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Bacteria Associated with a Tree-Killing Insect Reduce Concentrations of Plant Defense Compounds

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Abstract Bark beetles encounter a diverse array of constitutive and rapidly induced terpenes when attempting to colonize living conifers. Concentrations of these compounds at entry sites can rapidly reach levels toxic to beetles, their brood, and fungal symbionts. Large numbers of beetles can overwhelm tree defenses via pheromone-mediated mass attacks, but the mechanisms are poorly understood. We show that bacteria associated with mountain pine beetles can metabolize monoterpenes and diterpene acids. The abilities of different symbionts to reduce concentrations of different terpenes appear complementary. *Serratia* reduced concentrations of all monoterpenes applied to media by 55–75 %, except for α-pinene. Beetle-associated *Rahnella* reduced (−)- and (+)-α-pinene by 40 % and 45 %, respectively. *Serratia* and *Brevundimonas* reduced diterpene abietic acid levels by 100 % at low concentrations. However, high concentrations exhausted this ability, suggesting that opposing rates of bacterial metabolism and plant induction of terpenes are critical. The two major fungal symbionts of mountain pine beetle, *Grosmannia clavigera* and *Ophiostoma montium* were highly susceptible to abietic acid. *Grosmannia clavigera* did not reduce total monoterpene concentrations in lodgepole pine turpentine. We propose the ability of bark beetles to exert landscape-scale impacts may arise partly from micro-scale processes driven by bacterial symbionts.

Keywords Bark beetles · Conifers · Detoxification · Plant defense · Symbiosis · Terpenes

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Introduction

Conifers possess sophisticated chemical defenses against bark beetles (Faccoli and Schlyter 2007; Hamberger et al. 2011). In particular, monoterpenes and diterpene acids in bark can sometimes deter entry, and they also rapidly increase at the site of attack to concentrations exceeding the physiological tolerances of the beetles, their brood, and their symbiotic fungi (Raffa et al. 2005). Some species, such as mountain pine beetle, *Dendroctonus ponderosae* Hopkins, kill healthy trees by engaging in pheromone-mediated mass attacks (Blomquist et al. 2010). During outbreaks, they exert landscape-scale effects and pose significant socioeconomic challenges. The exact mechanisms by which high densities of beetles exhaust tree defenses are unknown.

Bark beetles have close associations with fungi (Six and Klepzig 2004). The principal ophiostomatoid fungi carried by *D. ponderosae*, *Grosmannia clavigera* and *Ophiostoma montium*, are nutritionally beneficial to *D. ponderosae*

(Bleiker and Six 2007), moderately pathogenic to pines (Lee et al. 2006), and moderately tolerant of monoterpenes (Adams et al. 2009). Their ability to tolerate diterpene acids is unknown.

Bacterial symbionts of bark beetles have received relatively little attention. Several species have been shown to enhance digestion (Morales-Jimenez et al. 2009, 2012) or inhibit antagonistic fungi (Cardoza et al. 2006; Scott et al. 2008). Additionally, bacterial communities associated with mountain pine beetle are highly enriched with genes involved in terpene metabolism (Adams et al. 2013).

We tested whether bacteria associated with mountain pine beetle reduce monoterpene and diterpene acid concentrations in amended media. We conducted analogous assays with the beetle's primary fungal associates.

Methods and Materials

Dendroctonus ponderosae adults constructing ovipositional galleries in newly attacked pine trees, their galleries, and phloem samples were collected in Alberta and British Columbia, Canada (Online Resource Table 1). We isolated bacteria and fungi as described in Adams et al. (2009). Potential bacterial bioassay candidates were cultured on media that were enriched with terpenes and provided minimal other sources of carbon (Online Resource Table 1). Morphotypes growing most abundantly under these conditions were selected for further investigation, and were identified by direct sequencing of the V5 region of rRNA 16S genes (Online Resource Table 2).

