Action of Douglas Fir Tussock Moth Larvae and Their Microflora on Dietary Terpenes

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A single type of bacterium, tentatively identified as a member of the genus Bacillus, was isolated from 2 of 20 midguts of Douglas fir tussock moth larvae being fed a diet of fir needles. No bacteria could be isolated from most midguts. Although spherically shaped bodies were present in the food bolus, these bodies, if microorganisms, could not be distinguished from spherical bodies associated with the plant tissue. The Douglas fir tussock moth dietary terpenes were altered during their passage through the insects, with two new terpenes being detected in the feces. One of these was identified as isoborneol. The relative significance of the insect and gut microflora with respect to terpene modification is unresolved. The well-established toxicity of terpenes may account for the near absence of common gut microflora in the insects.

The Douglas fir, Pseudotsuga menziesii, which is a preferred food source for Orgyia pseudotsugata, the Douglas fir tussock moth (DFTM), is rich in essential oils consisting mainly of terpene compounds. The composition of these volatile oils has been demonstrated to vary considerably, depending mainly upon the geographic location of the trees (12).

Toxicity to the terpenes found in essential oils has been shown with many living systems. Terpenes are known to be toxic to fish (9), humans (4), insects (11), and microbes (10). The terpenes which are found in conifers have been shown to have several effects on insect pests: (i) vapors of terpenes found in cortex oleoresins from pine trees make these trees more resistant to attack by bark beetles (11); (ii) terpenes are known to aid in the attraction of an insect to its preferred host (7, 8); and (iii) terpenes are known to supply raw material for the production of insect pheromones (2). The microflora of the gut has been implicated in terpene-related reactions. For example, microbes isolated from bark beetles have been shown to synthesize the pheromonal compound verbenol from pinene (2).

Other relationships between insects and microbes have been demonstrated. For example, Fogleston et al. (5) demonstrated that a variety of microorganisms are present in the hindguts of cockroaches. Barra (1) elucidated a symbiotic relationship between certain fungi and Southern pine beetles. It is apparent that there may be a variety of relationships among dietary terpenes, the insects, and insect gut microflora. To eluci-

date these interrelationships in the DFTM, a study describing the fate of dietary terpenes and the possible significance of gut microflora upon terpene modification was carried out.

MATERIALS AND METHODS

Chemicals. α-Pinene (practical grade) and limonene were obtained from Eastman Chemical Products, Inc. β-Pinene was obtained from K & E Fine Chemicals. Isoborneol and isobornyl acetate were obtained from Aldrich Chemical Co. Penicillin G (sodium salt), chlorotetracycline, chloramphenicol, and streptomycin sulfate were obtained from Sigma Chemical Co. Fir needles were collected on private land near Princeton, Idaho. To insure a relatively constant oleoresin content throughout the study, the needles were collected at one time and then stored as described by von Rudloff (12). No change in the terpene content of the needles was observed during the course of the study.

DFTM larvae. DFTM egg masses were collected and sent to us by David Holland, U.S. Forest Service, Albuquerque, N.M., from the Cibola National Forest in the fall of 1978. The egg masses were stored, and the larvae were reared as previously described (3). Larvae employed in experiments were reared to the third instar on an artificially prepared diet (6) and then placed on a fir needle diet until the fifth instar.

Measurement of terpenes. Plastic petri plates containing 2 g (wet weight; approximately 1 g, dry weight) of Douglas fir needles and 10 larvae were placed in a temperature-controlled incubator with a 16-h photophase. In addition, plates containing only fir needles were placed in the rearing chamber to serve as controls for the measurement of the level of unmodified dietary terpenes. Fir needles were collected in May 1979. It was determined that fecal pellets could be incubated for up to 2 weeks in a petri dish without loss of terpenes. The possible role of midgut microbes in terpene changes was also investigated by analyzing feces of larvae reared on needles from boughs that had
been dipped briefly in a solution containing penicillin, streptomycin, chloramphenicol, and chlorotetracycline, each at a concentration of 1 mg/ml. The antibiotic solution contained 0.01% Triton X-100 (vol/vol) to act as a wetting agent. It was determined that each gram of needles adsorbed 2.4 ml of the antibiotic-containing solution; thus, each gram of fir needles contained about 2.4 mg of each antibiotic.

