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Mountain Pine Beetles Colonizing Historical and Naïve Host Trees Are Associated with a Bacterial Community Highly Enriched in Genes Contributing to Terpene Metabolism

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The mountain pine beetle, Dendroctonus ponderosae, is a subcortical herbivore native to western North America that can kill healthy conifers by overcoming host tree defenses, which consist largely of high terpene concentrations. The mechanisms by which these beetles contend with toxic compounds are not well understood. Here, we explore a component of the hypothesis that beetle-associated bacterial symbionts contribute to the ability of D. ponderosae to overcome tree defenses by assisting with terpene detoxification. Such symbionts may facilitate host tree transitions during range expansions currently being driven by climate change. For example, this insect has recently breached the historical geophysical barrier of the Canadian Rocky Mountains, providing access to naïve tree hosts and unprecedented connectivity to eastern forests. We use culture-independent techniques to describe the bacterial community associated with D. ponderosae beetles and their galleries from their historical host, Pinus contorta, and their more recent host, hybrid P. contorta–Pinus banksiana. We show that these communities are enriched with genes involved in terpene degradation compared with other plant biomass-processing microbial communities. These pine beetle microbial communities are dominated by members of the genera Pseudomonas, Rahnella, Serratia, and Burkholderia, and the majority of genes involved in terpene degradation belong to these genera. Our work provides the first metagenome of bacterial communities associated with a bark beetle and is consistent with a potential microbial contribution to detoxification of tree defenses needed to survive the subcortical environment.

In addition to the well-established roles of pairwise symbioses, the importance of multipartite associations and microbial communities to multicellular organisms is becoming increasingly recognized (1–6). Despite this, our understanding of how symbiotic relationships contribute to large-scale processes, such as ecosystem dynamics and the structure and functioning of biomes, remains poorly understood. Insects serve as a particularly useful model for exploring these cross-scale interactions because of their widespread and diverse associations with microbiota, roles in driving ecosystem processes, influences on human socioeconomic values, rapid evolutionary adaptations, and shifting responses to anthropogenic forces, such as climate change, species invasions, and habitat alteration.

Bark beetles (Curculionidae: Scolytinae) have the ability to overcome host tree defenses and thus colonize and kill healthy conifers. Several species undergo intermittent landscape-scale outbreaks, which appear to be increasing in frequency, magnitude, and interspecific confluence as a result of a warming climate and habitat conversions. For example, bark beetles caused substantial mortality across 47 million acres of conifers in western North America from 1997 to 2007, and the ongoing mountain pine beetle (Dendroctonus ponderosae Hopkins) outbreak is predicted to deplete 1 trillion cubic meters of pine in British Columbia, Canada, alone by 2014 (7).

The historical range of D. ponderosae extends from northern Mexico to southern British Columbia and inland to western North Dakota in the United States and the Rocky Mountains in Canada (8). Its preferred host is lodgepole pine, Pinus contorta (Douglas ex. Loud.), which occurs throughout this range. As conditions have warmed, D. ponderosae has expanded to higher latitudes (9–11) and breached the geophysical barrier of the Canadian Rocky Mountains (12) (Fig. 1). It has now colonized the hybrid crossings of P. contorta and jack pine, Pinus banksiana Lamb., and also the pure P. banksiana trees in this region (13, 14). These trees are contiguous with P. banksiana forests throughout all of southern Canada east of the Rocky Mountains, connecting with eastern white pine, Pinus strobus L., and red pine, Pinus resinosa Sol. Ex Aiton, further to the east (15). These beetles therefore potentially threaten pines across North America.

An important feature of bark beetles is their reliance on symbiotic microbes, which likely assist with host colonization and utilization. Symbiotic relationships with fungi have been studied for several decades (4, 16, 17) and are known to contribute to larval nutrition. Additionally, some bacterial symbionts have been implicated in defense against antagonistic fungi (18–20) and in nutrient acquisition (21). Moreover, beetle-associated microbes...
have demonstrated community structures that reflect both host beetle and geographic zones (22).

Both bacterium-beetle and bacterium-fungus-beetle relationships are mediated by host tree chemistry (18, 23). Pines synthesize terpenoids that are toxic at high concentrations to a broad range of insects, including bark beetles and their symbiotic fungi (24). These compounds are present in constitutive resin and phloem and are synthesized and translocated in response to the early stages of beetle-microbe attack. Monoterpines can kill or repel bark beetles, and both monoterpenes and diterpene acids can inhibit fungal symbionts (25–27). Terpenoid-based defenses of healthy trees confront beetles with a significant barrier during periods when their populations are low, and thus colonization is restricted to highly stressed hosts. However, several species, including D. ponderosae, can exhaust tree defenses through pheromone-mediated mass attack when population densities are high (7, 28).

