アマモ根面における従属栄養細菌による窒素固定活性

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Nitrogenase Activity of Heterotrophic Bacteria Associated with Roots of Eelgrass *Zostera marina*

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Nitrogenase activity associated with the seagrass *Zostera marina* (eelgrass) was examined by the acetylene-reduction assay method. Most of the rhizome-root complex samples showed significant levels of nitrogenase activity after an initial lag period of 8 to 12 hours under aerobic or anaerobic conditions. However, separated rhizomes and roots rarely showed significant nitrogenase activity. Glucose addition had little effect on the activity of rhizomes but remarkably enhanced the activity of roots, which suggests that heterotrophic nitrogen-fixing bacteria are predominately associated with the roots rather than the rhizomes and also that the rhizome-root complexes show significant nitrogenase activity only when the rhizomes contain adequate energy source the for root-associated nitrogen-fixing bacteria. Most of the root-associated nitrogen-fixing bacteria were probably distributed on the surface and not inside the roots.

Zostera marina (eelgrass; Fig. 1) is a widely distributed seagrass, developing an extensive rhizome-root system beneath the sediment in temperate coastal waters. The occurrence of dense eelgrass beds in apparently nutrient-limited areas[1] stimulated the initial investigations of nitrogen fixation as a potential nitrogen source of this plant.[2-6] Nitrogenase activity of heterotrophic bacteria associated with the eelgrass underground parts (rhizome-root complexes) has been demonstrated in several eelgrass beds.[3,5,6] This suggests that root- and/or rhizome-associated nitrogen-fixing bacteria may indeed play a role in supplying the nitrogen demand by eelgrass. Recently, we reported some halophilic, facultatively anaerobic, nitrogen-fixing bacteria isolated from eelgrass roots.[7,8]

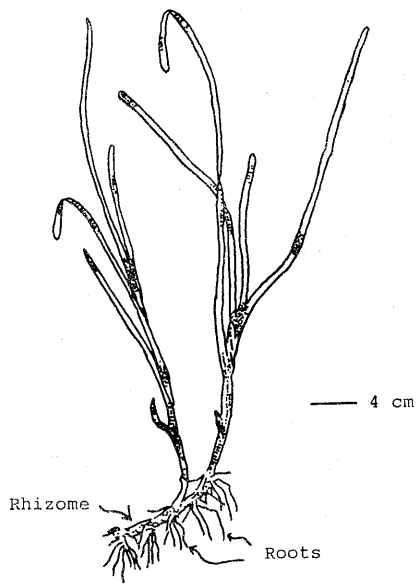

Fig. 1. Eelgrass (*Zostera marina*); separated roots and rhizome and rhizome-root complex were used in the present study.

The objectives of this paper were to determine: (a) whether the nitrogenase activity of the eelgrass-bed sediment is different from that of the eelgrass rhizome-root complexes; (2) whether the scale of root-associated nitrogenase activity is different from that of rhizome-associated nitrogenase activity; (3) the effect of O_2 and glucose addition (as an energy source) on the nitrogenase activity of various samples; (4) Whether the root-associated nitrogenase activity is exorhizal or endorhizal.

Materials and Methods

Collection and Preparation of Samples

Eelgrass rhizome-root complexes and sediment were collected from an eelgrass bed in Aburatsubo

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Inlet, Kanagawa, Japan 1 to 2 h before the experiments. The sediment was predominately composed of sand and gravel with some silt. Experiments were carried out once a month from April to August, 1987. The eelgrass rhizome-root complexes were washed free of sediment with sterile seawater. Roots, rhizomes, rhizome-root complexes, and sediment were used for the experiments. For the determination of root-associated nitrogenase activity, either the roots were used directly (untreated roots) or they were again washed 3 times vigorously in sterile seawater containing 1 ppm of Tween 80 for 3 min (surface-washed roots) or were sterilized in 70% ethanol for 20 s, followed by rinsing in 3 changes of sterile seawater (surface-sterilized roots). To measure the nitrogenase activity of the samples, 1-g (wet weight) of each sample was placed in a 20 m*l* test tube with either 2 m*l* of seawater buffer (20 mM Tris in 80% seawater, pH 8.0) or the buffer containing glucose at a concentration of 0.5%. All tubes were then sealed with rubber stoppers.

