is lethal to almost 100% of pine engraver adults within 48 h. (Raffa et al. 1995). Mountain pine beetles subjected to approximately the same levels of volatile monoterpenes lost about 15% water weight, and survivorship decreased by about 30% after 24 h of exposure (Manning and Reid 2013, Reid et al. 2017).

The fates of the monoterpenes degraded by these bacteria are unknown. No new peaks were detected in these chromatograms (Supp Figs. 4–6), indicating test compounds were not converted to other *n*-hexane soluble compounds. Two suggested fates of (–)-α-pinene degraded by bacteria have been proposed (Marmulla and Harder 2014): 1) oxidation to (–)-α-pinene oxide and ring cleavage to isonovalal, which is then isomerized to novalal or 2) degradation via limonene and pinocarveol. If (–)-α-pinene were oxidized by these bacteria to (–)-α-pinene oxide, or similarly to verbenone (Brand et al. 1975, Xu et al. 2015), those compounds would have been detected under our GC conditions. Transformation of α-pinene oxide to isonovalal has been demonstrated with *Pseudomonas fluorescens* (Fontanille et al. 2002), but it is unknown how novalal would affect beetles, as no toxicity studies have been performed. The second possibility, degradation via limonene, also appears unlikely in this system, as limonene would have been detected in the chromatograms (Supp Figs. 4–6 [Online only]), and from an adaptive standpoint is one of the more toxic monoterpenes to bark beetles (Raffa 1991, Seybold et al. 2006, Reid and Purcell 2011).

The members of the bacterial community identified in this study are consistent with the previous studies examining bacterial associates of pine engravers (Delalibera et al. 2007) and other bark beetles (Adams et al. 2010, 2013; Hulcr et al. 2011; Boone et al. 2013). The bark beetles appear to have convergent communities that are strongly influenced by the selective pressures imposed by tree defense chemistry (Aylward et al. 2014, Mason et al. 2015). Because there are an enormous number of potentially transient microbes present in this system (Adams et al. 2010, Mason et al. 2015), a prescreen was needed to reduce the number of microbe-monoterpene combinations. As mentioned previously, we selected (–)-α-pinene because it is the predominant monoterpene present in this system. Such prescreening can favor isolates that can reduce that compound. However, the patterns we observed of bacterial reduction of monoterpene concentrations are consistent with previous work (Adams et al. 2011, Boone et al. 2013), in particular the opposing trends between how bacteria contend with (–)-α-pinene versus 3-carene and myrcene. Therefore, these results appear reasonably robust.

Bacterial communities of pine engravers likely exhibit geographic and or host-based variation, as does *D. valens*, another transcontinentally distributed bark beetle that exploits some of the same tree species (Adams et al. 2010, Aylward et al. 2014). Even though the community members may be relatively plastic, however, the challenges beetles must overcome to successfully exploit various hosts are generally similar. Also interactions among community members (Mason et al. 2014) may allow some monoterpene-degrading activities to be missed or underestimated in our controlled, independent assays.

