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Responses of Bark Beetle-Associated Bacteria to Host Monoterpenes and Their Relationship to Insect Life Histories

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Abstract Bark beetles that colonize living conifers and their microbial associates encounter constitutive and induced chemical defenses of their host. Monoterpene hydrocarbons comprise a major component of these allelochemicals, and many are antibiotic to insects, fungi, and bacteria. Some bark beetle species exhaust these defenses by killing their host through mass attacks mediated by aggregation pheromones. Others lack adult aggregation pheromones and do not engage in pheromone-mediated mass attacks, but rather have the ability to complete development within live hosts. In the former species, the larvae develop in tissue largely depleted of host terpenes, whereas in the latter exposure to these compounds persists throughout development. A substantial literature exists on how monoterpenes affect bark beetles and their associated fungi, but little is known of how they affect bacteria, which in turn can influence beetle performance in various manners. We tested several bacteria from two bark beetle species for their ability to grow in the presence of a diversity of host monoterpenes. Bacteria were isolated from the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, which typically kills trees during colonization, and the red turpentine beetle, *Dendroctonus valens* LeConte, which often lives in their host without causing mortality. Bacteria from *D. ponderosae* were gram-positive Actinobacteria and Bacilli; one yeast also was tested. Bacteria from *D. valens* were Actinobacteria, Bacilli, and γ-Proteobacteria. Bacteria from *D. valens* were more tolerant of monoterpenes than were those from *D. ponderosae*. Bacteria from *D. ponderosae* did not grow in the presence of α-pinene and 3-carene, and grew in, but were inhibited by, β-pinene and β-phellandrene. Limonene and myrcene had little inhibitory effect on bacteria from either beetle species. Tolerance to these antibiotic compounds appears to have resulted from adaptation to living in a terpene-rich environment.

Key Words Actinobacteria · Bacillales · Bacteria · *Dendroctonus ponderosae* · *Dendroctonus valens* · Host defense · Monoterpenes · Polymerase chain reaction (PCR) · γ-Proteobacteria

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Introduction

Bark beetles are major sources of conifer mortality, in scales ranging from localized pockets (Klepzig et al., 1991) to entire landscapes (Aukema et al., 2006; Bentz et al., 2010). Individual trees within a beetle's host range and preferred size class can often resist attack by utilizing terpenoid-based defenses (Lewinsohn et al., 1991; Brignolas et al., 1995; Keeling and Bohlmann, 2006). In particular, monoterpenes can kill or repel bark beetles, and inhibit growth of their fungal symbionts (Smith, 1963; Raffa and Smalley, 1995; Wallin and Raffa, 2000). These compounds are present in

constitutive resin and undergo enhanced production in response to biotic injury, and to a lesser extent to mechanical wounds (Raffa and Berryman, 1983; Raffa and Smalley, 1995). These terpene-based defenses play an important role in constraining populations of eruptive species from transitioning from endemic to epidemic population densities (Boone et al., 2011).

The largest insect outbreak in recorded history currently is underway in western Canada and the U.S. Rocky Mountains (Bentz et al., 2009). Here the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, is causing high rates of mortality to lodgepole pine, *Pinus contorta* var. *latifolia* Dougl., including vigorously growing and well-defended trees (Boone et al., 2011). Elsewhere, they also kill large numbers of ponderosa pines, *Pinus ponderosa* Dougl. Ex Laws, and several other species, including high elevation pines like whitebark pine, *Pinus albicaulis* Engelmann (Logan et al., 2010). Once outbreaks have exhausted preferred hosts, *D. ponderosae* also may attack and kill spruce trees (Furniss and Schenk, 1969; Huber et al., 2009). The ability of these beetles to coordinate pheromone-mediated mass attack allows them to exhaust the resistance of vigorous, well-defended trees, and thereby provide their brood with a substrate with relatively low allelochemical concentrations. The resin content declines rapidly during successful mass attacks (Raffa et al., 1993). These mass attacks typically kill the tree, or in some cases result in strips of dead tissue (Rasmussen, 1974).

Recent warming trends are facilitating range expansion by populations of *D. ponderosae* into new regions and hosts (Carroll et al., 2003). On the east side of the Rocky Mountains in Alberta, mature stands of *P. contorta* are sympatric and hybridize with jack pine, *Pinus banksiana* Lamb. (Critchfield, 1985). Historically, *P. banksiana* has not been exposed to this insect due to the physical barrier of the Rocky Mountains and the cold climate in this region. In 2001, *D. ponderosae* breached this barrier and colonized *P. contorta*, *P. contorta*-*P. banksiana* hybrid, and later *P. banksiana* pines (Nealis and Peter, 2008; Cullingham et al., 2011). The extent to which *D. ponderosae* will impact this region and subsequently the boreal and eastern forests of North America is unknown (Carroll et al., 2003; Logan et al., 2003; Bentz et al., 2010; Safranyik et al., 2010). Similarly, *P. albicaulis* to a large extent historically escaped exposure to sustained *D. ponderosae* populations due to low temperatures of its subalpine habitat, but current warming trends are now facilitating continuous mortality by *D. ponderosae* (Bentz et al., 2010; Logan et al., 2010).

