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Relationship between Nitrogen-Fixing Sulfate Reducers and Fermenters in Salt Marsh Sediments and Roots of *Spartina alterniflora*

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A combination of inhibitors and carbon substrates was used to determine the relative contribution of sulfate-reducing bacteria (SRB) and fermenting bacteria to nitrogen fixation in a salt marsh sediment and on the roots of *Spartina alterniflora*. Because a lag period precedes acetylene-reducing activity (ARA) in amended sediments, an extensive analysis was done to be sure that this activity was due to the activation of dormant cells, not simply to cell proliferation. Since ARA was not affected by metabolic inhibitors such as rifampin, nalidixic acid, or methionine sulfoximine, it appeared that cell growth was not responsible for this activity. Instead, dormant cells were being activated by the added energy source. Molybdate inhibition studies with glucose-amended sediment slurries indicated that ARA in the upper 5 cm of the salt marsh was due primarily (70%) to SRB and that below that level (5 to 10 cm) it was due primarily (>90%) to fermenting bacteria. ARA associated with washed roots of intact *S. alterniflora* plants was not inhibited by molybdate, which indicates that bacteria other than SRB were responsible. However, when the roots were excised from the plant, the activity (per unit of root mass) was 10-fold higher and was severely inhibited by molybdate. While this high activity is probably an artifact, due to the release of oxidizable substrates from the excised roots, it indicates that SRB are present in high numbers on *Spartina* roots.

The high level of primary production in salt marshes is due in large part to the fact that nitrogen, although limiting, is constantly supplied by nitrogen-fixing bacteria residing primarily in the top few centimeters (the root zone) of the sediment (31, 34). The types of microbes responsible for this activity have been the topic of numerous investigations (3, 5, 12, 18, 20, 23). When carbon substrate is not limiting (due to in vitro amendments), over 90% of the nitrogen-fixing potential was associated with sediment particles and only 5 to 10% was associated with plant root material (38). Those N_2 fixers found to be active in the marsh sediment were *Azotobacter* species and cyanobacteria on the surface and sulfate-reducing bacteria (SRB) and fermenters such as clostridia beneath the surface (3, 5, 10, 12). Because the metabolizable carbon needed to support N_2 fixation in the salt marsh sediment is limited (8, 9, 12, 38), those N_2 fixers which play a major role in the anoxic decomposition of plant material would be expected to contribute most to the nitrogen economy of this ecosystem. In the salt marsh, anaerobic fermenting bacteria dominate the initial steps of mineralization, with SRB completing the process by further degrading the end products of fermentation (7, 26). While these two groups would be expected to carry out most of the N_2 fixation in the salt marsh, little is known about either their location in relation to one another or their relative rates of fixation. Before rates of acetylene reduction activity (ARA) by the various physiological groups of bacteria in the salt marsh can be assessed, however, the validity of the in vitro assay needs to be examined. The reason is that carbon-amended sediment slurries invariably show a lag period of 8 to 25 h before high rates of activity begin (2, 20, 22, 38), while ARA in situ is linear from time zero (34, 38). The possibility cannot be excluded that proliferation occurs during the lag period, which would account for the subsequent high rates of activity. The lag period was examined in this study, and it was concluded that cell proliferation was not occurring but that dormant bacteria were probably being activated by the added energy source.

Finally, fermenting bacteria are known to produce significant amounts of acetate in natural anoxic environments (11, 13, 14). Therefore, the isolation of SRB that oxidize this molecule was a significant finding (35, 37). The ability of SRB to couple acetate oxidation to N_2 fixation has just recently been reported (36). However, nothing is known about the role of acetate-utilizing SRB as contributors of fixed nitrogen in the marsh.

In this study, a combination of carbon amendments (including acetate) and metabolic inhibitors was used to determine the spatial relationship and relative potential of both active and dormant sulfate reducers and fermenters to contribute fixed nitrogen to the salt marsh ecosystem.