An important question emerging from the current climate-driven range expansion of these beetles is how they will perform in new tree species and whether they will persist in a relatively endemic state or engage in self-driving outbreaks (29–31). Here, we focus on the potential role that D. ponderosae-associated bacterial communities may play during the colonization of both lodgepole and hybrid pines. We first describe how we obtained and examined samples of beetles, galleries, and trees to broadly identify similarities and differences between the communities associated with these environments using denaturing gradient gel electrophoresis (DGGE). Then, using a community metagenomic approach, we provide a detailed analysis of the bacterial communities associated with D. ponderosae and their galleries in both native and hybrid tree hosts. Finally, we explore the hypothesis that bacteria play a role in the detoxification of host tree defenses by specifically analyzing genes involved in terpene degradation encoded by these bacteria.

**Sample collection.** We obtained samples of D. ponderosae adults, their galleries, and unattacked tree phloem from four sites in 2010 across a gradient ranging from historical to naïve tree species of P. contorta (Mackenzie, British Columbia, Canada, and Grande Prairie, Peace River, and Slave Lake, Alberta, Canada), hybrid P. contorta-P. banksiana (Whitecourt, Alberta), and P. banksiana (Athabasca, Alberta) (Table 1; Fig. 1). For DGGE analysis, 291 beetle, gallery, and unattacked tree samples were obtained from multiple sites in Alberta and British Columbia, and for the community metagenomes, sampling consisted of approximately 300 adults and 130 galleries from 20 trees from pines located at each of two sites in July of 2009. One site was located near McBride, British Columbia, in a P. contorta stand, and the other was near Grande Prairie, Alberta, in a mixed P. contorta and hybrid P. contorta-P. banksiana stand (32) (Table 1; Fig. 1). At the time of sampling, D. ponderosae beetles were not observed colonizing P. banksiana.

For both analyses, D. ponderosae adults and 0.5- by 2-cm sections of phloem that included egg galleries and frass were removed from recently attacked trees and placed on ice before being transported to the laboratory at the University of Wisconsin—Madison. Phloem tissue from trees with no visible signs of bark beetle attack or disease was also sampled for DGGE analysis, with a maximum of three beetles and galleries and two phloem samples from a single tree and no samples from the same gallery (see the supplemental material). Samples for the community metagenomes were collected in the same way and pooled after collection for a total of four samples representing beetles and galleries from P. contorta and hybrid P. contorta-P. banksiana stands.

**Community metagenome sequencing and analysis.** Total bacterial genomic DNA was extracted from all four pooled samples. Eukaryotic material was removed through a series of washes, as previously described (33). Briefly, materials were ground with a mortar and pestle and washed in 1× phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄) containing 0.1% Tween. Each sample was shaken for 15 min at 150 rpm and centrifuged for 5 min at 500 rpm. This resulted in a 3-layer mixture containing (from the top) bacterial cells, fungal material, and insect material. The bacterial layer was removed and subjected to this process two more times. The final bacterial suspensions were centrifuged for 30 min at 4,100 rpm, resuspended in 1× PBS con-
TABLE 1 Site location and sample sizes of the collection of *D. ponderosae*, galleries, and phloem of unattacked trees

<table>
<thead>
<tr>
<th>Site location(s)</th>
<th>Host</th>
<th>Sample type</th>
<th>Sample size</th>
<th>Method of community analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mackenzie, British Columbia</td>
<td><em>P. contorta</em></td>
<td><em>D. ponderosae</em></td>
<td>26</td>
<td>DGGE</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gallery</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phloem</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>McBride, British Columbia</td>
<td><em>P. contorta</em></td>
<td><em>D. ponderosae</em></td>
<td>300</td>
<td>Community metagenomics</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gallery</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>Grand Prairie, Peace River, and</td>
<td><em>P. contorta</em></td>
<td><em>D. ponderosae</em></td>
<td>52</td>
<td>DGGE</td>
</tr>
<tr>
<td>Slave Lake, Alberta</td>
<td></td>
<td>Gallery</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phloem</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Whitecourt, Alberta</td>
<td><em>P. contorta</em></td>
<td><em>P. banksiana</em> hybrid</td>
<td>41</td>
<td>DGGE</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>D. ponderosae</em></td>
<td>13</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gallery</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Grand Prairie, Alberta</td>
<td><em>P. contorta</em></td>
<td><em>P. banksiana</em> hybrid</td>
<td>300</td>
<td>Community metagenomics</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>D. ponderosae</em></td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>Athabasca, Alberta</td>
<td><em>P. banksiana</em></td>
<td><em>D. ponderosae</em></td>
<td>8</td>
<td>DGGE</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gallery</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phloem</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 2 Summary of sequencing statistics for the four *D. ponderosae*-associated metagenomes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Hybrid pine</th>
<th>Lodgepole pine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size of assembly (Mbp)</td>
<td>29</td>
<td>30.2</td>
</tr>
<tr>
<td>No. of protein-coding genes</td>
<td>42,426</td>
<td>53,181</td>
</tr>
<tr>
<td>No. of contigs</td>
<td>9,161</td>
<td>9,616</td>
</tr>
<tr>
<td>No. of singleton reads</td>
<td>24,220</td>
<td>47,597</td>
</tr>
<tr>
<td>Largest contig (bp)</td>
<td>142,661</td>
<td>59,558</td>
</tr>
</tbody>
</table>

