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Host-specific ecotype diversity of rhizoplane diazotrophs of the perennial glasswort *Salicornia virginica* and selected salt marsh grasses

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ABSTRACT: The degree of host specificity of most plant root associated bacteria is poorly understood. In this study we examined the physiological diversity of oxygen utilizing, culturable diazotrophs from the rhizoplane of the high marsh perennial glasswort *Salicornia virginica* and compared them to diazotrophs from other salt marsh plants (tall and short *Spartina alterniflora*, *Juncus roemerianus*, and *Spartina patens*) from the same ecosystem. Forty-six pure culture strains were recovered from the rhizoplane of *S. virginica* by stab inoculating freshly collected roots into combined nitrogen-free semi-solid media, followed by streak plating of clonal outgrowths. The majority of these strains were Gram-negative obligately aerobic or microaerophilic rods, but 3 Gram-positive strains were also isolated and characterized. API 20NE test strips were used for preliminary characterization of all strains, yielding 22 physiologically different API strain groups. One representative strain was selected from each API group and tested for the presence of *nifH*, denoting strains capable of N_2-fixation. Seventeen strains (14 Gram-negative, 3 Gram-positive) were *nifH*-positive and were characterized further using BIOLOG test plates. Four well-supported strain clusters were identified by bootstrapped cluster analysis of the BIOLOG substrate utilization profiles. These clusters differed in utilization of carbohydrates, carboxylic acids, and amino acids. *S. virginica* diazotrophs were physiologically quite different from rhizoplane diazotrophs from the low marsh plants *S. alterniflora* and *J. roemerianus*, but much more similar to diazotrophs from another high marsh plant, *S. patens*. We hypothesize that the observed physiological differentiation between high marsh and low marsh diazotrophs reflects differences in selection pressures in the rhizoplane microenvironment produced by plants with differing abilities to ventilate the rhizosphere. In addition, high and low marsh branches were further resolved into host-specific strain clusters, which also implies a strong impact of other host features, such as the suite of carbon exudate compounds produced, on the distributions of specific diazotroph strains. These findings imply endemic, host-specific distributions of salt marsh diazotrophs and are consistent with the great diversity of diazotrophs that have been observed in this ecosystem to date.

KEY WORDS: Diazotrophs · Rhizoplane · Physiological specialization · Rhizosphere ventilation

INTRODUCTION

The species diversity and distribution patterns of microorganisms in nature are strongly impacted by environmental heterogeneity and variability. Frequent and often extreme changes in key environmental parameters (physical, chemical, and biotic) occur, sometimes simultaneously, over quite small spatial and temporal scales in many systems (Revsbech & Ward 1984, Ramsing et al. 1996, Edgcomb et al. 1999, Piceno et al. 1999, Bagwell & Lovell 2000b). Several recent studies have demonstrated substantial shifts in bacterial species (or ecotype) composition along environmental gradients (McArthur et al. 1988, Ferris et al. 1996, 1997, Moore et al. 1998, Bagwell & Lovell 2000a) and in response to environmental variability (Ferris et al.

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1997, Stoffels et al. 1998, Fernandez et al. 1999, Nusslein & Tiedje 1999), suggesting that these organisms may have limited distribution patterns (i.e. endemism). Endemism would imply that microorganisms are physiologically restricted to defined niches *in situ* (e.g. Ferris et al. 1996, Moore et al. 1998, Bagwell & Lovell 2000a), with important implications for microbial diversity (Fenchel et al. 1997, Finlay et al. 1998). However, relatively little is known about the distribution patterns of most microorganisms.

Vegetated intertidal salt marshes provide an ideal setting in which to test hypotheses about environmental impacts on microbial species diversity and distribution patterns. Within the marsh, increasing elevation and distance from low marsh tidal creeks results in the formation of distinct environments differing in abiotic (i.e. sediment texture, soluble H_2S, pH, redox potential, salinity; Bagwell & Lovell unpubl. data) and biotic features (e.g. plant zonation, Tiner 1993; macrofaunal burrowing, Bertness 1985). Interspecies competition and differences in plant tolerance of various physicochemical variables dictate host plant distribution patterns in the intertidal zone (Bertness 1988, 1991a,b, Levine et al. 1998, Ungar 1998), presumably with substantial impacts on microbial diversity and distributions. However, the impacts that these different environmental features have on plant-associated microbial communities or important functional assemblages therein are not understood.

Diazotrophic bacteria are ubiquitous in salt marshes, serving as an ecologically significant source of 'new' biologically available nitrogen in these systems (Whittaker 1975, Patriquin & McClung 1978, Hanson 1983). Nitrogen fixation is carried out exclusively by certain species of *Bacteria* and *Archaea* (Postgate 1998), with the highest rates of activity occurring on and around plant root surfaces (the rhizoplane and rhizosphere respectively; Teal et al. 1979, McClung et al. 1983, Campbell & Greaves 1990). Microenvironmental heterogeneity in the rhizoplane and rhizosphere presumably results in the formation of many different niches *in situ* (Bowen 1980, Bagwell & Lovell 2000a), supporting an abundant and diverse diazotroph assemblage in these root microenvironments (Bagwell et al. 1998, Piceno et al. 1999, Lovell et al. 2000). The rhizoplane/rhizosphere diazotroph assemblage of *Spartina alterniflora* has been studied extensively (Lovell 2001 and references therein); however, we know relatively little about the diazotrophs associated with macrophytes inhabiting high intertidal environments, which are substantially different from the low intertidal environment occupied by *S. alterniflora*.

