

Physiological Diversity of the Rhizosphere Diazotroph Assemblages of Selected Salt Marsh Grasses

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Rhizosphere diazotroph assemblages of salt marsh grasses are thought to be influenced by host plant species and by a number of porewater geochemical parameters. Several geochemical variables can adversely affect plant productivity and spatial distributions, resulting in strong zonation of plant species and growth forms. This geochemically induced stress may also influence the species compositions and distributions of rhizosphere diazotroph assemblages, but little is currently known about these organisms. The diversity and key physiological features of culturable, O₂-tolerant rhizosphere diazotrophs associated with the tall and short growth forms of *Spartina alterniflora* and with *Juncus roemerianus* were examined. A total of 339 gram-negative strains were isolated by a root stab culture approach and morphologically and physiologically characterized by using API and BIOLOG tests. Eighty-six distinct groups composed of physiologically similar strains were identified. Of these groups, 72% were shown to be capable of N₂ fixation through molecular analyses, and a representative strain was chosen from each diazotroph group for further characterization. Cluster and principal-components analysis of BIOLOG data allowed the designation of physiologically distinct strain groupings. Most of these groups were dominated by strains that were not identifiable to species on the basis of API or BIOLOG testing. Representatives of several families including the *Enterobacteriaceae*, *Vibrionaceae*, *Azotobacteraceae*, *Spirillaceae*, *Pseudomonadaceae*, and *Rhizobiaceae* were recovered, as well as strains with no clear taxonomic affiliations. This study identifies numerous potentially important physiological groups of the salt marsh diazotroph assemblage.

Intertidal salt marshes along the Atlantic coast of temperate North America are dominated by *Spartina alterniflora* (smooth cord grass), which grows in extensive and often monophyletic stands (46). High rates of macrophyte primary production and microbially mediated nutrient cycling are characteristic of these systems, resulting in substantial contributions to global carbon (16, 44) and nitrogen (13) budgets. Whole-system nutrient budgets indicate a net export of nitrogen from *Spartina* marshes (15), and the consensus of numerous studies is that primary productivity (11, 24, 48), as well as decomposition processes (28, 49), in *Spartina* marshes is nitrogen limited. Thus, nitrogen input through nitrogen fixation (diazotrophy) is potentially very important to maintaining high levels of macrophyte primary production in this ecosystem.

Spartina shows a range of growth morphologies that reflect nitrogen limitation and other environmental stress factors (8, 10). In the North Inlet, S.C., salt marsh and elsewhere, highly productive tall-form plants (≥ 1 m high) are found primarily near tidal creek banks while less productive short-form plants (≤ 0.5 m high) occur higher in the intertidal zone. Transitional zones of medium-height plants are also observed between the tall and short zones at many locations. These major differences in plant morphology and productivity result from several stress factors, whose impacts correspond to variations in elevation and sediment texture. The low-marsh growth zone of tall *Spartina* is characterized by low average porewater hydrogen sulfide levels and salinity relative to the high-marsh zone dominated by short *Spartina*. Active porewater exchange occurs along the creek banks, regulating dissolved solute levels and allowing

some oxygen to penetrate these sediments (9). The high-marsh growth zone of short *Spartina* is more subject to interstitial porewater stagnation. Environmental stressors in the high-marsh sediments (high salinity and hydrogen sulfide concentrations, and low oxygen availability) reduce the efficiency of nitrogen uptake by *Spartina* (10), exacerbating nitrogen limitation in this zone. Porewater chemistry clearly has a strong impact on the growth and productivity of *Spartina*. These stressors may act as selective forces on the composition and diversity of rhizosphere microbial species including the diazotrophs. However, incomplete characterization of the rhizosphere microenvironment and its potential to shelter the resident microflora from such stresses limits our understanding of selection in the rhizosphere.

Most of the diazotrophic activity in *Spartina* marshes is closely associated with plant roots (30, 36, 51, 52), and this activity increases in response to treatments that stimulate plant primary production (21, 36, 51). Root exudates are thought to be the main source of carbon and energy for the microflora immediately surrounding active plant roots (20, 34), and diazotrophy in the *Spartina* rhizosphere is enhanced by amendment with extractable carbohydrates and carboxylic acids from *Spartina* tissues (7). In addition, *Spartina* transports significant quantities of oxygen into its rhizosphere, supporting aerobic respiration in sediments that would otherwise be anoxic and highly reduced (45). The rhizosphere of *Spartina* thus supports high levels of diazotrophic activity and fosters conditions (the "rhizosphere effect") that may be conducive to maintenance of substantial diazotroph diversity. This diversity is poorly characterized at present.