Bacteria were inoculated from single colonies into 20 % Tryptic Soy Broth (TSB) (All product details in Online Resource Table 3) and grown overnight at room temperature. To prime potentially relevant transcriptional programs that might be affected by laboratory culturing, bacteria were exposed to lodgepole pine turpentine (LPT) and its volatiles prior to conducting assays. The day after inoculation, we added LPT to the broth, bringing the terpene concentration to ~1 %. Cultures were shaken with LPT overnight at room temperature, and cultured on 10 % Tryptic Soy Agar (TSA) in Petrie dishes with a piece of filter paper containing ~100 μl LPT fixed to the lid. Plates were sealed, inverted, and incubated in darkness at room temperature until colonies formed. Bacterial suspensions from a single colony were inoculated into 10 % TSB, and shaken at room temperature until turbid.

Monoterpene assays were conducted in clear glass vials containing 15 ml 10 % TSB. Each assay comprised 100 μl bacterial suspension and 150 μl monoterpene treatment independently, and was shaken in darkness for 4 d at room temperature. Controls consisted of equivalent amount of 10 % TSB. Each assay was replicated five times.

On day four, 3 ml GC Resolv hexanes were added to vials, which were sealed and shaken for 3 h. Vials were removed, placed upright, and left overnight at −20 °C. After thawing at room temperature, 1 ml of hexanes-terpene phase was transferred to autosampler vials with open top caps and PTFE/rubber septa. Equipment was rinsed with hexanes between samples. The internal standard 5 μl of 50 % isobutylbenzene in GC Resolv hexanes was added to each sample. Samples were stored at −20 °C until analyzed by gas chromatography (Online Resource Table 3). Total and individual peak integrations were analyzed for each compound using a non-parametric Kruskal-Wallis test.

We used a similar procedure to test whether *S. marcescens* and *B. vesicularis* decrease concentrations of the diterpene abietic acid. We grew bacteria in media (Online Resource Table 3) containing 1.1, 2.2, 4.3, or 8.7 mg/ml of abietic acid dissolved in 30 ml 10 M NaOH or control, and measured subsequent abietic acid concentrations (N=2). The medium was titrated with HCl to pH 7 before autoclaving. An overnight culture of bacteria was diluted 1:50 in the broth to 500 ml. On day 20, 1 ml was transferred to a GC vial and stored at −20 °C until analysis.

We dried 100 μl of each bacterial solution in a vacuum centrifuge, added 500 μl of absolute EtOH, vortexed vigorously, and sonicated for 5 min. We transferred 200 μl of each solution to a flat bottom vial insert, spun 5 min, and withdrew 100 μl of clear supernatant. Abietic acid was analyzed by HPLC (Online Resource Table 3). Each abietic acid concentration and control was statistically analyzed separately using ANOVAs (Online Resource Table 3) with Scheffé *post hoc* tests.

We assayed effects of abietic acid on *G. clavigera* and *O. montium* growth as per Adams et al. (2009). Assays were performed in 100 mm diam plastic Petri dishes containing an agar medium with minimal salts and 0, 0.01, or 1.0 % abietic acid. One 3 mm diam leading-edge plug of fungi growing on Malt Extract Agar (MEA) was placed hyphae-side down onto the center of amended agar with 10 replicates per fungus-abietic acid concentration. ANOVAs with Scheffeé *post hoc* tests evaluated whether fungal growth varied with treatment.

We tested for putative fungal metabolism of monoterpenes using the same approach. Spore solutions were created by adding 6 ml sterile double distilled H_2O to *G. clavigera* growing on Yeast MEA. To initiate the bioassay, 100 μl of spore solution were added to 5 ml Malt Extract Broth (15 g/l) and 150 μl LPT. Assays were performed, extracted, and analyzed via gas chromatography as above. We used non-parametric Kruskal-Wallis Test to test whether *G. clavigera* growth varied with LPT.

Results

Four beetle-associated bacteria, most closely matching *Serratia marcescens, Pseudomonas mandelii, Pseudomonas migulae,* and *Rahnella aquatilis,* reduced concentrations of monoterpenes under controlled conditions (Fig. 1a). Of these, *S.*

marcescens (hereafter we omit the qualifier 'most closely matching' for brevity) had the largest effect, reducing concentrations of all but (−)- and (+)-α-pinene. This bacterium decreased mean concentrations of 3-carene and (−)-β-pinene by 49–79 %. *Pseudomonas mandelii* decreased concentrations of all monoterpenes by 15–24 %. *Rahnella aquatilis* decreased concentrations of all monoterpenes, including the greatest reduction of (−)-α-pinene (38 %) and (+)-α-pinene (46 %) of any bacterium tested. *Pseudomonas brenneri* and *Pantoea agglomerans* had no effect. No bacterium differentially affected α-pinene enantiomers.