In this study the physiological diversity of the oxygen utilizing, culturable fraction of the rhizoplane diazotroph assemblage of the perennial glasswort *Salicornia virginica* (*Salicornia* hereafter) was assessed. In addition, a quantitative comparison of the physiological capabilities of rhizoplane diazotrophs isolated from various intertidal host plants, including tall and short form *Spartina alterniflora* (Bagwell et al. 1998), *Juncus roemerianus* from an island located within the short *S. alterniflora* growth zone (Bagwell et al. 1998), *Spartina patens* (Bergholz et al. 2001) and *Salicornia*, was performed. Our results demonstrate substantial diazotroph host specificity (previously proposed in Bagwell & Lovell 2000a, Lovell et al. 2000) and highlights some key physiological adaptations of rhizoplane diazotrophs to host defined niches *in situ*.

MATERIALS AND METHODS

Sampling site description. This study was conducted in the North Inlet estuary, near Georgetown, South Carolina, USA (33° 20′ N, 79° 10′ W). Low elevations in the marsh are dominated by the smooth cordgrass *Spartina alterniflora*, existing largely as a monoculture stand. A variety of salt marsh grasses and plants grow in pure and mixed stands at higher elevations in the intertidal and along the terrestrial fringe, including *Juncus roemerianus*, *Salicornia*, *Spartina patens*, and *Borrichia frutescens*. *Salicornia* plants were collected from a pure stand on Goat Island in September 1999. A detailed description of this system and the study site are provided elsewhere (Dame & Kenny 1986, Morris & Haskin 1990).

Root culture inoculation and pure culture isolation. *Salicornia* plants were collected with intact roots and root zone sediments and transported to Columbia, South Carolina, USA. Due to the woodiness, very shallow depths of occurrence, and high density of emerging shoots on the rhizomes, only soft tissue, sediment-penetrating roots were used in this study. Roots were washed briefly with distilled water to remove loosely associated sediments, then stab inoculated into duplicate tubes of combined nitrogen-free semisolid media supplemented with glucose, sucrose, citrate, or malate and adjusted to either pH 7.0 or 7.5 (a total of 16 roots), loosely capped, and incubated (see Bagwell et al. 1998 for media recipes). All incubations were conducted in the dark at 28°C. Clonal outgrowths from the root surfaces were collected and stab inoculated into fresh culture tubes of nitrogen-free semisolid medium with the appropriate carbon source and pH. Pure cultures were obtained by streak plating on nitrogen-free media. Pure cultures were maintained on Bacto Marine Agar for morphological and physiological characterization. Broth cultures grown on Bacto Marine Broth were supplemented with dimethyl sulfoxide and glycerol (5% final concentration of each) and stored frozen at –70°C.

Strain characterization and grouping. Colony morphology, cell morphology, motility, Gram reaction, and endospore formation were determined for each strain using standard methods (Gerhardt et al. 1981). Oxygen requirements were determined in a semisolid medium based on Bacto Marine Broth supplemented with sodium thioglycolate as described previously (Gerhardt et al. 1981, Bagwell et al. 1998). Initial physiological characterization was by API 20NE (non-fermentative) test strips following the manufacturer's instructions (bioMerieux Inc., Hazelwood, MS, USA). Briefly, API test strips contain a series of biochemical reaction capsules, which were inoculated with a fresh cell suspension (0.85% saline). The strips were incubated at 28°C for 24 h, and test results scored on the basis of color reaction or observable cell growth. Specific tests included on the API 20NE test strips include: ADH (arginine dihydrolase), ADI (adipate assimilation), ARA (arabinose assimilation), CAP (caprate assimilation), CIT (citrate assimilation), ESC (esculin hydrolysis), GEL (gelatinase), GLU1 (glucose acidification), GLU2 (glucose assimilation), GNT (gluconate assimilation), MAL (maltose assimilation), MAN (mannitol assimilation), MLT (malate assimilation), MNE (mannose assimilation), NAG (N-acetyl-D-glucosamine assimilation), NO_3 (nitrate reduction), PAC (phenylacetic acid assimilation), PNPG (β-galactosidase), TRP (indole production from tryptophane), and URE (urease). The API data were supplemented with tests for cytochrome oxidase production following the manufacturer's instructions (bioMerieux Inc.). Strains differing by no more than 3 API test results were grouped to eliminate redundancy in the culture collection. The utility of API test strips for determination of redundant strains has been demonstrated previously (Bagwell et al. 1998, Bergholz et al. 2001). One representative strain was selected from each API group and used for the remainder of the study.