Some stem-colonizing bark beetle species do not engage in pheromone-mediated mass attacks or cause tree death, but rather have the ability to develop within living hosts without killing them (Coulson and Witter, 1984; Berryman, 1986). For example, the black turpentine beetle, *Dendroctonus terebrans* (Oliver) shows evidence of a mating, but not an aggregation, pheromone (Phillips et al., 1990). The red turpentine beetle, *Dendroctonus valens* LeConte, is native to much of North America, and colonizes a large number of pine species (Kelley and Farrell, 1998), and to a lesser extent *Abies*, *Larix*, and *Picea* (Wood, 1982). In the midwestern United States, its major hosts are red pine, *Pinus resinosa* Ait., and *P. banksiana*. A long-term study demonstrated that in Wisconsin, *D. valens* more commonly attacks living red pines prior to lethal attack by *Ips pini* (Say) than the opposite sequence (Aukema et al., 2010). Trees not attacked by *I. pini* the following year continued to live, and did not die until being attacked eventually by this stem-colonizing herbivore (Aukema et al., 2010). Fifty seven percent of these trees were attacked by only one pair of *D. valens* within a year, and 93% of the trees had four or fewer colonizing pairs. Unlike species that must mass attack and kill their hosts prior to reproducing, these beetles appear capable of surviving high monoterpene concentrations in living hosts throughout their development. This is evidenced by descriptions of *D. valens* that complete development in live trees without killing them, and the larvae remain in contact with large amounts of resin in such hosts (Hopkins, 1909; Smith, 1961; Schmid and Mata, 1991; Randall, 2006). In addition to live trees that are often stressed, and whose colonization can lead to subsequent lethal attack by tree-killing species, *D. valens* also colonize stumps from recently cut trees (Furniss and Carolin, 1977; Aukema et al., 2010; Owen et al., 2010). In the late 1990s, *D. valens* invaded parts of China, where it is attacking a new host, Chinese red pine, *Pinus tabuliformis* Carr., in higher numbers, and has become a primary mortality agent (Yan et al., 2005).

Beetles that colonize a diversity of hosts encounter variable monoterpene environments (Seybold et al., 2006). For example, the volatile fraction of *P. contorta* oleoresin is comprised primarily of β-phellandrene and to a lesser extent β-pinene (Zavarin et al., 1969; Raffa and Berryman, 1982a; Pureswaran et al., 2004). β-Phellandrene is rare or absent in other hosts of *D. ponderosae* (Smith, 2000). *Pinus ponderosa* oleoresin contains a more diverse monoterpene profile with relatively equal proportions of 3-carene and β-pinene, and substantial amounts of α-pinene, myrcene, and limonene (Sturgeon, 1979; Hobson et al., 1993). *Pinus albicaulis* oleoresin contains substantial amounts of 3-carene and myrcene (Smith, 2000). *Pinus banksiana* oleoresin contains primarily α-pinene, β-pinene, and 3-carene (Wallin and Raffa, 1999), whereas *P. resinosa* oleoresin contains primarily α-pinene and β-pinene (Raffa and Smalley, 1995).

Bark beetles are associated with a diversity of micro-organisms that facilitate their ability to exploit the subcortical environment (Whitney, 1982; Paine et al., 1997). Of these, fungi have been most thoroughly studied. Conifer mono-terpenes can strongly inhibit some of these fungi, although

toxicity varies among compounds and some fungi are relatively tolerant (Raffa et al., 1985; Paine and Hanlon, 1994; Klepzig et al., 1996; Hofstetter et al., 2005; DiGuistini et al., 2011). Recent studies have shown that some bacterial taxa are frequently associated with bark beetles (Adams et al., 2010; Hulcr et al., 2011) and fulfill important ecological functions. For example, Actinomycete bacteria associated with two beetle species inhibit the beetles' fungal antagonists (Cardoza et al., 2006; Scott et al., 2008), and Proteobacteria stimulate the growth and reproduction of beetle symbiotic fungi in the presence of the host compound α-pinene (Adams et al., 2009). Bacteria inhabiting guts of other wood-feeding insects, such as termites and cerambycid beetles, degrade plant-cell wall components, such as cellulose and lignin, and contribute to acquisition of nutrients from these generally recalcitrant woody-substrates (Warnecke et al., 2007; Geib et al., 2008). Some bacteria associated with bark beetles tolerate myrcene (Skrodenyte-Arbaciauskiene et al., 2006) and α-pinene (Adams et al., 2009), but their tolerance to other monoterpene components of conifer defense is unknown.