Measurement of Nitrogenase Activity

The nitrogenase activity of the samples was measured by the acetylene-reduction assay method. To measure aerobic nitrogenase activity, 2.0 m*l* of the air in the 20-m*l* test tube was removed and then the same volume of acetylene gas was injected using a gas-tight syringe; for anaerobic nitrogenase activity, all the air in the test tube was replaced with Ar/C_2H_2 (90%: 10%, v/v). All the samples were incubated at 20°C in the dark. A 0.1-m*l* gas sample from each tube was removed every 4 h with a gas-tight syringe, and analyzed for ethylene and acetylene using a Shimadzu G-4CM gas chromatograph equipped with a flame ionization detector (Porapak R column 3 mm × 50 cm, 50°C, nitrogen carrier gas, 40 m*l*/min). Time course experiments showed that ethylene production rates in the present experimental system were linear (or nearly linear) up to 20 h tested after a lag period of 8 to 12 h; no or negligible ethylene production was observed during the lag period. Specific nitrogenase activities of all samples were calculated from ethylene production between 12 and 16 h of incubation. The samples that showed nitrogenase activities of not more than 1.2 nmol ethylene/g wet wt/4 h (7.2 nmol ethylene/g wet wt/day) were recorded as trace activity, and their activities were treated as insignificant. Control samples incubated under air or 100% Ar did not show detectable ethylene production after incubation for 2 days.

Microscopy

Eelgrass roots were washed, excised, and fixed in 2% glutaraldehyde in 200 mM phosphate buffer (pH 7.0). They were post-fixed with 1% osmium tetraoxide in phosphate buffer, and dehydrated in a graded series of ethanol. The dehydrated roots were transferred into isoamylacetate as a transitional fluid, critical-point dried with liquid carbon dioxide, coated with gold, and examined with an Akiashi Alpha 25A Scanning Electron Microscope (SEM). Cross sections (2 μm) were made with an LKB 2088 Ultrotome after embedding the dehydraded roots with Shell Epon 812. They were stained with a 0.5% aqueous solution of methylene blue, and examined under a compound microscope (Nikon Type 114).

Results and Discussion

Tables 1 and 2 show the specific nitrogenase activities of sediment and eelgrass samples de-

Table 1. Nitrogenase activities of duplicate samples without glucose supplement

Sample	nmol ethylene/g wet wt/day				
	April	May	June	July	August
Aerobic					
Sediment	0	0	0	0	0
Rhizome	0	0–Trace	0	0	0
Roots	0	0–28	0	0	0–Trace
Rhizome-root complex	64–99	28–127	Trace–142	0–42	35–92
Anaerobic					
Sediment	0	0	0	0	0
Rhizome	0	0	0–Trace	0	0
Roots	0	0	0	0–Trace	0
Rhizome-root complex	56–64	Trace-42	0–28	0–21	Trace–63

Trace, <7.2 nmol ethylene/g wet wt/day.

Table 2. Nitrogenase activities of duplicate glucose-amended samples

Sample	nmol ethylene/g wet wt/day				
	April	May	June	July	August
Aerobic					
Sediment	0–Trace	0	Trace–26	0–Trace	Trace
Rhizome	0	0	0	0–35	0–Trace
Roots	268–303	423–536	155–367	169–183	204–205
Rhizome-root complex	42–71	28–169	28–99	43–113	116–134
Anaerobic					
Sediment	Trace	0–Trace	Trace	0–Trace	Trace–28
Rhizome	0–Trace	0	0	0–Trace	0–Trace
Roots	113–148	71–148	92–120	324–427	102–261
Rhizome-root complex	42–85	28–85	42–71	92–195	95–212

Trace, <7.2 nmol ethylene/g wet wt/day.

termined under various conditions. All glucose-amended samples of roots and rhizome-root complexes and more than half of the unamended rhizome-root complexes showed significantlev els of nitrogenase activity after a lag period of 8 to 12 h under either aerobic or anaerobic conditions. The lag period might be attributed to O_2 inactivation of nitrogenase as suggested by Patriquin[9] since handling of the samples may allow diffusion of oxygen into the micro-sites and might have inactivated the oxygen-labile nitrogenase system. The lag period would then be the time needed to reduce oxygen by the respiration of microorganisms and eelgrass roots (or rhizome-root complexes) until the oxygen level is suitable for nitrogen fixation. Studies on marsh grass *Spartina alterniflora*,[10] the seagrass *Thalassia testudinum*,[11] and several terrestrial grasses[10] have shown that oxygen exposure may indeed produce a lag period, and this lag period can be shortened or avoided by minimizing the exposure of samples to O_2 during sample preparation. The use of a liquid phase in the present experimental system might have also delayed the appearance of nitrogenase activity; it might have required several hours for the diffusion and equilibration of acetylene to the sites of activity under these conditions.

Although no nitrogenase activity was detected with unamended sediment, the trace activity in most of the glucose-amended sediment samples indicates that nitrogen-fixing bacterria exist in the sediment. We have isolated a variety of halophilic, facultatively anaerobic, nitrogen-fixing bacteria from the sediment collected from the same eelgrass bed, and some of these isolates have been reported.[8,12] All glucose-amended sediment samples showed significant levels of nitrogenase activity after incubation for more than 40 h (data not shown). This could be attributed to the growth of nitrogen-fixing bacteria during the long period of nitrogenase activity determination.