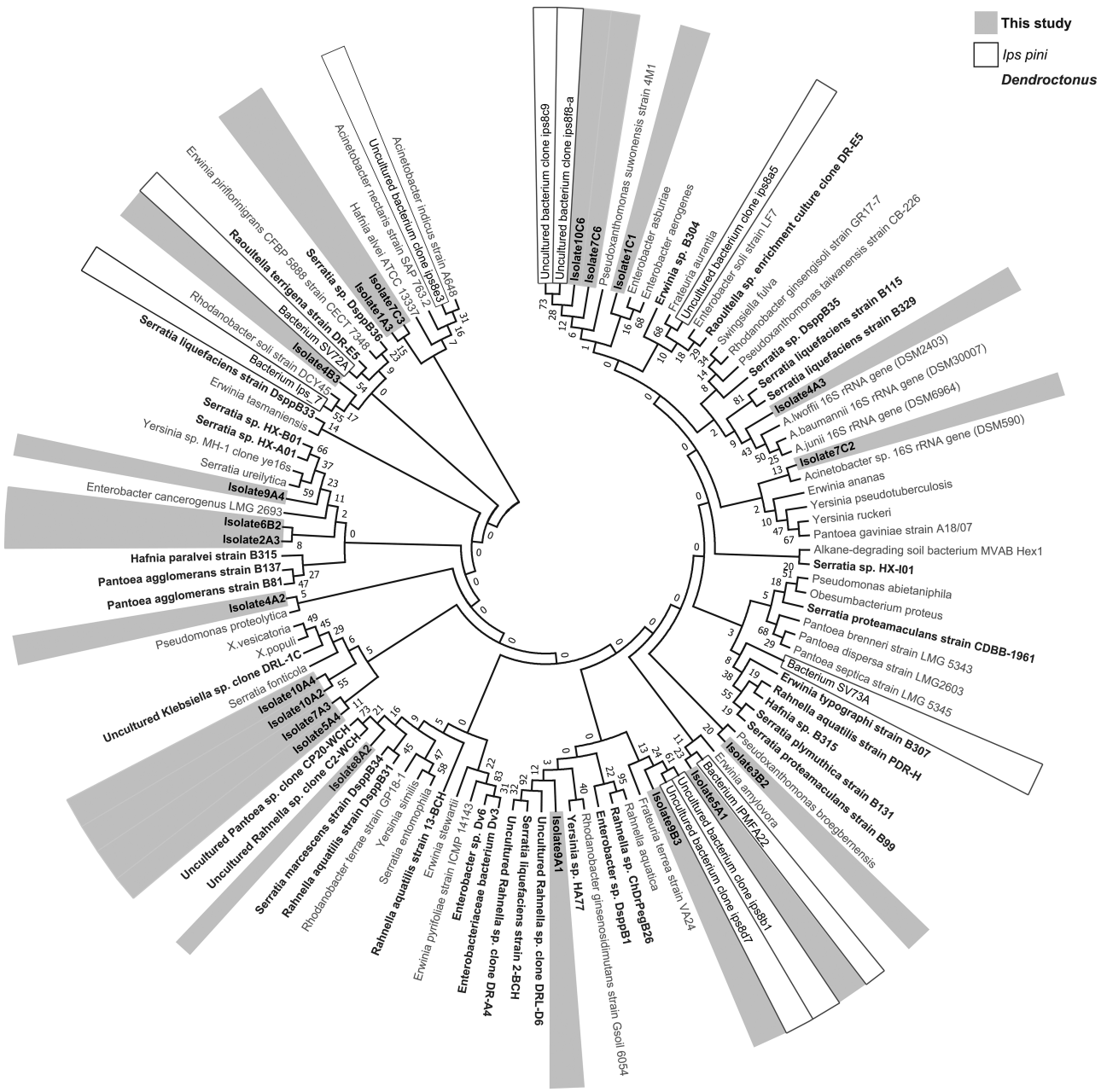

Fig. 5. Unrooted phylogenetic tree of sequences with isolates (gray shading) detected in this study. Outlined isolates represent strains isolated from pine engravers, bolded isolates represent strains isolated from *Dendroctonus*, and type strain sequences are light gray. Numbers represent bootstrapped consensus. Isolates used in this study are broadly similar to pine engravers and *Dendroctonus* spp. associated bacteria from previous studies. Information about known sequences used to construct the tree is in Supp. Table 1 (Online only).

These results contribute to our overall understanding of how bacteria associated with insects mediate various components of plant-insect interactions. Functionally redundant bacterial communities likely ensure bark beetles can contend with a myriad of different chemical defenses in numerous host trees. The model of functional redundancy in bacterial communities needs to be tested on a greater scale and related to frequency of selective pressure, e.g., bark beetles that attack healthy trees may differ from those that attack trees with compromised defenses.

Future work should also examine whether host tree species, and additional monoterpenes and diterpenes, affect the composition and functionality of bark beetle bacterial communities and the fate of metabolized compounds. Future work should also examine how the functionalities of communities compare with those of individual isolates and how they affect insect performance (Mason et al. 2014). In addition, interactions between bacterial and fungal associates can be particularly important to bark beetle success (Cardoza et al. 2008, Scott et al. 2009, Six 2013, Raffa 2014) and must be studied further. Additional attention is needed to scaling-up these relations to better understand in vivo performance (Therrien et al. 2015).

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

Acknowledgments

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