Identification of strains capable of N_2-fixation. API group representatives were screened for *nifH*, the gene encoding the nitrogenase iron protein, by dot blot hybridization with a biotinylated *nifH* specific probe (Bagwell et al. 1998). Genomic DNA was extracted from 2 ml broth cultures grown overnight at 28°C using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer's instructions. Dot blot preparation and hybridization conditions have been described previously (Bagwell et al. 1998). Probe detection utilized the Southern-Light chemiluminescence detection kit (Tropix, Bedford, MS, USA) following the manufacturer's instructions. Film was exposed for 72 h and experimental dots were scored against dots of positive (*Azospirillum lipoferum*) and negative (*Staphylococcus aureus*) control DNA samples in triplicate hybridizations.

Diazotrophic strain physiological characterization by BIOLOG. API group representatives that were scored positive for *nifH* by hybridization were characterized further using GN and GP BIOLOG test plates (Hayward, CA, USA). Overnight cultures were grown on Bacto Marine Agar plates and used to inoculate BIOLOG test plates following the manufacturer's instructions. BIOLOG plates were incubated in the dark at 28°C for 24 h and color development in each test well was measured using an automated plate reader. Increases in absorbance were converted to percent change values, relative to the negative control, by the accompanying MICROLOG software (version 3.5), and standardized by hand into 5 threshold range values, each assigned an arbitrary 'amino acid' designation (A, C, D, E, and F respectively) (Bagwell & Lovell 2000a). These artificial 'amino acid' sequences were used to construct a dendrogram from Euclidean distances with bootstrap support values (500 resamplings) in MEGA (version 1.01) (Kumar et al. 1993). BIOLOG substrate utilization profiles, evaluated as artificial 'amino acid' sequences, were reproducible among replicates of selected strains (data not shown).

RESULTS AND DISCUSSION

Salicornia root stab cultures yielded 46 pure culture isolates, 3 of which stained Gram positive. All strains were short rods and 17 were motile. Endospore formation was observed in 13 strains. Four strains were obligately aerobic while all others were microaerophilic. Fourteen strains tested positive for the production of cytochrome oxidase. Yellow, red, or orange pigmentation was produced by 7 strains; the rest were colorless.

API 20NE test strips were used in the preliminary characterization of all strains. Strains isolated on combined nitrogen-free media supplemented with carboxylic acids (citrate [n = 11] and malate [n = 10]) showed similar results and were positive for more API tests than those isolated on media supplemented with carbohydrates (glucose [n = 17] and sucrose [n = 8]). The majority of all strains (actual percentages are provided for each test) isolated on carboxylic acid supplemented media tested positive for the following API tests: ESC (100%), GEL (100%), GNT (100%), MLT (100%), NO_3 (100%), TRP (100%), GLU2 (86%), MAL (86%), MNE (76%), MAN (67%), NAG (67%), and PNPG (62%). Many of the strains isolated on glucose supplemented media tested positive for ESC (100%), PNPG (85%), GLU1 (57%), MAN (57%), and NO_3 (57%), while most of the strains isolated on sucrose supplemented media tested positive for GLU2 (100%), MAL (100%), MAN (100%), GNT (88%), GEL (75%),

and NAG (75%). Medium pH did not substantially impact the total number of strains isolated (22 and 24 strains from pH 7.0 and 7.5 media respectively) or the number of strains isolated on a specific carbon source (citrate, pH 7.0 [n = 4], citrate, pH 7.5 [n = 7], malate, pH 7.0 [n = 5], malate, pH 7.5 [n = 5], glucose, pH 7.0 [n = 9], glucose, pH 7.5 [n = 8], sucrose, pH 7.0 [n = 4], and sucrose, pH 7.5 [n = 4]). Overall, API profiles for all strains isolated on pH 7.0 and 7.5 media were similar (data not shown).

Glucose was a more useful substrate for enrichment of rhizoplane diazotrophs from *Salicornia* than from low intertidal grasses (Bagwell et al. 1998). The carbon sources selected for the enrichment media used in this study are major constituents of root exudates and internal carbon pools in a variety of grasses including *Spartina alterniflora* (Livingstone & Patriquin 1980, Boyle & Patriquin 1981), as well as in other plants (Street et al. 1978, Bokhari et al. 1979, Newman 1985, Whipps & Lynch 1986). Presumably, these carbon sources are important compounds in the root microenvironments of *Salicornia* as well. The qualitative differences in diazotroph isolation efficiency among these carbon compounds may reflect meaningful differences in labile carbon availability in the rhizoplanes of different host plants and/or the impact of differential edaphic conditions on host plant physiology. Biases associated with pure culture studies are well known (Staley & Konopka 1985, Amann et al. 1995), but there was great consistency between these results and those from previous studies utilizing the same isolation media and growth conditions (Bagwell et al. 1998, Bergholz et al. 2001).