METHODS AND MATERIALS

Sediment samples. Sediments were obtained from the short *Spartina* zone near Clambank Landing at North Inlet salt marsh at Georgetown, S.C., during 1987. Tubes (polyvinylchloride) with beveled edges to cut through roots were used to remove sediment cores from between *Spartina* culms. The tubes were immediately stoppered at both ends to keep the cores anoxic, and they were transported back to the laboratory for use within 24 h. Root material was removed by pressing the sediment through a 2-mm-mesh sieve in a large container under a continuous stream of argon. Only the top 5 cm of sediment was used, unless indicated otherwise. Samples (2 g [wet weight]) were placed into 22-ml culture tubes containing a degassed solution of

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filtered seawater plus carbon source and inhibitors (as required). The tubes were sealed with serum bottle stoppers and were again evacuated and refilled with argon. Acetylene was added to give a final concentration of 2.6% for assaying nitrogenase activity by monitoring its reduction to ethylene, which was quantitated by flame ionization gas chromatography. Acetylene levels were kept low to prevent any possible inhibition of SRB (24). It must be noted that intact sediments have numerous microenvironments, but ARA is measured only in those bacteria most capable of taking advantage of an anaerobic slurry provided in these in vitro experiments.

Root samples. *S. alterniflora* was cultured in the greenhouse in pots (7.5 by 8 cm) as described previously (33). Nitrogenase activity of intact plant roots was assayed in a chamber that provided airtight separation between the leaves and roots. The root chamber (see Fig. 1 in reference 33) contained 300 ml of seawater and had a gas space of 100 ml above the hydroponic solution, which contained 90% argon and 5% acetylene from which gas samples could be withdrawn for analysis of ethylene. Sodium molybdate was added as required (see the legend to Fig. 5).

Excised roots of *S. alterniflora* were prepared by first washing away the sand from the cultured plants under tap water for 3 to 4 min. The roots were then cut from the plant, washed in filtered seawater, and cut into 1-cm segments. After the segments were mixed, 20 mg (wet weight) of the root pieces were placed in Fernbach vessels (6 ml) containing 1.5 ml of anoxic filtered seawater. Vessels were again degassed and filled with the same gas phase as that used for the roots of intact plants. Sodium molybdate (20 mM) was added from an anaerobic solution, as indicated in the legend of Fig. 5. Gas samples (25 ml) were taken periodically for analyses of ethylene and acetylene. These gases were analyzed on a Varian (model 1400) gas chromatograph with an aluminum column (maintained at 195°C) and a flame ionization detector. Peaks were integrated on an HP 3390A integrator (Hewlett-Packard Co., Palo Alt., Calif.).

RESULTS

Activation of dormant cells. To determine if the rapid rise seen in ARA after 10 to 15 h of sediment incubation was due to activation of dormant cells by the added energy source or was due to cell growth, metabolic inhibitors were added to the slurries to prevent cell division. Methionine sulfoximine (MSX), an inhibitor of a glutamine synthetase (a key enzyme in procaryotic nitrogen metabolism [4, 30]), is highly effective in inhibiting growth (19). Instead of being inhibited by 250 μM MSX, ARA was actually stimulated, while the lag period remained unchanged (Fig. 1).

Further indications that cell growth was not responsible for nitrogenase activity in these sediments were the facts that neither rifampin, a well-known inhibitor of transcription, nor nalidixic acid, an inhibitor of DNA synthesis (29), had a significant effect on ARA (Table 1). Chloramphenicol, which stops translation, did inhibit ARA, but similar effects are also seen in nongrowing pure cultures (see Discussion). We observed that ARA inhibition in sediments started within 15 min after the addition of chloramphenicol to the sediment, which is consistent with this interpretation (data not shown). These observations gave us some degree of assurance that cell proliferation was not responsible for the ARA observed in sediment slurries but that dormant cells were apparently being activated.

Interrelation between SRB and fermenters in sediment. We examined slurries from two zones, 0 to 5 and 5 to 10 cm, to determine (i) the relative contribution of the fermenters compared with that of the SRB in the top 2 or 3 cm of sediment and (ii) if the ratio of the activity contributed by these two groups of bacteria changes with the depth of sediment. ARA supported by endogenous carbon sources, with and without added molybdate, was examined first (Fig. 2). Although the activity was low, it is clear that SRB (which are molybdate inhibited) (21, 25) predominate in the upper 5 cm of the sediment (Fig. 2A) but are less active in the 5- to 10-cm zone (Fig. 2B). Furthermore, the activity was linear with time, indicating that a small population of cells is active in vitro.