RESULTS

Microbial community analysis. Principle component analysis of the DGGE data revealed no distinct clustering among beetle, gallery, or tree samples from either location or tree species (see Fig. S1 in the supplemental material). We sequenced bands corresponding to the 12 most commonly identified operational taxonomic units (OTU) in our DGGE analyses and found that 10 were most similar to *Gammaphotobacteria*, 1 to *Betaproteobacteria*, and 1 to *Actinobacteria* (see Table S1 in the supplemental material).

Community metagenome sequencing and phylogenetic binning. The four metagenomes generated using shotgun 454 pyrosequencing comprised 27.1 to 58.8 Mbp of sequence after assembly (Table 2). These were constructed from (i) whole *D. ponderosae* beetles from infested *P. contorta*, (ii) gallery material from infested *P. contorta*, (iii) whole *D. ponderosae* beetles from infested hybrid *P. contorta-P. banksiana*, and (iv) gallery material from infested hybrid *P. contorta-P. banksiana*.

Nucleotide sequence accession numbers. Raw pyrosequencing data for the community metagenomes have been deposited in the National Center for Biotechnological Information’s (NCBI) short read archive under accession no. SRA4088237, SRA4088238, SRA4088239, and SRA4088241. Assembled community metagenomes are on the Joint Genome Institute’s Integrated Microbial Genomes/Genomes/Genomes (IMG/M) database (47) under project identification (ID) no. 2032320008, 2032320009, 2029527007, and 2035918003. Sequences of all DGGE bands obtained in this study are available in GenBank under accession no. JF810915 to JF810926.
ning analysis revealed similar taxonomic patterns across all four community metagenomes. Broadly, **Gammaproteobacteria** comprised the majority of all sequences in each sample, although **Betaproteobacteria**, **Alphaproteobacteria**, **Firmicutes**, and **Actinobacteria** were also represented (see Fig. S3 in the supplemental material). Analysis at the genus level identified abundant sequences matching those of **Pseudomonas**, **Rahnella**, **Serratia**, **Erwinia**, **Stenotrophomonas**, and **Pantoea** (Fig. 2A; for details, see Data Set S1 in the supplemental material).

**Analysis of genes putatively involved in terpene degradation.** We performed a metabolic reconstruction analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (48) (Table 3). An analysis of proteins involved in the limonene and pinene degradation pathway yielded between 90 and 198 proteins in each community metagenome (Table 3; see Data Set S2 in the supplemental material). This comprises 0.17 to 0.27% of the predicted proteins in these data sets. Of the 20 enzymes comprising this pathway, 5 were found to be enriched in the pine beetle metagenomes compared to metagenomes of other plant biomass-associated microbial communities (Table 3; \( P < 0.05 \), Fisher’s exact test). These included an aldehyde dehydrogenase (EC 1.2.1.3), an oxidoreductase (EC 1.14.13.-), an enoyl-coenzyme A (CoA) hydratase (EC 4.2.1.17), and two 3-hydroxyacyl-CoA epimerases (EC 1.1.1.35, 4.2.1.17, 5.1.2.3, and 5.3.3.8). All enzymes involved in the conversion of \( \alpha \)-pinene to 3-isopropylbut-3-enoic acid, myrtenic acid, or pinocarvone, as described in KEGG, were detected. The beetle metagenome from the hybrid pine contained the highest proportion of potential terpene degradation enzymes (113 proteins, 0.27% of proteins), while the gallery metagenome from the hybrid pine contained the smallest (90 proteins, 0.17% of proteins).