In this study, we tested how bacteria isolated from two bark beetle species that exhibit different host defense tolerances associated with their different host colonization behaviors (i.e., killing trees by mass attack vs. colonizing live trees without mass attacks or causing mortality) are affected by a diversity of monoterpenes from pine oleoresin. Specifically, we isolated bacteria from *D. ponderosae* and *D. valens* and exposed these bacteria to monoterpenes that comprise the major resin and phloem constituents of *P. contorta*, *P. banksiana*, *P. resinosa*, and *P. albicaulis*. We hypothesized that bacteria from both beetle species will grow in the presence of many monoterpenes, and that bacteria associated with *D. valens* will be more capable of tolerating these compounds given its life history.

Methods and Materials

Isolation of Microorganisms *Dendroctonus ponderosae* adults were collected in July 2008 from under the bark of naturally attacked trees in a stand of *P. contorta* and *P. contorta—P. banksiana* hybrid pines, near Grande Prairie, Alberta, Canada (54°43′N, 119°39′W). Beetles of both sexes were actively tunnelling in the egg gallery at the time of collection. *Dendroctonus valens* adults of both sexes were collected in May 2008 from under the bark of naturally colonized *P. resinosa* stumps in a spring-thinned plantation in Black River State Park, Black River Falls, WI, USA (44°14'57 N, 90°34'07"W). These beetles were actively tunnelling in egg galleries as well. For all beetles collected in this study, galleries were clear of resin and appeared to represent successful colonization of the host. During sampling, each adult was placed into individual, sterile vials, placed on ice, and transferred to the lab and stored at 4°C.

Within 24 hr, at least 25 beetles from each species were surface washed in phosphate buffered saline, pH 7.4 (Sigma-Aldrich Co., St. Louis, MO, USA) (PBS) with 1% Tween 20 (ICI America Inc., Wilmington, DE, USA) by vortexing for 30 sec, and rinsed in PBS. Bacteria were isolated by crushing beetles in PBS and plating serially diluted aliquots of the samples on 10% tryptic soy agar (TSA; Difco, Sparks, MD, USA). Petri dishes were stored at room temperature for up to 2 week, and colonies from a diversity of morphologies were selected from multiple beetles and obtained in pure culture for bioassay.

All bacteria were identified by direct sequencing of regions V5 of the rRNA 16S gene. Polymerase chain reaction (PCR) was used to amplify partial ribosomal RNA (rRNA) gene sequences (Holben et al., 2002), with the exception that PCR amplicons were used in direct sequencing reactions rather than for cloning. The primers 27f (5'-GAGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') (based on the *Escherichia coli* numbering system) were used for PCR. PCR reaction mixtures contained 25 µl GoTaq Green Mastermix (Promega, Madison, WI, USA), 2 µl of each primer 20 µl of water, and 1 µl of culture. PCR reactions were performed by using an initial denaturation of 5 min at 94°C, followed by 30 cycles of denaturation for 1 min at 94°C, primer annealing for 1 min at 55°C, and primer extension at 72°C for 2 min. This was followed by a final extension reaction at 72°C for 10 min. Sequencing of PCR products was performed by the University of Wisconsin Biotechnology Center (Madison, WI, USA). Sequences were deposited in GenBank (Table 1). National Center for Biotechnology Information (NCBI) similarity scores were obtained by using the NCBI search of previously deposited sequences in GenBank (www.ncbi.nlm.nih.gov).

Effects of Monoterpenes on Growth of Bacteria We quantified the effects of monoterpenes on growth of bacteria by using methods described by Middelbeek et al. (1992) and Adams et al. (2009). Briefly, 10 µl of actively growing culture were inoculated into 200 µl of 10% tryptic soy broth in 96-well plates (Becton Dickinson and Co., Franklin Lakes, NJ, USA). Wells were amended with 0%, 1%, or 5% concentrations (*v*/*v*) of one of 6 monoterpenes for isolates from *D. ponderosae*, and 5 monoterpenes for isolates from *D. valens*. The monoterpenes were racemic α-pinene (98%, Aldrich, Milwaukee, WI, USA), (−)-β-pinene (technical grade, approx. 86%, Aldrich), (*R*)-(+)-limonene (98%, Aldrich), myrcene (tech. grade, approx. 78%, Aldrich), and (+)-3-carene (90%, Aldrich), which were tested against all isolates, and β-phellandrene (Glidco