When all needles had been consumed in the larval plates at the end of 3 days, both fecal pellets and control needles were collected, frozen in liquid nitrogen, ground, and then placed in CS2 (5 ml/g of needles). The suspensions were then refluxed for 30 min, followed by filtration through Whatman no. 41 filter paper. Pooled filtrates from three successive refluxes were concentrated by distilling away the CS2.

The extracts were fractionated by using a Hewlett-Packard series 5700 gas chromatograph fitted with a 30-m OV-1 glass capillary column. Chromatograms were generated by using total ions and quantitated by using a Hewlett-Packard standard computer program. The oven temperature was programmed for 60°C for 5 min, followed by an 8°C/min increase to a final temperature of 180°C. This was coupled to a Hewlett-Packard 5900 mass spectograph with a 5930 data system.

Microbial examination of midguts. Midguts from larvae of needle-fed DFTM were removed aseptically, placed in 1 ml of brain heart infusion broth, and blended in a Vortex mixer. A 0.01-ml amount of broth was used immediately to inoculate thioglycolate broth and brain heart infusion agar. A loopful of the broth was used to streak eosin methylene blue agar plates for colony isolation and to streak a 1.5% agar-based medium containing 1% glucose and 10% blended fir needles, and then the broth itself was incubated to permit possible microbial growth. Brain heart infusion agar plates were incubated both aerobically and anaerobically, whereas the other media were incubated only aerobically. All media were incubated at 30°C for 72 h. An incubation temperature of 30°C was selected to duplicate the temperature employed in the cultivation of the insect larvae. Wet mounts of midgut contents were examined with a phase-contrast microscope.

Scanning electron microscope techniques. DFTM midguts were fixed for 12 h in 0.1 M cacodylate buffer containing 3% glutaraldehyde, postfixed for 1 h in 1% osmium tetroxide in water, and dehydrated to 100% ethanol. They were then sliced medially with a razor blade. The halves were critical point dried, mounted on aluminum stubs, and coated with a 30-nm layer of gold. Fir needles were cut into 4-mm segments and then slit longitudinally. They were fixed by the same procedure as were the DFTM midguts, critical point dried, mounted, and gold coated. Critical-point drying was done in a Bowman 1500 critical-point device, and gold coating was done in a Technics Hummer V sputtering device. Samples were viewed on an Eltec Autoscan U-1 scanning electron microscope at 20 kV and with a viewing angle of approximately 30°.

RESULTS

Figure 1 shows a gas chromatogram of the dietary terpenes extracted from the Douglas fir needles employed in this study. The compounds found in these needles at more than 1% of the total were santene, α-pinene, camphene, Δ3-carene, β-pinene, limonene, and isobornyl acetate.

The terpenes found in the feces were both qualitatively and quantitatively different from those found in the original needles (Fig. 1 and 2). Two new, nondiet terpenes, labeled 1A and 2A, were detected in the feces. The mass spectra for these compounds are shown in Fig. 3 and 4. One of the terpenes was identified as isoborneol (Fig. 4). Although it remains unidentified, the similarity of the spectrum of the other compound (Fig. 3) to that of α-pinene suggests that it may be an α-pinene derivative. The isoborneol and unknown terpene made up approximately 10 and 20% of the total fecal terpenes (Table 1).