Comparisons of API profiles resulted in the identification of 22 physiologically different API groups, 3 of which contained one each of the Gram-positive strains. The remaining 19 API groups contained from 1 to 4 Gram-negative strains each. Multiple isolations of the same or physiologically similar strains are not unexpected (Bagwell et al. 1998), and strains representing API groups were derived from groups of physiologically redundant isolates. Grouping physiologically redundant strains, defined as strains that differ by fewer than 3 API tests, into an API group may mask small, but meaningful differences among strains. However, this treatment facilitates examination of physiologically different organisms. While the number of pure cultures recovered and characterized from *Salicornia* is relatively small, these organisms cover a broad range of physiological features and capabilities.

Dot blot hybridization revealed that 17 of 22 (77%) API group representatives contained *nifH*, including all 3 of the Gram-positive strains. Key morphological and physiological characteristics of the *nifH* containing API group representative strains are provided in Table 1. The combined nitrogen-free media used here are clearly well suited for selectively enriching diazotrophs. Nitrogen efficient strains are also common in

Table 1. Key physiological features and preliminary taxonomic affiliations of the diazotroph API group representatives. Strains are designated by host plant origin (SV = *Salicornia virginica*), carbon source used for isolation (C = citrate, M = malate, G = glucose, S = sucrose), pH of isolation medium (1 = pH 7.0, 2 = pH 7.5), and strain number. Physiological characteristics include Gram reaction (GN = Gram negative, GP = Gram positive), cytochrome oxidase production (ON = oxidase negative, OP = oxidase positive), endospore formation (EN = endospore negative, EP = endospore positive), oxygen requirements (A = obligate aerobe, M = microaerophile). Substrate utilization potentials were scored as the number of positive test results out of the total number of substrates tested within each substrate class (CH = carbohydrates, CA = carboxylic acids, AA = amino acids)

Strain	Key physiological features	Substrate utilization potential			Closest taxonomic group
		CH	CA	AA	
SV-M1-4	GN, OP, EN, M	21/28	16/26	14/20	*Azotobacteraceae*
SV-C2-1	GN, OP, EP, A	22/28	18/26	14/20	*Flavobacterium*
SV-M2-1	GN, ON, EN, M	22/28	16/26	13/20	Unknown
SV-M1-1	GN, OP, EN, M	22/28	18/26	15/20	*Flavobacterium*
SV-G2-4	GN, ON, EN, A	22/28	15/26	12/20	Unknown
SV-M2-2	GN, OP, EN, M	21/28	14/26	13/20	*Azotobacteraceae*
SV-S2-1	GN, ON, EP, M	13/28	17/26	14/20	Unknown
SV-G2-3WHT	GN, ON, EN, M	14/28	17/26	13/20	*Pseudomonadaceae*
SV-G1-4	GN, ON, EN, M	25/28	19/26	14/20	Unknown
SV-G1-3	GN, ON, EN, M	24/28	20/26	16/20	Unknown
SV-C1-3	GN, ON, EP, M	12/28	9/26	12/20	Unknown
SV-S2-3	GN, ON, EP, M	17/28	16/26	18/20	Unknown
SV-G1-2ORG	GN, ON, EN, M	13/28	16/26	13/20	*Pseudomonadaceae*
SV-C2-6	GN, ON, EN, M	13/28	11/26	13/20	*Pseudomonadaceae*
SV-G1-1	GP, ON, EN, M	38/52	10/18	9/10	*Aeromicrobium*
SV-S1-4	GP, ON, EP, M	37/52	8/18	7/10	Unknown
SV-G2-1	GP, ON, EN, A	37/52	10/18	9/10	*Aeromicrobium*

the salt marsh environment (Bagwell et al. 1998), implying that nitrogen limitation may select for organisms proficient at scavenging low levels of nitrogen.

Four clusters of *Salicornia* rhizoplane diazotrophs were defined by bootstrapped cluster analysis of the BIOLOG physiological profiles (Fig. 1). Strains falling outside these clusters were physiologically divergent from any other strains. Cluster 1 (SV-C2-6, SV-C1-3) and Cluster 2 (SV-G1-3, SV-G1-4) were reasonably well supported by bootstrap analysis (62 and 67%