The unamended root samples rarely showed significant nitrogenase activity; only 1 of the 20 (aerobic or anaerobic) unamended root samples showed a nitrogenase activity of 28 nmol ethylene/g wet wt/day under aerobic conditions. However, all glucose-amended root samples showed relatively high levels of nitrogenase activity without exception (155–536 nmol ethylene/g wet wt/day under aerobic conditions and 71–427 nmol ethylene/g wet wt/day under anaerobic conditions). These results indicate that the number of root-associated nitrogen-fixing bacteria was constantly sufficient for the expression of nitrogenase activity throughout the sampling period, however, separated roots were usually short of utilizable substrates for energy. In contrast, the number of rhizome-associated nitrogen-fixing bacteria was insufficient on most occasions because glucose addition had little effect on the nitrogenase activity of rhizome samples; none of the unamended rhizome samples showed significant nitrogenase activity and with the addition of glucose, only one rhizome sample showed significant nitrogenase activity (35 nmol/g wet wt/day) under aerobic conditions (Table 2). The rhizome, being a storage organ may contain a variety of organic compounds. Some of these compounds might be transported to and excreted from the roots.[13] From the above reasons, it is concluded that nitrogen-fixing bacteria were predominately associated with roots rather than rhizomes. However, most of the unamended rhizome-root complexes tested here might have shown significant nitrogenase activity (Table 1) only when rhizomes contained utilizable organic compounds for the energy of root-associated

Table 3. Effects of surface washing and surface sterilization on the nitrogenase activity of glucose-amended roots. All results were expressed as ranges of duplicate samples

Sample	nmol ethylene/g wet wt/day		
	June	July	August
Aerobic			
Untreated roots	155–367	169–183	204–205
Surface-washed roots	35–42	43–85	Trace
Surface-sterilized roots	28–35	Trace–28	Trace–21
Anaerobic			
Untreated roots	92–120	324–427	102–261
Surface-washed roots	Tace–28	42–56	Trace–28
Surface-sterilized roots	0–Trace	Trace–21	0–Trace

Trace, <7.2 nmol ethylene/g wet wt/day.

nitrogen-fixing bacteria. The statement of Capone and Budin[3] that the bulk of nitrogenase activity was associated with rhizomes and not roots was not confirmed by the present results.

Smith and Hayasaka[6] hypothesized that aerobic conditions would be essential for the nitrogenase activity of eelgrass rhizosphere. They deduced that rhizosphere nitrogen-fixing bacteria would cease fixing nitrogen during the plant's summer dormancy due to the lack of oxygen transport to the roots. However, their deduction could not explain the results obtained here, since anaerobically, all the present glucose-amended root samples, including those collected during the summer months (July and August) showed significant levels of nitrogenase activity. Furthermore, we have isolated a variety of facultative nitrogen-fixing bacteria from eelgrass roots in the summers of 1985 and 1986.[7,8] All these isolates showed the highest nitrogenase activity under anaerobic conditions while their nitrogenase activities were negligible or very weak under aerobic conditions. Clearly, the inability of the present unamended roots to show significant levels of nitrogenase activity was primarily due to the lack of substrates for energy and not because of the lack of oxygen.

The enhanced effect of glucose on the root-associated nitrogenase activity was totally or severely inhibited when the root surface was vigorously washed with seawater containing 1 ppm Tween 80 or sterilized with 70% ethanol (Table 3). The results suggest that most of the root-associated nitrogen-fixing bacteria are distributed on the surface and not inside the roots. Scanning electron microscopy showed a dense covering or root hairs on the mature zone of the eelgrass roots. However, nodular-like structures were not found on the surface of roots or root hairs. No endosymbiotic-like bacteria could be observed within the root cells using the cross sections of the roots. This also suggests that the root-associated nitrogenase activity is not attributed to endorhizal nitrogen-fixing bacteria.

We have estimated the MPN values of nitrogen-fixing bacteria distributed in the eelgrass bed using anaerobic and microaerobic enrichment culture methods.* For eelgrass-root rinse water, the values ranged from 2.2×10^5 to 1.2×10^7 cells/g wet wt roots, which confirmed that a large number of nitrogen-fixing bacteria are distributed on the surface of eelgrass roots. We have also isolated a variety of halophilic, facultatively anaerobic, nitrogen-fixing bacteria from eelgrass roots and some of the isolates have been reported.[7,8] Attempts were also made to isolate aerobic or microaerobic nitrogen-fixing bacteria from eelgrass roots but none of these bacteria could be isolated. Our recent works suggest that facultatively anaerobic nitrogen-fixing bacteria may possibly be the most important nitrogen fixers associated with eelgrass roots.

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