It can also be seen that between approximately 80 and 95% of the terpenes ingested were accounted for in the feces. The relative quantities

![Fig. 1. Gas chromatographic profile of fir needle terpenes. The major terpenes and their absolute concentrations (milligrams of terpene per gram of needles) include: 1, santene (55.3); 2, α-pinene (210); 3, camphene (369); 4, Δ3-carene (46.4); 5, β-pinene (17.6); 6, limonene (90.8); and 7, isobornyl acetate (372).](http://aem.asm.org/)

![Fig. 2. Gas chromatographic profile of terpenes in the feces of fir needle-fed larvae.](http://aem.asm.org/)
of the two fecal terpenes not present in the needle diet accounted for approximately 30% of the total (Table 1). Several methods were employed to detect the presence of microorganisms and the possible significance of such organisms to the alterations observed during passage of the terpenes through the insects. Larvae fed on the fir needle diet, but containing the antibiotics, produced feces containing virtually the same concentrations of the same major terpenes (Table 1). The larvae were reared from the third instar to pupation with no noticeable difference between treated and untreated larvae in weight gain, amount eaten, and rate of development. The lack of antibiotic effect on the fecal terpene pattern suggested that gut bacteria were not significant to terpene metabolism in the DFTM larvae. Nevertheless, bacteria might still exist in the untreated midguts. It is even conceivable that bacteria were still present in the midguts of larvae on the anti-

**Table 1. Terpene content of needles and feces**

<table>
<thead>
<tr>
<th>Compound no.</th>
<th>Compound</th>
<th>Distribution in needles (%)</th>
<th>Distribution in feces (%)</th>
<th>Recovery in feces (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>On normal diet</td>
<td>On antibiotic diet</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Santene&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.8</td>
<td>3.6</td>
<td>3.2</td>
</tr>
<tr>
<td>2</td>
<td>α-Pinene&lt;sup&gt;d&lt;/sup&gt;</td>
<td>17.0</td>
<td>9.4</td>
<td>9.8</td>
</tr>
<tr>
<td>3</td>
<td>Camphene&lt;sup&gt;d&lt;/sup&gt;</td>
<td>32.3</td>
<td>14.0</td>
<td>16.0</td>
</tr>
<tr>
<td>4</td>
<td>Δ&lt;sub&gt;2&lt;/sub&gt;-Carene&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.0</td>
<td>1.1</td>
<td>1.0</td>
</tr>
<tr>
<td>5</td>
<td>β-Pinene&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.6</td>
<td>2.9</td>
<td>2.8</td>
</tr>
<tr>
<td>6</td>
<td>Limonene&lt;sup&gt;d&lt;/sup&gt;</td>
<td>7.0</td>
<td>2.0</td>
<td>3.2</td>
</tr>
<tr>
<td>7</td>
<td>Isobornyl acetate&lt;sup&gt;d&lt;/sup&gt;</td>
<td>34.2</td>
<td>34.9</td>
<td>35.5</td>
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<td>1A</td>
<td>Unknown</td>
<td>0.4</td>
<td>6.0</td>
<td>6.0</td>
</tr>
<tr>
<td>2A</td>
<td>Isoborneol&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.0</td>
<td>9.2</td>
<td>9.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Absolute amounts of terpenes are shown in the legend to Fig. 1.<n<sup>b</sup> Percentage of ingested terpenes recovered in feces.<n<sup>c</sup> Compound identified by comparison with published mass spectra.<n<sup>d</sup> Compound identified by comparison with mass spectra obtained from standard compound.

FIG. 3. Mass spectrum of fecal terpene no. 1A.

FIG. 4. Mass spectrum of fecal terpene no. 2A, identified as isoborneol.