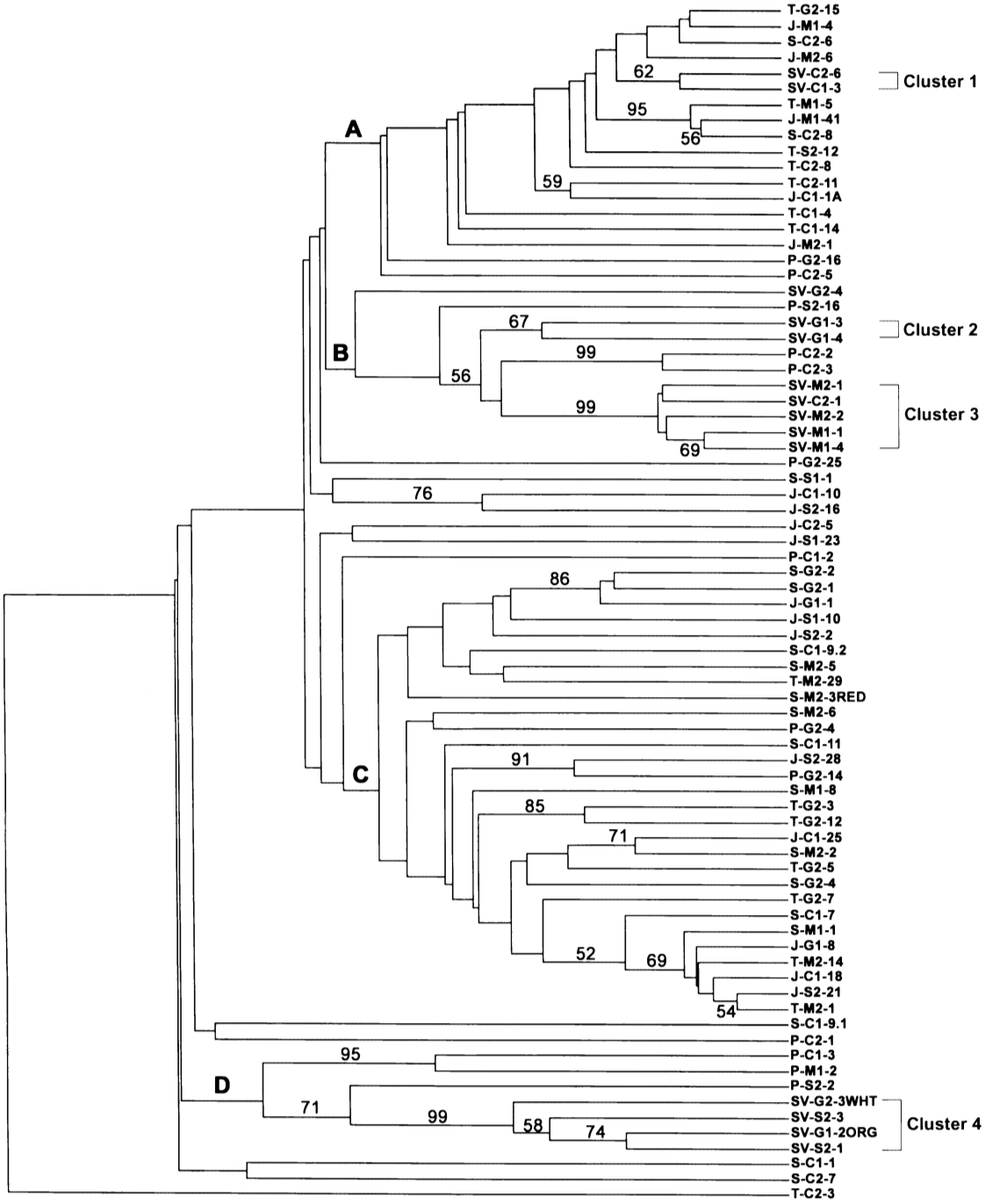

Fig. 1. Dendrogram of physiological profiles, represented as arbitrarily defined artificial 'amino acid' sequences, of Gram-negative diazotrophic strains isolated from the rhizoplane of *Salicornia virginica*, tall and short form *Spartina alterniflora*, *Juncus roemerianus*, and *Spartina patens*. Strain identifications denote the host plant from which the strain was isolated (Low marsh: TS and SS = tall and short *S. alterniflora* respectively, J = *J. roemerianus*, and High marsh: SV = *S. virginica*, P = *S. patens*), enrichment medium carbon source and pH (C = citrate, M = malate, S = sucrose, G = glucose, 1 = pH 7.0, 2 = pH 7.5), and strain number. Major branch divisions are denoted by letter (A, B, C, and D), and bootstrap support values (500 resamplings) ≥50% are shown

bootstrap support values respectively), while Cluster 3 (SV-M2-1, SV-C2-1, SV-M2-2, SV-M1-1, SV-M1-4) and Cluster 4 (SV-G2-3WHT, SV-S2-3, SV-G1-2ORG, SV-S2-1) were strongly supported (99% bootstrap support). Cluster 1 strains did not utilize many of the carbohydrate or carboxylic acid substrates included in the BIOLOG test plates (45 and 39% of carbohydrates and carboxylic acids respectively), but utilized a broader range of amino acids (63%). Cluster 2, 3, and 4 strains utilized >50% of the carbohydrate, carboxylic acid, and amino acid substrates included on the BIOLOG test plates. However, Cluster 2 strains demonstrated much more physiological versatility, these strains utilized 1 to 10 more substrates from each of these substrate classes than Cluster 3 or Cluster 4 strains. Cluster 2 and Cluster 3 strains utilized more different carbohydrates than carboxylic acids or amino acids, while Cluster 4 strains utilized more different amino acids.