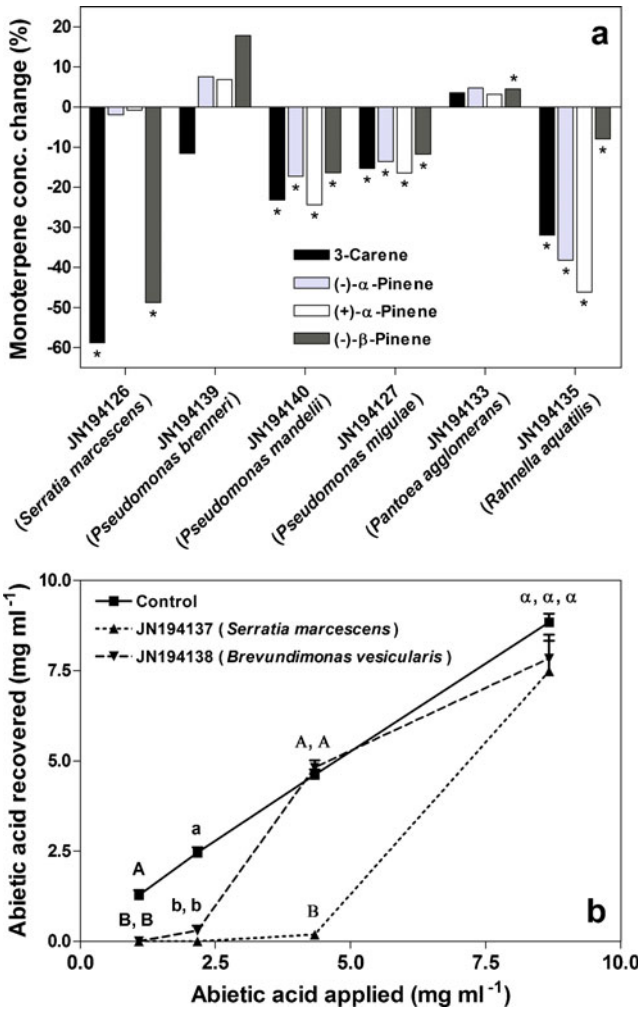

Fig. 1 Effects of bacteria associated with mountain pine beetle on concentrations of pine terpenes added to media. **a** Monoterpenes. Percent change in monoterpene concentration in presence of bacteria relative to controls. *Asterisks* indicate a statistically significant difference ($P<0.05$) between media with and without bacteria; **b** Diterpene, abietic acid. The Y axis shows the quantity recovered for each concentration of abietic acid applied, for two bacteria plus blank control. Complete recovery generates a 45-degree slope; complete loss generates a flat line. Error bars represent ±1 SE. 1.1 mg/ml abietic acid: $F_2=81.0$, $P=0.003$; 2.2 mg/ml abietic acid: $F_2=129.4$, $P=0.001$; 4.3 mg/ml abietic acid, $F_2=328.1$; $P<0.001$; 8.7 mg/ml abietic acid: $F_2=1.1$, $P=0.432$. Bacteria with *different letters* within an abietic acid treatment are different at $P<0.05$

Serratia marcescens and *B. vesicularis* greatly decreased abietic acid concentrations (Fig. 1b), with each eliminating 100 % of the diterpene acid at low doses. This ability diminished with increasing concentrations of terpene, with the threshold varying between bacteria. *Serratia marcescens* caused no reduction at 0.43 % abietic acid, whereas the ability of *B. vesicularis* to decrease concentrations was lost at 0.87 % abietic acid.

Abietic acid strongly inhibited *G. clavigera* and *O. montium* (Table 1). *G. clavigera* growth was inhibited by 54 %, and *O. montium* growth was inhibited by 85 %, at 0.01 % abietic acid. Abietic acid at 1 % reduced *G. clavigera* growth by 67 %, and completely inhibited *O. montium*. *Grosmannia clavigera* did not reduce monoterpene concentrations in LPT [mean mg/m, (sem): LPT Control: 55.78 (0.85); LPT+*G. clavigera* exposed to UV: 53.77 (0.33); LPT+*G. clavigera* (54.91 (0.59)].

