

ROLE OF THREE RODENTS IN FOREST NITROGEN FIXATION IN WESTERN OREGON: ANOTHER ASPECT OF MAMMAL-MYCORRHIZAL FUNGUS-TREE MUTUALISM

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ABSTRACT.—To determine the role of the California red-backed vole (*Clethrionomys californicus*), the northern flying squirrel (*Glaucomys sabrinus*), and the deer mouse (*Peromyscus maniculatus*) in the nitrogen cycle of forest stands in western Oregon, bacterial colonies were isolated and purified from feces, and their nitrogen-fixing ability measured by acetylene-reduction assay. The ability of the bacterial species *Azospirillum* sp. to withstand freezing was also tested. Fecal extracts were used to test whether fecal pellets can provide the nutrients necessary for growth of the bacteria. All the feces tested contained viable nitrogen-fixing bacteria, and both species can survive drying and one can survive freezing. *Azospirillum* colonies grew well on liquid medium but required yeast extract for growth and nitrogenase activity. Fecal extracts from flying squirrels and chickarees (*Tamiasciurus douglasi*) were as effective as the yeast. The results suggest another link in the chain of mutualism that unites small mammals, mycorrhizal fungi, and forest trees.

Some small forest-dwelling rodents help maintain productivity of forested ecosystems by disseminating viable spores of hypogeous, mycorrhizal fungi (Kotter and Farentinos 1984, Maser et al. 1978, McIntire 1984, Rothwell and Holt 1978, Trappe and Maser 1976). Nitrogen-fixing bacteria have recently been found in fungal sporocarps eaten by small mammals (Hunt and Maser 1985, Li and Castellano 1985, Maser et al. 1978). To determine whether small mammals play a role in the nitrogen balance of the forest by eating hypogeous fungal sporocarps and thereby dispersing the bacteria, we sought answers to these questions:

- Do feces of forest-dwelling rodents contain nitrogen-fixing bacteria?
- Are these nitrogen-fixing bacteria viable after passage through a rodent's intestinal tract?
- Can these bacteria survive freezing, drying, and high temperature?
- Do the fecal pellets provide the nutrients necessary for growth of nitrogen-fixing bacteria?

We studied three common and widely distributed forest rodents: the deer mouse (*Peromyscus maniculatus*), the California red-backed vole (*Clethrionomys californicus*), and the northern flying squirrel (*Glaucomys sabrinus*).

The deer mouse, ubiquitous throughout most of North America, feeds on fruits, seeds (including conifer seeds), and hypogeous, mycorrhizal fungi (Hunt and Maser 1985, Maser et al. 1978, 1981). The red-backed vole ranges south of the Columbia River, throughout the forested areas of western Oregon into northwestern California, and from the Pacific Coast to the crest of the eastern Cascade Range (Maser et al. 1981). It eats mostly hypogeous fungal sporocarps (Maser et al. 1978, Ure and Maser 1982). The flying squirrel, a nocturnally active inhabitant of most coniferous forests throughout temperate North America, also eats mostly fruiting bodies of hypogeous, mycorrhizal fungi (Maser et al. *Food habits*, 1985; Maser et al. *Northern flying squirrel*, 1985).

We recognize that more quantitative data may be desired than is found in this paper. Our data are the first reported for the following interactions, however, and we are only now determining what quantitative questions can and need to be asked. Further, we have not found a way or person to identify the unknown bacteria and yeasts that we encounter.

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TABLE 1. Acetylene reduction by bacteria isolated from small mammal feces.

Bacterium isolated	Source	Acetylene reduction (nmole ethylene/mg protein per hr)
<i>Azospirillum</i> sp.	California red-backed vole	167.0 ¹
<i>Azospirillum</i> sp.	Northern flying squirrel	88.6 ²
<i>Clostridium butyricum</i>	Deer mouse	344.0 ¹

¹Average of 5 replicates²Average of 3 replicates

METHODS

To determine if nitrogen-fixing bacteria survive passage through rodent digestive tracts, techniques for fecal collection and laboratory techniques were developed to isolate and purify bacterial colonies from rodent feces (Li and Maser, 1986). Acetylene-ethylene assay for nitrogenase activity as an indication of nitrogen-fixing ability was as described by Hardy et al. (1968). Yeast populations in feces were determined by dilution-plating on sodium albumenate agar (Waksman and Fred 1922).

To test the ability of *Azospirillum* sp. to withstand freezing, flying squirrel feces were held at -17 C (1 F) for three months. The feces were thawed and the bacteria isolated, grown, and tested for nitrogenase activity (Li and Maser, in press; additional details on file, Forestry Sciences Laboratory, Corvallis, Oregon 97331).

Azospirillum sp. isolated from feces of the flying squirrel were used to test whether fecal pellets provide the nutrients necessary for growth of nitrogen-fixing bacteria. We used fecal extracts from both the flying squirrel and the chickaree (*Tamiasciurus douglasi*), a diurnal tree squirrel that shares the flying squirrel's habitat in the Pacific Northwest. Fecal extracts were prepared by homogenizing the fecal pellets in deionized water (2:100 w/v) in a Polytron with a saw-tooth generator, 165 x 12 mm, at maximum speed for 1 min. Debris was removed by centrifugation for 30 min at 14,500 x g. The supernatant was filter-sterilized, and 0.2 ml was added to 20 ml of Döbereiner's nitrogen-free liquid medium (Döbereiner and Day 1976).