Six Gram-positive diazotrophs have been isolated and characterized from the rhizoplanes of *Salicornia* and *Spartina patens*, 3 strains per plant (this study and Bergholz et al. 2001). Overall, utilization profiles for carbohydrate, carboxylic acid, and amino acid substrates on the GP BIOLOG test plates were 63% similar for all 6 strains, but *Salicornia* Gram-positive strains utilized 5 to 10 more substrates from each of these substrate classes than *S. patens* strains. Interestingly, Gram-positive strains originating from the same host plant habitat were physiologically more similar to each other than to strains originating from a different host plant site. *S. patens* strains were 77% similar and *Salicornia* strains were 88% similar for utilization of carbohydrate, carboxylic acid, and amino acid substrates. Several different Gram-positive strains have been described in association with seagrasses (Kurtz et al. 1998), but Bagwell et al. (1998) did not isolate any Gram-positive organisms from the rhizoplanes of tall or short *Spartina alterniflora* or from *Juncus roemerianus* growing in close proximity to the *Salicornia* stand sampled in this study. Clearly, Gram positives were not the dominant organisms in any of these culture collections and too few strains were recovered to support broad conclusions concerning their distributions. Their occurrence, however, may suggest a greater prevalence of Gram-positive diazotrophs in the high intertidal, but further work will be required to adequately test this hypothesis.

Physiological separation of high intertidal rhizoplane diazotrophs from those of low intertidal host origins was clearly demonstrated in the cluster analysis by the formation of distinct strain groupings (Fig. 1). Branch A consists of diazotroph strains from all host origins, and clusters among these strains are not particularly well supported by bootstrapping (except T-M1-5, J-M1-41, S-C2-8 at 95%). These strains do fall within this major grouping and consequently some physiological similarities are shared by all. However, low bootstrap values within this group and higher order branch separation within the major cluster suggests that placement of some of these strains is somewhat ambiguous, likely due to significant physiological differences. Branches B and D form distinct groupings of *Salicornia* and *Spartina patens* diazotroph strains to the exclusion of diazotrophs originating from the low marsh. High bootstrap values also support host-specific strain clusters within these major branches, demonstrating that some key physiological differences exist among diazotrophs from the 2 different high marsh host plants. Branch C is the major cluster in the dendrogram and is composed mostly of low marsh diazotroph strains (tall and short *Spartina alterniflora* and *Juncus roemerianus* island). Two high marsh strains fall within this cluster, both from *S. patens*, but only one of these strains (P-G2-14) was very similar to any diazotroph from the low marsh. Several mixed strain clusters of low marsh diazotrophs are distributed throughout this branch grouping and are well supported by bootstrap analysis. Higher order branching in the dendrogram demonstrates physiological separation between low marsh versus high marsh diazotroph strains; however, bootstrap analysis also supports the designation of host-specific strain clusters.

Another key physiological feature that seems to differentiate strains originating from low intertidal (tall and short *Spartinaalterniflora* and *Juncus roemerianus* island) versus high intertidal (*Salicornia* and *Spartina patens*) origins is oxygen preference. The apparent predominance of obligately aerobic and microaerophilic strains in the rhizoplane of *Salicornia* and *S. patens* may reflect greater (or more consistent) oxygen availability at these locations. In the high intertidal, infrequent tidal flooding results in sediment exposure for extended periods of time. Consequently, anoxia in the shallow sediments may only occur for relatively short time intervals. In addition, *Salicornia* and *S. patens* are not proficient at oxygenating the root microenvironment due to an absence (*Salicornia*) or low quantity (*S. patens*) of aerenchymatous tissue (Anderson 1974). Consequently, roots and rhizomes of these plants, and presumably diazotrophs inhabiting their rhizoplanes, likely depend on oxygen diffusion across the air-sediment interface to meet their oxygen demands. This idea is supported by the concentration of root structures of both *Salicornia* and *S. patens* just below the sediment surface. This is in contrast to *S. alterniflora* and the *J. roemerianus* islands in the low intertidal, where frequent tidal flooding promotes cyclic changes from oxic or suboxic to anoxic conditions. Lacunal transport is the major source of oxygen to roots, rhizomes, and the rhizoplane/rhizosphere (via diffusive

losses from roots and rhizomes) at these locations (Teal & Kanwisher 1966, Armstrong 1978, Hwang & Morris 1991). However, oxygen availability in the root microenvironment is likely intermittent and generally low (Lovell 2001 and references therein). An overwhelming prevalence of facultatively anaerobic diazotrophs was isolated from the roots of these grasses (Bagwell et al. 1998) and these organisms would seem well adapted to conditions of intermittent oxygen supply.