We also investigated genes involved in diterpene degradation using the well-characterized \( \text{dit} \) gene cluster found in the diterpene-degrading bacterium **Pseudomonas abietaniphila** BKME-9 (39, 49). Of the 20 proteins annotated as belonging to the \( \text{dit} \) gene cluster, 17 to 19 were present in each of the community metagenomes sequenced in our study (Fig. 3A). Of these, 12 to 16 were found to be enriched compared to metagenomes of other plant
TABLE 3 Identification of genes involved in the KEGG limonene and pinene degradation pathway within the four mountain pine beetle metagenomes compared to a combined data set of publicly available metagenomes

<table>
<thead>
<tr>
<th>Product annotation</th>
<th>KEGG Orthology (KO) identifier</th>
<th>No. of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hybrid pine</td>
<td>Lodgepole pine</td>
</tr>
<tr>
<td></td>
<td>D. ponderosae gallery</td>
<td>D. ponderosae gallery</td>
</tr>
<tr>
<td>OXIDOREDUCTASES</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidoreductases</td>
<td>K00120</td>
<td>0</td>
</tr>
<tr>
<td>Aldehyde dehydrogenase</td>
<td>K00128</td>
<td>43</td>
</tr>
<tr>
<td>Oxidoreductases</td>
<td>K00155</td>
<td>0</td>
</tr>
<tr>
<td>Oxidoreductases</td>
<td>K00149</td>
<td>12</td>
</tr>
<tr>
<td>Oxidoreductases</td>
<td>K00517</td>
<td>4</td>
</tr>
<tr>
<td>Acyltransferases with transferring groups other than amino-acetyl groups</td>
<td>K00680</td>
<td>1</td>
</tr>
<tr>
<td>HYDROLASES acting on ester bonds</td>
<td>K01076</td>
<td>0</td>
</tr>
<tr>
<td>Enoyl-CoA hydratase</td>
<td>K01692</td>
<td>32</td>
</tr>
<tr>
<td>Carbon-oxygen lyases</td>
<td>K01726</td>
<td>0</td>
</tr>
<tr>
<td>Hydroxycyclo-CoA dehydrogenase</td>
<td>K01782</td>
<td>10</td>
</tr>
<tr>
<td>Hydroxycyclo-CoA dehydrogenase</td>
<td>K01825</td>
<td>10</td>
</tr>
<tr>
<td>Ligation of carbon-sulfur bonds</td>
<td>K01913</td>
<td>0</td>
</tr>
<tr>
<td>(S)-Limonene 6-monoxygenase</td>
<td>K01381</td>
<td>0</td>
</tr>
<tr>
<td>(S)-Limonene 7-monoxygenase</td>
<td>K01349</td>
<td>0</td>
</tr>
<tr>
<td>Limonene 1,2-epoxide hydratase</td>
<td>K01382</td>
<td>0</td>
</tr>
<tr>
<td>trans-Carveol dehydrogenase</td>
<td>K01275</td>
<td>0</td>
</tr>
<tr>
<td>trans-Carveol dehydrogenase</td>
<td>K01243</td>
<td>0</td>
</tr>
<tr>
<td>Monoterpene epsilon-lactone hydratase</td>
<td>K01383</td>
<td>0</td>
</tr>
<tr>
<td>Limonene hydroxylase</td>
<td>K01378</td>
<td>0</td>
</tr>
<tr>
<td>Limonene hydroxybutyl-CoA epimerase</td>
<td>K01473</td>
<td>0</td>
</tr>
<tr>
<td>Limonene 1,2-monooxygenase</td>
<td>K01474</td>
<td>0</td>
</tr>
<tr>
<td>Total no. of proteins</td>
<td>42,427</td>
<td>53,182</td>
</tr>
</tbody>
</table>

a The publicly available metagenomes consist of plant biomass-degrading microbial communities associated with the termite hindgut, wallaby foregut, cow rumen, panda gut, leafcutter ant fungus garden, switchgrass compost community, and poplar biomass bioreactor (for details see Materials and Methods and Data Set S2 in the supplemental material). Results representing genes enriched relative to other plant biomass-degrading metagenomes are highlighted in boldface (P < 0.05, Fisher’s exact test).

The publicly available metagenomes consist of plant biomass-degrading microbial communities associated with the termite hindgut, wallaby foregut, cow rumen, panda gut, leafcutter ant fungus garden, switchgrass compost community, and poplar biomass bioreactor (for details see Materials and Methods and Data Set S2 in the supplemental material). Results representing genes enriched relative to other plant biomass-degrading metagenomes are highlighted in boldface (P < 0.05, Fisher’s exact test).