RESULTS AND DISCUSSION

Feces of all the small mammals we tested contained viable nitrogen-fixing bacteria.

Azospirillum sp.—a microaerophilic, nitrogen-fixing bacterium (Lakshmi et al. 1977)—was isolated from feces of one red-backed vole and five flying squirrels. *Clostridium butyricum*—an anaerobic, nitrogen-fixing bacterium (Buchanan and Gibbons 1974)—was isolated from feces of seven deer mice. The bacteria not only survived passage through the digestive tracts but also grew and reduced acetylene in vitro (Table 1).

Most bacteria do not survive freezing without desiccation because they rupture on thawing; thus, bacteria to be stored are usually freeze-dried (Ghera 1981). *Azospirillum* sp. can survive freezing in the feces, however, and *C. butyricum* forms an endospore stage that should also be able to survive freezing.

Azospirillum sp. survived at least 15 years in air-dried soil at 28 C ± 2 C (82 ± 4 F) (Lakshmi et al. 1977). In our study *C. butyricum* survived 3 months in air-dried feces, presumably in the endospore form, and it retained the capacity to reduce acetylene.

Many workers have used yeast extract or yeast extract combined with vitamins to promote nitrogenase activity of acetylene-reducing bacteria (Barber and Evans 1976, Hahtela et al. 1981, Murray and Zinder 1984, Rennie 1981, Tyler et al. 1979). The *Azospirillum* colonies grew well on nutrient agar or trypticase soy agar, and they reduced acetylene when grown under conditions of 99% nitrogen and 1% oxygen. The bacterium required yeast extract for growth and nitrogenase activity (Table 2). Controls without acetylene were also assayed with negative results. Extracts from the feces of flying squirrels and chickarees were as effective as yeast extract for inducing nitrogenase activity. Addition of vitamins into the fecal extract proved unnecessary for growth and nitrogenase activity of *Azospirillum* sp. (Table 2).

Yeast extract, a component of many standard culture media (Tuladhar and Rao 1985),

TABLE 2. Influence of additives on acetylene reduction by *Azospirillum* sp. in Döbereiner's nitrogen-free liquid medium (Döbereiner and Day 1976).

Growth condition	Acetylene reduction ^{1/} (nmoles ethylene/mg protein per hr)
Medium with:	
Yeast extract	71.4
Yeast extract and vitamins	88.6
Vitamins	0
Flying squirrel fecal extract	75.2
Chickaree fecal extract	109.7
Medium without:	
Yeast extract and vitamins	0

^{1/}Average of 3 replicates each

is also necessary to the nitrogen-fixing bacteria in vitro (Table 2). We found (in three replications) that fecal pellets of deer mice contained yeast populations that ranged from 33,000 to 40,000 propagules per fecal pellet. A pure culture of yeast and a purified culture of *Azospirillum* sp., both isolated from feces of the flying squirrel, were placed in a nitrogen-free medium and incubated for five days at 30 C (86 F). Results (two replicates each) indicated that the yeast propagules promoted growth and nitrogenase activity of the bacterium. *Azospirillum* sp. alone or yeast propagules alone in Döbereiner's liquid medium exhibited no nitrogenase activity, but *Azospirillum* sp. and yeast propagules together in Döbereiner's liquid medium formed 43 nmoles from acetylene per sample per 17 hr.

Viable nitrogen-fixing bacteria, yeast, and spores of hypogeous, mycorrhizal fungi all survived passage through the digestive tracts of rodents. (Viability of the mycorrhizal fungus spores was tested in studies with seedlings and will be published elsewhere.) The fecal pellets contained the complete nutrients for the nitrogen-fixing bacteria. These findings have several implications for forest habitats. Inoculation of soil with organisms carried in rodent feces is probably common in forest ecosystems. For example, *Azospirillum* sp. can penetrate plant roots (Lakshmi et al. 1977, Patriquin and Döbereiner 1978) and is able to survive for 15 years in stored, air-dried soil (Lakshmi et al. 1977). The fossorial red-backed voles and arboreal flying squirrels are obligate forest-dwellers. When they dig at the bases of trees, the organisms in their feces can inoculate rootlets with nitrogen-fixing bacteria, yeast, and spores of mycorrhizal fungi.

The deer mouse is one of the first small mammals to occupy clearings after logging or fire, so it could inoculate the soil, even soil that has been severely altered by a hot fire. Although the spores of mycorrhizal fungi may not survive high surface temperatures in openings, they could survive under large woody debris on the soil surface where deer mice are active or below the soil surface in rodent burrows. Unlike the fungal spores, the nitrogen-fixing bacterium *C. butyricum* has a built-in survival mechanism (the endospore) by which it can withstand temperatures up to 80 C (176 F) (Simbert and Krieg 1981).

Small rodents have often been seen as detrimental to timber management (Campbell 1982, Crouch and Radwan 1975, Hooven 1975, Sullivan 1979, 1980), and poisons and habitat manipulation have been used against them. But the more forests are altered by human actions, the more evident becomes the need to understand the interactions of all the organisms in the ecosystem. How each component functions is often far more complex than might be anticipated, and the role it plays may be essential in maintaining ecosystem health.

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