Classical cultivation methods reveal a limited view of the actual species diversity that exists in many environments relative to molecular biological techniques (Amann et al. 1995); however, this approach facilitates identification of key physiological traits of recoverable strains and provides insight into how microorganisms interact with and respond to their environment. The physiological adaptations of plant-associated diazotrophs are expected to reflect the combined effects of host plant and edaphic environmental selection pressures in the root microenvironment, and clearly these selection pressures and the physiological adaptations of diazotrophs differ between high and low marsh plants. Physiological separation of low marsh and high marsh rhizoplane diazotrophs is consistent with differences in host plant ventilation of the rhizosphere microenvironment, but the formation of host-specific strain clusters suggests that additional host traits can also strongly influence the physiological capabilities and distribution patterns of diazotrophs. It is difficult to distinguish the relative impacts of abiotic and biotic environmental parameters on rhizoplane diazotrophs, but these findings suggest that the host plant is a strong determinant of diazotroph distributions. If so, endemic distribution may be the rule for salt marsh diazotrophs, which would be consistent with the great diversity of diazotrophs that have been observed in this ecosystem to date.

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LITERATURE CITED

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59:143–169

Anderson CE (1974) A review of structure in several North Carolina salt marsh plants. In: Reimold RJ, Queen WH (eds) Ecology of halophytes. Academic Press, New York, p 307–344

Armstrong W (1978) Root aeration in the wetland condition. In: Hook DD, Crawford RM (eds) Plant life in anaerobic environments. Ann Arbor Science Series, Ann Arbor, MI, p 269–298

Bagwell CE, Lovell CR (2000a) Microdiversity of culturable diazotrophs from the rhizoplanes of the salt marsh grasses *Spartina alterniflora* and *Juncus roemerianus*. Microb Ecol 39:128–136

Bagwell CE, Lovell CR (2000b) Persistence of selected *Spartina alterniflora* rhizoplane diazotrophs exposed to natural and manipulated environmental variability. Appl Environ Microbiol 66:4625–4633

Bagwell CE, Piceno YM, Ashburne-Lucas A, Lovell CR (1998) Physiological diversity of the rhizosphere diazotroph assemblages of selected salt marsh grasses. Appl Environ Microbiol 64:4276–4282

Bergholz PW, Bagwell CE, Lovell CR (2001) Physiological diversity of rhizoplane diazotrophs of the saltmeadow cordgrass, *Spartina patens*. Implications for host specific ecotypes. Microb Ecol (in press)

Bertness MD (1985) Fiddler crab regulation of *Spartina alterniflora* production on a New England salt marsh. Ecology 66:1042–1055

Bertness MD (1988) Peat accumulation and the success of marsh plants. Ecology 69:703–713

Bertness MD (1991a) Interspecific interactions among high marsh perennials in a New England salt marsh. Ecology 2: 125–137

Bertness MD (1991b) Zonation of *Spartina patens* and *Spartina alterniflora* in a New England salt marsh. Ecology 72:138–148

Bokhari UG, Coleman DC, Rubink A (1979) Chemistry of root exudates and rhizosphere soils of prairie plants. Can J Bot 57:1473–1477

Bowen GD (1980) Misconceptions, concepts, and approaches in rhizosphere biology. In: Ellwood DC, Latham MJ, Hedger JN, Lynch JM, Slater JH (eds) Contemporary microbial ecology. Academic Press, New York, p 283–304

Boyle CD, Patriquin DG (1981) Carbon metabolism of *Spartina alterniflora* Loisel in relation to that of associated nitrogen-fixing bacteria. New Phytol 89:275–288

Campbell R, Greaves MP (1990) Anatomy and community structure of the rhizosphere. In: Lynch JM (ed) The rhizosphere. John Wiley and Sons Ltd, New York, p 11–34

Dame RF, Kenny PD (1986) Variability of *Spartina alterniflora* primary production in the euhaline North Inlet estuary. Mar Ecol Prog Ser 32:71–80

Edgcomb VP, McDonald JH, Devereux R, Smith DW (1999) Estimation of bacterial cell numbers in humic-acid rich salt marsh sediments with probes directed to 16S ribosomal DNA. Appl Environ Microbiol 65:1516–1523

Fenchel T, Esteban GF, Finlay BJ (1997) Local versus global diversity of microorganisms: cryptic diversity of ciliated protozoa. Oikos 80:220–225

Fernandez A, Huang S, Seston S, Xing J, Hickey R, Criddle C, Tiedje J (1999) How stable is stable? Function versus community composition. Appl Environ Microbiol 65: 3697–3704

Ferris MJ, Muyzer G, Ward DM (1996) Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Appl Environ Microbiol 62:340–346

Ferris MJ, Nold SC, Revsbech NP, Ward DM (1997) Population structure and physiological changes within a hot spring microbial mat community following disturbance. Appl Environ Microbiol 63:1367–1374

Finlay BJ, Esteban GF, Fenchel T (1998) Protozoan diversity: converging estimates of the global number of free-living ciliate species. Protist 149:29–37

Gerhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR, Phillips GB (1981) Manual of methods for general bacteriology. American Society for Microbiology, Washington, DC

Hanson RB (1983) Nitrogen fixation activity (acetylene reduction) in the rhizosphere of salt marsh angiosperms, Georgia, U.S.A. Bot Mar 26:49–59

Hwang YH, Morris JT (1991) Evidence for hygrometric pressurization in the internal gas space of *Spartina alterniflora*. Plant Physiol 96:166–171