Table 1 GenBank accession numbers for bacteria isolated from *Dendroctonus ponderosae* and *D. valens*

Isolate source	Isolate name	Class of closest match in GenBank	Closest named match in GenBank (accession #)	Sequence length (bp)	Percent similarity	Accession No. of isolate
D. ponderosae	A7-1a	Actinobacteria	*Micrococcus terreus* (HQ009859)	1216	99	HQ728081
	A3-1a	Bacilli	*Paenibacillus xylanexedens* (HM439462)	758	100	HQ728079
	A5-1	Bacilli	*Paenibacillus* sp. (GQ920787)	383	99	HQ728082
	A3-1b	Bacilli	*Bacillus simplex* (FJ544333)	551	98	HQ728080
	A9-4[a]	Saccharomycetes	*Candida piceae* (EU011713)	526	100	HQ728083
D. valens	Dv3	γ-Proteobacteria	*Enterobacter* sp. (AY082447)	1320	98	HQ728086
	Dv6	γ-Proteobacteria	*Enterobacter amnigenus* (DQ481471)	1320	100	HQ728087
	Dv25	γ-Proteobacteria	*Pseudomonas* sp. (AM913961)	942	99	HQ728088
	Dv26	Actinobacteria	*Frigoribacterium* sp. (AF157479)	1350	100	HQ728084
	Dv34	Bacilli	*Paenibacillus provencensis* (EF212893)	849	100	HQ728085

[a] Fungus

Organics Corp., Jackson, MS, USA), which was tested against isolates from *D. ponderosae*. Individual wells were sealed by using VIEWseal pressure sensitive adhesive (Greiner Bio-one, Maybachstraße, Germany). Cultures were shaken at medium speed (500 revolutions per min) and orbital directionality at 24°C, and optical density was measured every hour for up to 48 h by using a DXT 880 Multimode Detector (Beckman Coulter, Corona, CA, USA) at absorbance of 595 nm. Each isolate was exposed to each monoterpene and tested an average of two times to verify consistent growth patterns (Table 2).

Data Analysis We compared the doubling times of bacteria with and without monoterpene addition during the exponential phase of growth (Middelbeek et al., 1992). To determine the time period in which the exponential phase took place, growth of bacteria were monitored for up to 48 h, a period that encompassed the growth phase. Next, absorbance values were log transformed and the time interval with the most linear log-transformed absorbance values was selected by calculating the r^2 values of each possible time interval (StatView version 4.57, Abacus Concepts, Inc., Berkeley, CA, USA). During this period of exponential growth, an index of monoterpene impact on bacterial growth rate was calculated by using methods described by Middelbeek et al. (1992): the difference in absorbance values at the beginning and end of the exponential growth phase (representing the number of cell divisions) was divided by the $\log_2$ of the duration (number of hours) of the exponential growth phase. The reciprocal of this value is then taken to provide the number of hours per generation, or doubling time. The doubling time of each bacterium growing in each terpene concentration was calculated relative to controls by subtracting the doubling time of the isolate growing without terpene from the doubling time of the isolate growing with the terpene, and dividing this value by the doubling time of the isolate growing without terpene, x 100. Relative doubling times were grouped into five categories: −75% to −25%, stimulated growth; −25% to 25%, growth not affected; 25% to 100%, growth inhibited; > 100%, growth strongly inhibited; and no growth, complete inhibition.

Results

Isolation and Identification of Microorganisms Bacteria closely related to gram-positive actinobacterium *Micrococcus terreus* (isolate A7-1a), and Bacillales *Paenibacillus* spp. (isolates A3-1a and A5-1) and *Bacillus simplex* (isolate A3-1b) were isolated from *D. ponderosae* and tested in this study (Table 1). One yeast isolate (A9-4) most closely related to *Candida piceae*, also was obtained in culture and used in bioassays (Table 1).

Bacteria most closely related to an actinobacterium *Frigoribacterium* sp. (isolate Dv26) and Bacillales *Paenibacillus provencensis* (isolate Dv34) were isolated from *D. valens* and tested (Table 1). Three other isolates were most closely related to gram-negative γ-Proteobacteria *Enterobacter* spp. (isolates Dv3 and Dv6) and *Pseudomonas* sp. (isolate Dv25) (Table 1).