FIG. 5. Scanning electron micrographs of the midgut food bolus from larvae on a fir needle diet. Micrographs reveal spherical bodies (a) and corrugated structures (b). Bar = 1 μm in (a) and 10 μm in (b).
biotic-containing diet, although they would have to have been resistant or otherwise protected from the antibiotic supplements. To determine the presence of bacteria in untreated larvae, the midgut contents were analyzed. The isolation procedures, designed to recover most common bacteria, yielded only one type of bacterium, found only in the brain heart infusion broth tubes, which was isolated from 2 of 20 midguts analyzed. The isolates from both midguts appeared to be identical in the tests run and were tentatively identified as members of the genus *Bacillus*. These isolates grew up in and were isolated from the original brain heart infusion broth tubes, which had been inoculated with the larval midgut. Both isolates were large, motile rods, were aerobic, formed spores, and were gram positive and catalase positive. The isolates grew on the surface of the thioglycolate broth subculture but not in the original thioglycolate tubes, suggesting that they were present in very low numbers in the original inoculum.

Wet mounts of midgut material from insects on a fir needle diet did not reveal any motile microorganisms. By contrast, it has been frequently observed in this laboratory that insects growing on an artificial diet without microbial inhibitors contain motile bacterial forms. Scanning electron microscopy of the midgut contents of insects feeding on fir needles revealed spheres that were of a size corresponding to that of bacteria (Fig. 5a). Corrugated structures with adherent spherical bodies were also found in the gut material (Fig. 5b). To determine whether these structures were fir needle components, a brief scanning electron microscope examination of fir needles was performed. The surface structure of an uningested needle is shown in Fig. 6a. The corrugated structures found in the midgut were identified as broken portions of needle surface. Spheres (Fig. 6c), indistinguishable by scanning electron microscopy from those found in DFTM guts, were found inside the sectioned cells (Fig. 6b) of fir needles. Both spherical particles and the corrugated needle surface structure, indistinguishable from those found in larvae fed on untreated fir needles, were also found in the midguts of insects feeding on antibiotic-treated needles.

**DISCUSSION**

Although common bacteria have not been detected in the midgut, the existence of other less common microorganisms may play a role in the metabolism of terpenes. It is also possible that

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*individual cells of the needle. Bar = 10 μm in (a), 20 μm in (b), and 1 μm in (c).*
Microbes may be present in the foregut or hindgut and that these are causing changes in the terpene content. Standard microbial isolation procedures indicated that a few DFTM larvae raised on Douglas fir needles contained a single common microorganism tentatively identified as a member of the genus Bacillus. Scanning electron microscopy revealed spherical bodies in the midgut bolus that might be bacteria. However, since similarly sized and shaped bodies were also found associated with the plant tissue, the identity of these bodies remains unresolved. The apparent lack of a variety of midgut bacteria was surprising. Should these observations prove to be correct, the DFTM midgut would seem to represent an unusual microenvironment which is relatively free of common microorganisms. However, these observations still do not preclude the existence of highly fastidious or other unusual forms of microorganisms. Although insect growth conditions in the laboratory were similar to those in nature, larvae grown completely in the wild may have more contact with microorganisms that could survive in the gut.

Two terpenes which were not present in the original diet were found in the feces. The first, which was identified as isoborneol, is probably formed by the hydrolytic cleavage of the acetate moiety from isobornyl acetate. The second compound has not been identified yet. Since its mass spectral peaks most closely matched those of α-pinene, it is possible that it is derived from the latter terpene. The mechanism of formation and the function, if any, of the two nondietary terpenes found in the feces remain to be determined.

The terpene profile of the Douglas fir needles reported here is similar to that reported by von Rudloff for the Rocky Mountain strains of Douglas fir (12). At least two explanations may be offered for the low recovery of the dietary terpenes from the feces. First, some of the terpenes may have volatilized during digestion. This possibility is supported by the observation that, in general, there was less recovery of terpenes with lower boiling points. The fact that unconsumed control needles show no such drop is an indication that the digestive process might somehow have facilitated volatilization. The second alternative would be to assume that the insect, perhaps aided by the gut microflora, disposes of the terpenes by metabolic or other processes. Since at least two nondietary terpenes were detected in the feces, it is apparent that some metabolic or physiochemical conversion of the dietary terpenes occurs in the gut.

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LITERATURE CITED