Kumar S, Tamura K, Nei M (1993) MEGA: molecular evolutionary genetics analysis, version 1.0. The Pennsylvania State University, University Park, PA

Kurtz JC, Yates DF, Genthner FJ, Devereux R (1998) Characterization of bacteria associated with seagrass rhizospheres, abstr N-31. In: Abstracts of the 98th General Meeting of the American Society for Microbiology. American Society for Microbiology, Washington, DC, p 371

Levine JM, Brewer JS, Bertness MD (1998) Nutrients, competition and plant zonation in a New England salt marsh. J Ecol 86:285–292

Livingstone DC, Patriquin DG (1980) Nitrogenase activity in relation to season, carbohydrates and organic acids in a temperate zone root association. Soil Biol Biochem 12: 543–546

Lovell CR (2001) Plant-microbe interactions in the marine environment. In: Bitton G (ed) Encyclopedia of environmental microbiology. John Wiley and Sons, New York (in press)

Lovell CR, Piceno YM, Quattro JM, Bagwell CE (2000) Molecular analysis of diazotroph diversity in the rhizosphere of the smooth cordgrass, *Spartina alterniflora*. Appl Environ Microbiol 66:3814–3822

McArthur JV, Kovacic DA, Smith MH (1988) Genetic diversity in natural populations of a soil bacterium across a landscape gradient. Proc Natl Acad Sci USA 85:9621–9624

McClung CR, van Berkum P, Davis RE, Sloger C (1983) Enumeration and localization of N_2-fixing bacteria associated with roots of *Spartina alterniflora* Loisel. Appl Environ Microbiol 45:1914–1920

Moore LR, Rocap G, Chisholm SW (1998) Physiology and molecular phylogeny of coexisting *Prochlorococcus* ecotypes. Nature 393:464–467

Morris JT, Haskin B (1990) A 5-yr record of aerial primary production and stand characteristics of *Spartina alterniflora*. Ecology 71:2209–2217

Newman EI (1985) The rhizosphere: carbon sources and microbial populations. In: Fitter AH (ed) Ecological interactions in soil, plants, microbes, and animals. British Ecological Society Special Publication 4. Blackwell Scientific Publications Ltd, Oxford, p 107–121

Nusslein K, Tiedje JM (1999) Soil bacterial community shift correlated with change from forest to pasture vegetation in a tropical soil. Appl Environ Microbiol 65:3622–3626

Patriquin DG, McClung CR (1978) Nitrogen accretion, and the nature and possible significance of N_2 fixation (acetylene reduction) in a Nova Scotian *Spartina alterniflora* stand. Mar Biol 47:227–242

Piceno YM, Noble PA, Lovell CR (1999) Spatial and temporal assessment of diazotroph assemblage composition in vegetated salt marsh sediments using denaturing gradient gel electrophoresis analysis. Microb Ecol 38:157–167

Postgate J (1998) Nitrogen Fixation, 3rd edn. Cambridge University Press, Cambridge

Ramsing NB, Fossing H, Ferdelman TG, Andersen F, Thamdrup B (1996) Distribution of bacterial populations in a stratified fjord (Mariager Fjord, Denmark) quantified by in situ hybridization and related to chemical gradients in the water column. Appl Environ Microbiol 62:1391–1404

Revsbech NP, Ward DM (1984) Microelectrode studies of interstitial water chemistry and photosynthetic activity in a hot spring microbial mat. Appl Environ Microbiol 48: 270–275

Staley JT, Konopka A (1985) Measurement of *in situ* activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annu Rev Microbiol 39:321–346

Stoffels M, Amann R, Ludwig W, Hekmat D, Schleifer KH (1998) Bacterial community dynamics during start-up of a trickle-bed bioreactor degrading aromatic compounds. Appl Environ Microbiol 64:930–939

Street HE, Elliott MC, Fowler MW (1978) The physiology of roots. In: Dommergues YR, Krupa SV (eds) Interactions between non-pathogenic soil microorganisms and plants. Elsevier Scientific Publishing Co, New York, p 69–130

Teal JM, Kanwisher JW (1966) Gas transport in the marsh grass, *Spartina alterniflora*. J Exp Bot 17:355–361

Teal JM, Valiela I, Berlo D (1979) Nitrogen fixation by rhizosphere and free-living bacteria in salt marsh sediments. Limnol Oceanogr 24:126–132

Tiner R (1993) Field guide to coastal wetland plants of the southeastern United States. The University of Massachusetts Press, Amherst, MA

Ungar IA (1998) Are biotic factors significant in influencing the distribution of halophytes in saline habitats? Bot Rev 64:176–199

Whipps JM, Lynch JM (1986) The influence of the rhizosphere on crop productivity. In: Marshall KC (ed) Advances in microbial ecology, Vol 9. Plenum Publishing Corp, New York, p 187–244

Whittaker RH (1975) Communities and ecosystems, 2nd edn. MacMillan Publishing Co Inc, New York

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