When these sediment slurries were amended with glucose and tested for ARA supported by SRB, a similar molybdate inhibitory pattern was observed (Fig. 3). Glucose was chosen as a substrate because it supports both the fermenters and the SRB, which use the glucose fermentation products (7, 26). In the top 5 cm of sediment, molybdate initially had no effect on ARA, but the log phase of ARA that began at 25

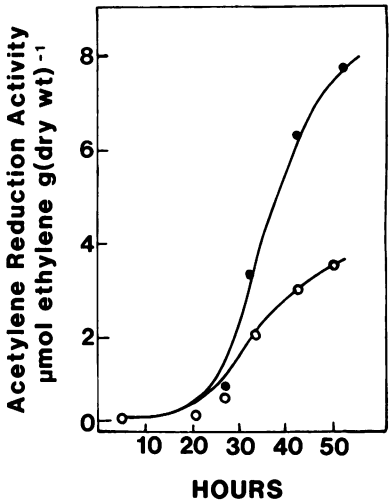

FIG. 1. Effect of MSX on ARA in slurries of salt marsh sediment. Activity was measured under an argon atmosphere (containing 2.6% acetylene) in the presence (●) and absence (○) of 250 μM MSX. Slurries were amended with 15 mM sodium lactate and were made from the top 5 cm of sediment taken in July 1987. The datum points represent the means of five replicas; the activities of the samples with and without MSX are significantly different at $P = 0.05$.

TABLE 1. Effect of inhibitors on lactate-supported ARA in salt marsh sediments slurries[a]

Inhibitor (μg ml^{-1})	Nmol of ethylene produced by ARA[b] (SE)
None	559 (35)
Rifampin (100)	798 (97)
(200)	785 (200)
Nalidixic acid (100)	663 (203)
(200)	788 (236)
Chloramphenicol (100)	53 (28)
(200)	49 (27)

[a] Sample preparation and assay conditions were the same as those described in the legend to Fig. 1.

[b] The numbers represent the means of five replicas. The samples contained 1.1 g (dry weight) of sediment and were incubated for 30 h. None of the inhibitors (except chloramphenicol) showed a significant effect ($P > 0.05$) on ARA.

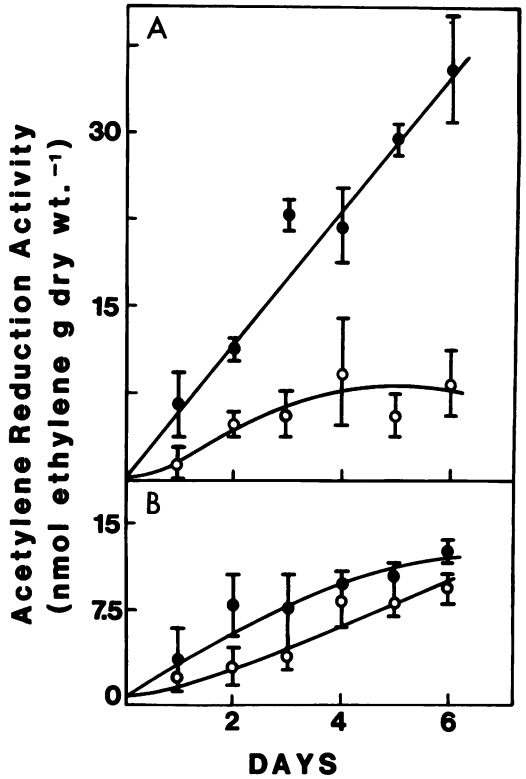

FIG. 2. Effect of molybdate on ARA supported by endogenous energy sources in sediments at depths of (A) 0 to 5 cm and (B) 5 to 10 cm. Slurries were amended with 20 mM molybdate (○) or had no addition (●). The ARA is the mean of five replicas; the bar represents ±1 standard deviation. The cultures were made anoxic as described in the legend to Fig. 1. Sediments were taken in November 1987.