DISCUSSION

This study provides the first community metagenomic analysis of a bark beetle. The results of both our community metagenomic and DGGE analyses indicate that Gammaproteobacteria are prevalent in both D. ponderosae beetles and their galleries from both lodgepole and hybrid lodgepole-jack pines (see Table S1 and Fig. S2 and S3 in the supplemental material). In particular, Gamma-proteobacteria belonging to the genera Pseudomonas, Stenotrophomonas, Erwinia, and Serratia were particularly abundant in these metagenomes (Fig. 2A), indicating these groups are consistently associated with D. ponderosae or their host trees. Our DGGE-based analysis did not resolve differences between these environments (see Fig. S1 in the supplemental material), indicating that bacterial communities may be broadly similar among all of them. Moreover, no distinct differences were observed in the composition of metagenomes from the beetle and gallery samples from Alberta and British Columbia. Taken together, this suggests that a relatively consistent bacterial community is associated with D. ponderosae and its microenvironment and that the recent expansion of this insect’s range will not be impeded by a lack of appropriate bacterial communities.

Conifers produce monoterpenes and diterpenes that are toxic to bark beetles and their fungal symbionts, both constitutively and in response to attack. We identified numerous bacterial genes associated with degradation of these compounds, including well-represented KEGG pathways for limonene and pinene degradation in each of the four metagenomes (Table 3). Moreover, we identified numerous genes homologous to the dit gene cluster of Pseudomonas abietiphila BKME-9 (Fig. 3A), which is known to be involved in diterpene degradation. We found a significantly higher proportion of these genes in our beetle metagenomes than...
those from other plant biomass-associated microbial communities (Fig. 3B). The biomass-degrading communities used for comparison originate from a diversity of different environments where plant toxins would be likely encountered, suggesting that bacteria associated with pine beetles are particularly well adapted to metabolize the aromatic plant toxins in their environment.

The majority of genes involved in terpene degradation belong to bacteria in the genera *Pseudomonas* and *Rahnella* (Fig. 2B; see Fig. S4 in the supplemental material). The genus *Pseudomonas* contains numerous species that degrade a wide range of aromatic compounds, including plant toxins, xenobiotics, and pollutants (39, 50–52). The biodegradative capacities of *Rahnella* isolates have been less intensively studied, but numerous *Enterobacteriaceae* closely related to this genus are known to degrade a diversity of aromatic compounds (53, 54). Our finding that these genera are both associated with *D. ponderosae* and possess numerous genes putatively involved in terpenoid degradation suggests they may contribute to *D. ponderosae*’s ability to attack live conifers. Future transcriptomic and culture-based work confirming the ability of these bacteria to degrade plant toxins is required.

These results suggest several testable models to explain associations of bacterial communities with *D. ponderosae* and their galleries. One hypothesis is that *D. ponderosae* vector terpene-degrading microbiota between trees. Bark beetles are known to consistently vector both fungi (16) and several genera of bacteria, including *Streptomyces* (19, 55), thereby explaining the similar composition of all metagenomes analyzed here. This would also be consistent with the high representation of *Pseudomonas* and *Rahnella* genes associated with terpene degradation in all four *D. ponderosae*-associated metagenomes we described. A second non-exclusive model is that these communities are associated primarily with host trees rather than the beetles themselves. Beetle colonization of a tree might induce proliferation of specific bacteria, such as *Pseudomonas* or *Rahnella*, that can exploit terpenoids and other carbon sources.

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**FIG 3** Representation of genes involved in diterpene degradation in mountain pine beetle-associated metagenomes. (A) In the diterpene degradation gene cluster *dit*, each gene is colored according to its representation within the four mountain pine beetle metagenomes. (B) Total copy numbers for each gene are shown, with those in blue enriched (Fisher’s exact test, \( P < 0.05 \)) relative to other plant biomass-degrading metagenomes.
sources present in resin. Thus, even if not vectored by the beetles, the colonization attempts and subsequent tree responses may create an environment that promotes the growth of terpenoid-metabolizing bacteria. According to this model, resident microbial populations may influence bark beetles, with trees harboring fewer terpene-degrading bacteria posing more resistance to colonization.

This work provides insights into host colonization and range expansion of *D. ponderosae* by characterizing the microbiome associated with these beetles and host conifers. A combination of methods suggests that a relatively consistent bacterial community is associated with these beetles in lodgepole and hybrid lodgepole-jack pines. Our results identify bacteria of the genera *Pseudomonas* and *Rahnella* that may directly or indirectly contribute to the ability of beetles to overcome tree defenses. Future studies confirming and quantifying the ability of bacteria to degrade tree defenses are needed to understand the influences they may have on bark beetle biology.

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