Effects of Monoterpenes on Growth Monoterpenes had substantial effects on bacterial growth (doubling time), and these varied with compound, isolate, and insect host (Table 2). Overall, growth of bacteria was most inhibited by (+)-3-carene and (±)-α-pinene, and was least inhibited by myrcene and limonene. Six of the ten isolates grew in the presence of α-pinene (Table 2, Fig. 1). The four isolates that

Table 2 Doubling time (h) of microbial isolates from *Dendroctonus ponderosae* (MPB) and *D. valens* (RTB) adults in the presence and absence of monoterpenes

Doubling time ± 90% C.I. (*N*)

Source	isolate	Conc. (%)	α-Pinene	β-Pinene	Limonene	Myrcene	3-Carene	β-Phellandrene
MPB	A7-1a	0	4.7±0.03 (2)	8.8±0.71 (4)	5.2±0.63 (4)	5.5±0.27 (2)	6.9±0.56 (4)	6.9±0.73 (3)
		1	11.1±0.48 (4)	12.7±0.24 (3)	8.7±2.54 (3)	4.0±0.01 (2)	CI (4)	6.6±0.51 (3)
		5	13.3±1.67 (2)	40.4±11.31 (4)	8.3±2.90 (3)	4.4±0.25 (2)	CI (4)	9.4±0.64 (3)
	A3-1a	0	5.3±0.07 (2)	6.6±0.09 (4)	5.0±0.16 (2)	3.6±0.10 (2)	4.9±0.49 (3)	14.9±2.37 (4)
		1	CI (2)	31.3±3.11 (2)	3.5±0.12 (2)	3.8±0.34 (2)	CI (3)	20.0±1.80 (3)
		5	CI (2)	20.6±1.13 (2)	3.1±0.03 (2)	2.8±0.25 (2)	CI (3)	21.7±3.52 (4)
	A3-1b	0	5.1±0.18 (2)	6.7±0.01 (3)	7.2±1.00 (4)	15.5±0.01 (2)	10.3±0.17 (2)	13.4±0.50 (2)
		1	CI (2)	9.1±0.02 (3)	8.1±0.19 (3)	17.7±0.45 (2)	17.8±0.06 (2)	17.5±1.81 (2)
		5	CI (2)	CI (3)	7.8±1.03 (3)	16.3±0.71 (2)	CI (2)	19.4 (1)
	A5-1	0	4.7±0.08 (2)	6.7±0.28 (2)	4.8±0.11 (2)	5.3±0.25 (2)	8.2±0.11 (2)	9.9±0.48 (4)
		1	CI (2)	10.3±0.37 (2)	4.9±0.01 (2)	5.3±0.20 (2)	CI (2)	17.4±2.10 (2)
		5	CI (2)	10.5±0.45 (2)	5.1±0.03 (2)	7.6±0.56 (2)	CI (2)	14.4±1.18 (2)
	A9-4	0	3.9±0.02 (2)	8.4±0.05 (2)	5.6±0.52 (4)	11.0±0.48 (2)	10.5±0.04 (2)	9.4±0.62 (4)
		1	CI (2)	8.2±0.69 (2)	6.4±0.42 (3)	4.7±0.23 (2)	13.2±0.71 (2)	13.3±0.91 (4)
		5	CI (2)	7.6±0.38 (2)	7.7±0.53 (2)	7.6±0.64 (2)	CI (2)	16.5±1.75 (3)
RTB	Dv3	0	5.0±0.31 (2)	3.3±0.12 (2)	no data	3.1±0.12 (2)	4.1±0.02 (2)	
		1	6.6±0.80 (2)	5.0±1.17 (2)	3.3 (1)	3.3±0.80 (2)	3.7±0.20 (2)	
		5	26.4±4.45 (2)	3.1±0.43 (2)	3.3±0.24 (2)	2.5±0.09 (2)	8.9±1.02 (2)	
	Dv6	0	4.1±0.02 (2)	4.2±0.08 (2)	3.4±0.17 (2)	4.0 (1)	4.3±0.05 (2)	
		1	3.5±0.01 (2)	5.4 (1)	3.3±0.34 (2)	3.5 (1)	5.7±0.02 (2)	
		5	4.8±0.00 (2)	4.4±0.38 (2)	3.7±0.42 (2)	4.9±0.22 (2)	5.7±0.31 (2)	
	Dv25	0	3.7±0.09 (2)	3.5±0.03 (2)	4.7±0.01 (2)	3.1±0.12 (2)	4.7±0.04 (2)	
		1	3.8±0.25 (2)	5.4±0.49 (2)	5.1±0.23 (2)	2.6±0.14 (2)	3.9±0.01 (2)	
		5	4.3±0.17 (2)	4.7±0.03 (2)	5.9±0.58 (2)	3.1±0.06 (2)	4.5 (1)	
	Dv26	0	2.9±0.10 (2)	3.0±0.23 (2)	5.1±0.22 (2)	3.5 (1)	3.0±0.14 (2)	
		1	3.3±0.17 (2)	4.4±0.25 (2)	3.6 (1)	3.2 (1)	3.0±0.10 (2)	
		5	4.8±0.19 (2)	3.8±0.00 (2)	8.6±0.52 (2)	3.4±0.26 (2)	4.5 (1)	
	Dv34	0	5.4±0.00 (2)	4.6±0.15 (2)	3.4±0.17 (2)	3.3±0.14 (2)	4.7±0.02 (2)	
		1	5.0±0.23 (2)	5.2±0.13 (2)	3.3±0.34 (2)	3.2±0.47 (2)	4.0±0.28 (2)	
		5	2.9±0.08 (2)	4.0±0.01 (2)	4.0±0.40 (2)	3.8±0.24 (2)	4.6±0.02 (2)	