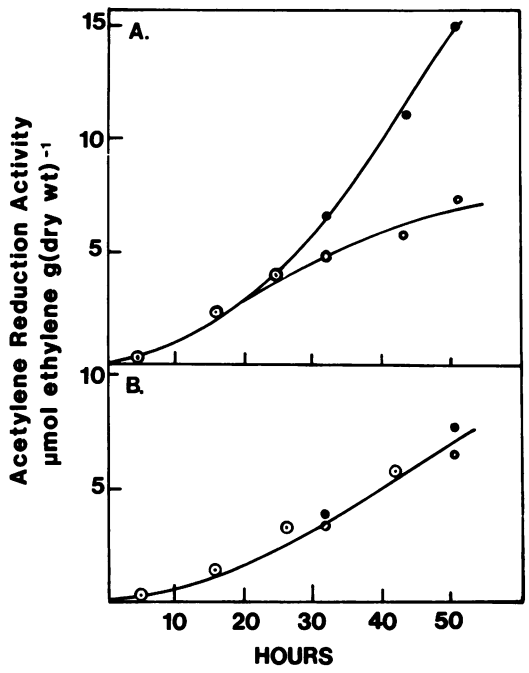

FIG. 3. Effect of molybdate on ARA in glucose-amended slurries made from sediments at depths of (A) 0 to 5 cm and (B) 5 to 10 cm. Slurries contained 15 mM glucose with no further additions (○) or with 20 mM sodium molybdate (●). The ARA is the mean of five replicas. The condition were anoxic; the gas phase was described in the legend to Fig. 1. Identical results were obtained from samples in June and December 1987.

h was blocked by molybdate (Fig. 3A). These data indicate that in the top 5 cm of sediment, SRB are responsible for about 75% (0.348 µmol of C_2H_4 h^{-1} g [dry weight]$^{-1}$) of the nitrogenase activity, with the remaining 25% (0.119 µmol of C_2H_4 h^{-1} g [dry weight]$^{-1}$) due to the anaerobic fermenters. ARA in sediments between 5 and 10 cm was lower and was not inhibited by molybdate (Fig. 3B), indicating that SRB were not active here.

N_2 fixation by acetate-oxidizing SRB. In the salt marsh ecosystem, we routinely observed acetate-dependent ARA in the top 5 cm, with rates being linear up to 15 mM acetate. From 10 experiments, the mean rate was 55 nmol of ethylene formed g (dry weight)$^{-1}$ h^{-1} in sediment (n = 80), with a range of 0 to 155 nmol. The strong inhibition of ARA by molybdate (in some experiments it was >90%) indicates that the activity supported by acetate was due to the action of SRB (Fig. 4). In the same set of experiments, lactate supported ARA in sediments was 80 nmol of ethylene formed g (dry weight)$^{-1}$ h^{-1} (n = 80; range of the means, 15 to 177 nmol). Glucose and other fermentable sugars support higher rates of ARA in marsh sediments than do organic acids (8, 23). We report similar results here, with mean rates of glucose-supported ARA being 213 (range, 61 to 354) nmol of ethylene formed g (dry weight)$^{-1}$ h^{-1} (n = 75). Although the range of activities observed was quite large with all substrates, no seasonal pattern was observed.

Root-associated SRB and fermenting bacteria. It is well established that roots of *S. alterniflora* harbor N_2-fixing bacteria both on their surface (17, 18, 22, 34) and in the

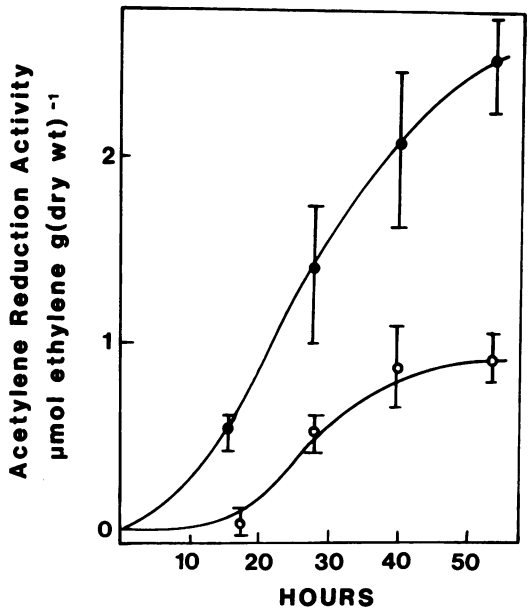

FIG. 4. Effect of molybdate on acetate-supported ARA in sediment slurries. Two sets of samples (five replicas) containing 15 mM sodium acetate as an energy source were incubated anaerobically with (○) 20 mM sodium molybdate, and one set was incubated without 20 mM sodium molybdate (●). Bars represent ±1 standard error of the mean. Sediments were taken in June 1987.