Discussion

These results provide the first direct evidence that bacteria associated with bark beetles can reduce concentrations of conifer defense chemicals. Different bacteria appear to perform complementary reactions: *Serratia marcescens* greatly reduce concentrations of most monoterpenes, whereas *B. vesicularis* greatly decrease the diterpene abietic acid. Within monoterpenes, *S. marcescens* decrease all compounds except (−)- and (+)-α- pinene, complemented by *R. aquatilis*, which most effectively decrease those two compounds. Complementary biochemical functions could be ecologically important, as monoterpenes are toxic mostly to the beetles, whereas diterpene acids are toxic mostly to their symbiotic fungi (Table 1) (Raffa et al. 2005). Even within a particular terpenoid class, individual compounds vary greatly in behavioral repellency, toxicity to beetles, inhibition of mycelial growth, and inhibition of spore germination (Raffa et al. 2005). Redundancy among various bacteria may likewise be important, as there is substantial between-individual and between-population variability in communities accompanying a beetle species. Complementarity and

Table 1 Effect of varying concentrations of abietic acid on linear growth (mm) of *Ophiostoma montium* (Rumbold) von Arx and *Grosmannia clavigera* (Rob.-Jeffr. & R.W. Davidson) Zipfel, Z.W. deBeer & Wingf

Abietic acid concentration (%)	Linear growth (mm): Mean (SE)	
	G. clavigera	*O. montium*
0	54.0 (2.1)^{a}	41.7 (1.2)^{A}
0.01	8.2 (0.7)^{b}	19.3 (0.5)^{B}
1.0	0.0 (0)^{c}	13.7 (0.6)^{C}
$F_{2,20}$	602.535	306.110
P	<0.001	<0.001

Means within a column followed by a different superscript letter are significantly different (α=0.05)

redundancy likely extend to the full insect-microbial complex. That is, we propose bacteria add to the collective physical and metabolic depletion of terpenes performed by the beetles (Sandstrom et al. 2006) and other symbionts (Davis and Hofstetter 2011; Wang et al. 2013).

The high enrichment of the *D. ponderosae* bacterial community with genes associated with terpene metabolism (Adams et al. 2013), as well as the utility of terpene-metabolizing bacteria such as other *Serratia* and *Pseudomonas* for commercial bioprocessing (Bicas et al. 2008), further suggest that bacteria assist bark beetles in overcoming tree defenses. The pronounced metabolism of (−)-α-pinene by *R. aquatilis*, relative to the high susceptibility of other *D. ponderosae* - associated bacteria to this compound (Adams et al. 2011), is noteworthy because (−)-α-pinene is a biosynthetic precursor to *trans*-verbenol (Blomquist et al. 2010), the primary aggregation pheromone of *D. ponderosae*. One bacterium appeared to increase β-pinene, consistent with terpene synthesis documented in some bacteria (Cane and Ikeda 2012).

The dose-dependent relationship with abietic acid illustrates the critical role of opposing rate reactions in conifer-bark beetle-microbial interactions (Raffa et al. 2005). Specifically, beetle-associated bacteria appear able to metabolize the quantities present in constitutive but not induced phloem tissue; in mature red pine, these are 0.1 % and 20 %, respectively. Diterpene acids in mature lodgepole pine phloem have not been quantified, but are approximately 0.1 % – 0.2 % dry weight in seedlings (Hall et al. 2013). Thus, unless inducible reactions were compromised, as occurs during mass attacks (Raffa et al. 2005), diterpenes would likely overwhelm bacterial metabolism and thereby prevent fungal establishment (Table 1).

Future work is needed to characterize biochemical pathways, identities, and bioactivities of end products, and *in vivo* effects of bacteria-terpene interactions on beetles and fungi. Ecological context also needs elaboration. In particular, these bacteria are not necessarily strict beetle symbionts, but can also inhabit conifer tissues (Adams et al. 2013). This raises the possibility that some resident bacteria might be activated upon beetle penetration of conifer tissues, use terpenes as a carbon source, and thereby benefit the herbivore.

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