For table entries labeled “CI”, the isolates were completely inhibited

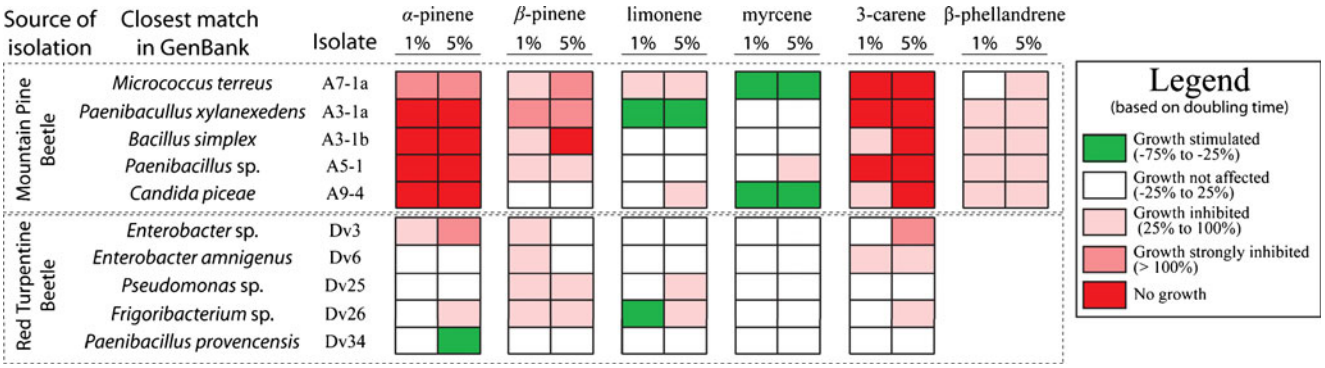

Fig. 1 Heat map representing the growth of bacterial isolates growing in the presence of monoterpenes relative to growing in the absence of monoterpenes. Percentages listed in the legend represent doubling time of each isolate growing with monoterpenes at 1% and 5% concentrations relative to each isolate growing in culture without terpenes

did not grow in any concentration of (±)-α-pinene were all isolated from *D. ponderosae*. One isolate, A7-1a, from *D. ponderosae* grew in, but was strongly inhibited by, both concentrations of α-pinene. All isolates from *D. valens* grew at 1% α-pinene, with no effect on four isolates and inhibition of one, isolate Dv3. All isolates also grew at 5% α-pinene, with an increase in growth of Dv34, no effect on Dv6 and Dv25, inhibition of Dv26, and strong inhibition of Dv3.

All isolates grew in the presence of (*S*)-(−)-β-pinene (Table 2, Fig. 1). All isolates from *D. ponderosae* grew at 1% β-pinene, and all but one isolate, A3-1b, grew at 5% β-pinene. Three isolates, A7-1a, A3-1b, and A5-1, were inhibited and isolate A3-1a was strongly inhibited at 1%. Isolate A5-1 was inhibited and isolates A7-1a and A3-1a were strongly inhibited at 5%. Growth of isolate A9-4 was not affected, and isolate A3-1b did not grow at 5%. All isolates from *D. valens* grew in both concentrations of β-pinene. All but isolate Dv34 were inhibited at 1%. At 5%, three isolates were unaffected, and Dv25 and Dv26 were inhibited.

All isolates grew in the presence of (*R*)-(+)-limonene (Table 2, Fig. 1). Isolate A3-1a from *D. ponderosae* was stimulated at both concentrations, isolate A7-1a was inhibited at both concentrations, and isolate A9-4 was inhibited at 5%. Three isolates from *D. valens* were unaffected by either concentration, and growth of isolate Dv26 was stimulated at 1%, and growth of isolates Dv25 and Dv26 was inhibited at 5%.