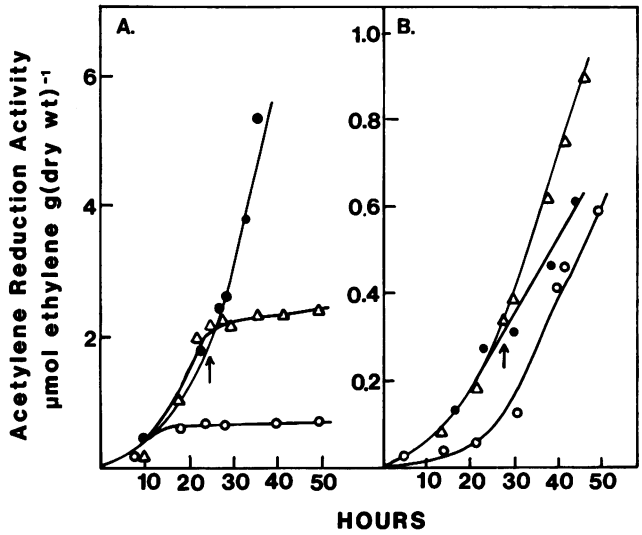

FIG. 5. Effect of molybdate on ARA of (A) excised roots and (B) roots of intact *S. alterniflora* plants. Roots were assayed anaerobically in filtered seawater (●), seawater plus 20 mM molybdate (○), or seawater with 20 mM molybdate added at the time indicated by the arrow (△). The intact plant was illuminated with 100 microeinsteins of light m^{-2} s^{-1}; the gas phase flowing through the leaf chamber was air amended with 0.25% CO_2. The rates of ARA observed in the root chamber are from a single experiment, but the results are similar to those from a number of other experiments done earlier. The rates shown for excised roots are the mean of five replicas.

cortex tissue (1, 18). While a microaerophile, a *Campylobacter* sp., has been isolated from surface-sterilized root tissue (17, 18), the types of bacteria that make up the surface population remain unknown. It was somewhat unexpected, however, to find that >90% of the ARA associated with washed excised roots was inhibited by molybdate, suggesting that SRB were responsible for this activity (Fig. 5A). Unlike ARA in sediments, root activity required no added carbon source. It should be pointed out that the young greenhouse plants were inoculated with SRB and other marsh bacteria from sediment slurries to ensure normal root colonization. Cell proliferation appears not to have been essential for this activity, since ARA was not inhibited when 250 µM MSX was included in the solution that bathed the roots (data not shown). The fact that there was no stimulation of ARA by MSX, as there was in sediments (Fig. 1), also suggests that *nif* genes in the root-associated SRB were already fully derepressed (6).

When ARA was measured on washed roots of intact plants, molybdate had no effect when it was added, either at time zero or after activity was fully established (Fig. 5B). It should be noted that much lower specific activities (based on root dry weights) were measured with intact plant roots than with excised roots (18.6 versus 224 nmol ethylene g [dry weight]$^{-1}$ h^{-1}, respectively). This difference probably results from the leakage of substrates from the excised roots and is discussed below.