All bacterial isolates grew in the presence of myrcene (Table 2, Fig. 1). Isolates A7-1a and A9-4 from *D. ponderosae* were stimulated at both concentrations, and growth of isolates A3-1a and A3-1b was not affected at either concentration. Isolates A3-1a and A3-1b were unaffected at either concentration of myrcene and isolate A5-1 was inhibited at 5%. Growth of all isolates from *D. valens* was unaffected by myrcene.

All but two isolates were inhibited by (+)-3-carene under some condition (Table 2, Fig. 1). Isolates A7-1a, A3-1a, and A5-1 from *D. ponderosae* did not grow at either concentration, and isolates A3-1b and A9-4 were inhibited at 1% and did not grow at 5%. All bacteria from *D. valens* grew at both concentrations of (+)-3-carene. Isolate Dv6 was inhibited at 1% and 5%, and isolate Dv26 was inhibited and isolate Dv3 was strongly inhibited at 5%.

β-Phellandrene inhibited the growth of all bacteria from *D. ponderosae*, and all but isolate A7-1a were inhibited at 1%. However, no bacteria were totally or even strongly inhibited at either concentration (Table 2, Fig. 1).

Discussion

Bacteria isolated from *D. ponderosae* were more strongly inhibited by exposure to monoterpenes than those isolated from *D. valens*. This appears to correspond with a major difference in their life histories, namely that *D. ponderosae* deplete host monoterpene concentrations by mass attack, whereas *D. valens* in Wisconsin often conduct single or low density attacks and complete development within a terpene-rich environment. This pattern occurred even though similar bacterial taxa were tested. For example, Actinobacteria and Bacillales isolates from *D. valens* were more tolerant than those from *D. ponderosae* (Table 1). Actinobacteria occur in associations with both pine phloem (Adams et al., 2008; Hulcr et al., 2011) and bark beetles (Cardoza et al., 2006, 2009; Scott et al., 2008), so some tolerance to monoterpenes would seem necessary for their survival. Some bacteria in the Bacillales are capable of degrading various monoterpenes (Chang and Oriel, 1994; Savithiry et al., 1998) and similar hydrocarbons synthesized by plants (Wright et al., 1986; Jiménez et al., 2010). Unlike the isolates from *D. ponderosae*, three of the isolates from *D. valens* were γ-Proteobacteria.

The tolerance to monoterpenes by the yeast isolate from *D. ponderosae* is not entirely unexpected, given associations of yeasts with bark beetles (Shifrine and Phaff, 1956; Boone et al., 2008b; Davis et al., 2011). The adaptation of yeasts to the terpenoid-rich environment may be particularly beneficial for some species of bark beetles, as yeasts have been proposed to contribute to pheromone synthesis (Brand et al., 1977) and favor advantageous fungi (Davis et al., 2011). However, the extent to which yeasts contribute to pheromone communication in nature is uncertain, and some *Ips*-associated yeasts are relatively sensitive to some terpenes (Leufvén et al., 1988; Seybold et al., 2000). Yeast survival also could adversely affect larvae, as some natural enemies of bark beetles are attracted to yeast metabolites (Boone et al., 2008b).

Bacteria from *D. ponderosae* were more tolerant of β-phellandrene than (±)-α-pinene and (*S*)-(−)-β-pinene. β-Phellandrene is the predominant monoterpene of the primary host of *D. ponderosae*, whereas α- and β-pinene are the predominant monoterpenes of *P. banksiana*, which is the host into whose range populations of *D. ponderosae* are expanding. Further, these bacteria were highly tolerant of, and their growth was stimulated by, myrcene, which *D. ponderosae* exploits as a synergist of its aggregation pheromone to mass attack trees (Miller and Lindgren, 2000; Boone et al., 2008a; Borden et al., 2008). This compound also is an important component of *P. albicaulis* resin (Smith, 2000).

The antibacterial activity of 3-carene is particularly noteworthy. Specifically, this compound is correlated with resistance of conifers to *D. ponderosae* and the white pine weevil, *Pissodes strobi* (Peck) (Coleoptera: Curculionidae) (Ott, 2009; Robert et al., 2010), yet paradoxically frequently proves to be among the least bioactive in insect and fungal assays (Raffa et al., 1985; Klepzig et al., 1996; Lu et al., 2010). The antibacterial activity of 3-carene may be a mechanism that assists in tree resistance. Although 3-carene has a strong antibiotic effect on bacteria isolated from *D. ponderosae*, it caused little inhibition of bacteria isolated from *D. valens*. 3-Carene is a major volatile attractant (kairomone) for the flight response of *D. valens* adults (Hobson et al., 1993; Erbilgin et al., 2007). It is possible that bacteria closely associated with *D. valens* have adapted to be tolerant of this compound, given their inevitable exposure once the beetle penetrates the host.

α-Pinene, which occurs in *P. contorta* at low concentrations (Zavarin et al., 1969; Raffa and Berryman, 1983) but is a more predominant monoterpene in other hosts of *D. ponderosae* such as *P. ponderosa* and *P. albicaulis* (Sturgeon, 1979; Smith, 2000), was strongly antibiotic to microbes from *D. ponderosae*. In another study, however, *D. ponderosae*-associated bacteria tolerated α-pinene (Adams et al., 2009). This distinction may be due to difference among bacterial taxa tested, specifically *Pseudomonas* sp. that are tolerant of a diversity of aromatic compounds (Jiménez et al., 2010). α-Pinene also inhibits growth and germination of fungi associated with *D. valens* (Klepzig et al., 1996).

Two monoterpenes that all microbes tolerated were limonene and myrcene. These monoterpenes are found in relatively low concentrations in volatile profiles of many conifers, with an exception in *P. albicaulis* in which myrcene is a dominant component (Smith, 2000). Myrcene increases the attraction of the aggregation pheromone of *D. ponderosae* (Miller and Lindgren, 2000; Boone et al., 2008a; Borden et al., 2008) but has no or very little significant effect on the flight response of *D. valens* (Hobson et al., 1993; Sun et al., 2004). No evidence exists for a role of limonene in communication in *Dendroctonus*, but it is an attractant of *Ips typographus* (Reddemann and Reinhard, 1996). Myrcene and limonene inhibit growth of several bark beetle-associated fungi that colonize *P. jeffreyi* and *P. contorta* and grand fir, *Abies grandis* (Raffa et al., 1985; Paine and Hanlon, 1994), hosts with low concentrations of myrcene (Raffa and Berryman, 1982a,b, 1983; Smith, 2000). Myrcene also inhibits growth of fungi associated with *D. frontalis* Zimmerman to some extent; however, limonene has little effect on the same fungi (Hofstetter et al., 2005). Further work is necessary to fully characterize the antibiotic effects of limonene and myrcene on bark beetle-associated microorganisms.

Two of the most frequently reported bacteria associated with *Dendroctonus* beetles include *Bacillus* spp. and γ-Proteobacteria such as *Enterobacter* spp. and *Pseudomonas* spp., which have been detected with *D. valens*, *D. ponderosae*, spruce beetle (*D. rufipennis* Kirby), and *D. frontalis* (Cardoza et al., 2009; Morales-Jiménez et al., 2009; Adams et al., 2010). The other bacteria used in this study also have been detected in bark beetles, including *Micrococcus* spp. from *D. rufipennis* and *D. ponderosae* (Adams et al., 2008; Cardoza et al., 2009), and *Paenibacillus* sp. and *Frigoribacterium* sp. from *I. pini* (Delalibera et al., 2007; Cardoza et al., 2009). Yeasts such as *Candida* spp. previously have been isolated from a diversity of bark beetles, including *D. ponderosae* (Adams et al., 2008). Future studies are needed to determine the activities of blends of compounds, better quantify the frequencies of association of various bacterial taxa with bark beetles, better partition geographic ranges and life history strategies, and construct comparisons with outlier groups to improve our evolutionary understanding. With increasing focus on bacteria associated with bark beetles and insects in general, and new tools such as next-generation sequencing, our understanding of these ecological relationships will likely improve.

The chemical defenses of conifers provide strong resistance mechanisms to colonization by biotic agents, as is evident from the relatively few insects and pathogens that

colonize the phloem of living trees. We hypothesized that the ability of bark beetles that colonize living hosts to establish in new environments and hosts, which are facilitated by climate warming trends and human-facilitated transportation (Sun et al., 2004; Yan et al., 2005; Logan et al., 2010), may be in part due to activities of their microbial symbionts. Microbial symbionts perform a variety of functions important to the success of their animal hosts (Zilber-Rosenberg and Rosenberg, 2008), so their ability to survive a harsh environment, such as within trees, likely is critical to their animal host. In this study, we show that microbes associated with two species of bark beetle survived and grew in the presence of antimicrobial components of the chemical defense of conifers. Future studies are needed to investigate the metabolic capabilities of bacteria growing in the presence of individual resin components and blends.

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