DISCUSSION

To evaluate the relative potential of SRB and fermenting bacteria for N_2 fixation in the various salt marsh habitats in vitro, it had to be determined whether cell proliferation was occurring during a lag period which always precedes ARA in sediment slurries or whether dormant cells were simply being activated by the added carbon substrate. Similar unexplained lag periods preceding ARA are commonly seen in soils (15) and estuarine sediments (10). The potential for nitrogen fixation activity in soils and sediments can be assessed in vitro only if one can distinguish between cell proliferation (which would make the data less meaningful) and the activation of dormant cells (28, 32). We were able to gain some insight into this lag period by using metabolic inhibitors along with the addition of an energy source. When the effect of the glutamine synthetase inhibitor, MSX (27), was examined on sediment ARA, it was found to stimulate rather than inhibit nitrogenase activity (Fig. 1). The stimulation proved that the drug reached and entered the cells; this is shown by the depletion of the intracellular glutamine pool which leads to the complete derepression of nitrogenase (6). The conclusion, therefore, is that growth of SRB in the presence of MSX would have been impossible, as it is in enteric bacteria (19), because growing cells cannot be deprived of glutamine, a key intermediate of procaryotic nitrogen metabolism and that activation of dormant cells must account for this high ARA following the lag. The lack of inhibition of ARA by rifampin or nalidixic acid (Table 1) substantiates this conclusion because it has been shown that of seven *Desulfovibrio* strains tested, all were Rif^s (at 100 µg ml^{-1}) and all but one were Nal^s (at 200 µg ml^{-1}) (Judy Wall, personal communication). These antibiotics, therefore, would have inhibited ARA if cell proliferation had been a prerequisite. Chloramphenicol did inhibit ARA (Table 1), but this inhibition is explained by the slow buildup of amino acid pools which occur even when low rates of protein synthesis are inhibited (Wall, personal communication). Since these pools include glutamine, the inhibition of ARA is readily explained by the nitrogenase switch-off regulatory system (16).

The lag period probably reflects the time that dormant sulfate reducers in the sediment slurry need to react to the added energy source. The linearity in ARA in the absence of added substrate (Fig. 2) indicates that a small population is fully active from time zero, as one would expect. We believe that the rate of ARA following the lag period is indicative of both the active and dormant cells that have been activated by the energy source and is not due to cell proliferation. This means that rates of ARA in the presence of specific substrates and inhibitors can be used to determine the relative abundance of SRB and fermenters in complex communities.

The work of Herbert (10) and Nedwell and Aziz (20) led them to the conclusions, based on bacterial counts, that fermenting bacteria play a relatively minor role in N_2 fixation in the salt marsh and that SRB predominate. We used a methodology that determined the nitrogen-fixing potential of these two physiological groups instead of the number of bacteria. Consistent with previous estimations (10, 20), our finding was that in glucose-amended sediment slurries, most (75%) of the ARA in the top 5 cm could be attributed to SRB, and only 25% could be attributed to fermentative bacteria. Interestingly, no SRB were supported by glucose fermentation products below 5 cm, as indicated by the lack of molybdate inhibition of ARA (Fig. 3B). Since we observed both lactate- and acetate-oxidizing SRB below 5 cm, the results shown in Fig. 2B and 3B may be explained if non-SRB are outcompeting SRB for the glucose fermentation products or if the fermenter population changes with depth and products are formed that are not utilized by SRB.

The relationship between fermenters and SRB on the roots of *S. alterniflora* was also examined by the inhibitor amend-

ment approach. The ARA on the roots of intact plants, which is supported by plant photosynthate (33), was not inhibited by molybdate (Fig. 5), which suggests that SRB were not involved. However, when the roots were excised, the activity increased by 10-fold and was severely inhibited by molybdate, but not by metabolic inhibitors like MSX (unpublished observations). Apparently the SRB are tightly attached to the root surface and are not dislodged by 5 min of washing with tap water before the roots are cut into segments. It is surmised that SRB do not normally receive photosynthate from the plant, but by cutting up the roots, plant cell contents were released, supporting the ARA of the numerous sulfate reducers that reside on the roots.

Finally, this is the first report showing nitrogenase activity coupled to acetate utilization by natural populations of sulfate reducers (Fig. 4). Ammonia switch-off of acetate-dependent ARA (data not shown) was comparable to that of lactate oxidizers (38). This rapid inhibition by NH_4^+ in SRB is in distinct contrast to the slow response to NH_4^+ we reported previously for mannose-amended sediments (38). This fermentable sugar obviously supports ARA in a different population of microbes, such as the clostridia, enterbacteria, and others which apparently do not have the NH_4^+-activated nitrogenase switch-off system.

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