Close associations between different species of diazotrophs and grasses are commonly observed. A variety of enterics (53), pseudomonads (5, 19, 25), and vibrios (39) have been isolated from the roots of assorted grasses. Clearly, numerous different

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diazotrophs can associate with the roots of grasses. Recent studies have highlighted the diversity of rhizosphere diazotrophs and illustrate the abundance of uncharacterized species associated with wetland grasses. Ueda et al. (47) constructed and screened a clonal library of PCR-amplified *nifH* sequences recovered from the rice rhizosphere and rhizoplane. Analysis of the *nifH* sequences revealed loosely defined clusters that contained sequences homologous to those of known diazotroph species (*Clostridium pasteurianum*, *Klebsiella pneumoniae*, and *Azotobacter* spp.). However, all of the *nifH* sequences recovered were unique, indicating a great diversity of uncharacterized diazotrophs in the rice rhizosphere. Resolution of PCR-amplified *nifH* sequences from tall and short *Spartina* rhizospheres by denaturing gradient gel electrophoresis has also revealed complex diazotroph assemblages (37). Denaturing gradient gel electrophoresis banding profiles show both overlapping and differentiating *nifH* sequences between the two zones. These molecular biological approaches demonstrate the occurrence of numerous diazotroph species in the rhizospheres of wetland grasses, including *Spartina*, but provide little and only inferential information on key ecophysiological characteristics of these organisms. To understand the relevant physiological features, distributions, and stress responses of these organisms, they must be isolated and characterized.

We have examined the diversity and key physiological characteristics of culturable oxygen-tolerant diazotrophs isolated from the rhizospheres of tall- and short-form *Spartina*. We also examined diazotrophs from *Juncus roemerianus* (black needle-rush), which occurs near the terrestrial fringe and in small islands of slightly higher elevation in the short-*Spartina* zone. Comparison between culturable diazotrophs from *Spartina* and *Juncus* facilitates a preliminary assessment of the importance of plant host species versus environmental parameters in dictating diazotroph species distributions. These efforts have resulted in a detailed physiological examination of the culturable rhizosphere diazotroph assemblages in these salt marsh grasses and have revealed a large number of unidentified diazotroph species (3, 4).

MATERIALS AND METHODS

Materials, reference cultures, and media. API test strips were obtained from Fisher Scientific (Pittsburgh, Pa.). BIOLOG test plates were purchased from BIOLOG (Hayward, Calif.). Reference cultures of *Azotobacter chroococcum* (ATCC 9043), *Acetobacter diazotrophicus* (ATCC 49037), and *Vibrio diazotrophicus* (ATCC 33466) were purchased from the American Type Culture Collection (Rockville, Md.). *Azospirillum brasilense* Sp7, *Azospirillum lipoferum* Sp59b, *Rhizobium leguminosarum* bv. *viciae* (USDA 2370), and *R. meliloti* (USDA 1025) were provided by Peter van Berkum, U.S. Department of Agriculture. *Azotobacter vinlandii* UW was provided by Robert Robson, University of Reading, Reading, United Kingdom, and *Klebsiella pneumoniae* M5a1-UW was provided by Gary Roberts, University of Wisconsin. The cultivation medium and incubation conditions for the *Azotobacter* spp. have been described previously (14). *Azospirillum* spp. were grown on PYE (peptone yeast extract [33]) at 30°C. *Rhizobium* spp. (30°C) and *K. pneumoniae* (37°C) were grown on full-strength tryptic soy agar (Difco, Detroit, Mich.). *Acetobacter diazotrophicus* was grown at 30°C on mannitol agar (1), which contains the following (per liter of distilled water): Bacto Yeast Extract, 5.0 g; Bacto Peptone, 3.0 g; mannitol, 25.0 g; and Bacto Agar, 15.0 g. *Vibrio diazotrophicus* was grown in tryptic soy broth at 26°C. The isolation media were based on the mineral salts recipe of Shieh et al. (40) and contained the following (per liter of distilled water): NaCl, 28 g; MgSO₄, 0.5 g; CaCl₂, 0.01 g; Na₂MoO₄, 0.01 g; and Trizma base, 6.06 g. Basal salts solutions were adjusted to pH 7.0 or 7.5, and 0.5 or 1.5% (wt/vol) Noble Agar (Difco) was added for semisolid tube and solid plating media, respectively. After being autoclaved, the media were supplemented with autoclaved FeCl₃ (0.05 mM) and K₂HPO₄ (3 mM) and sterile-filtered citrate (60 mM [final concentration]), glucose (70 mM), malate (90 mM), or sucrose (30 mM).

Root culture inoculation and pure-culture isolation. Short-form *S. alterniflora* plants were collected in January 1994, and tall-form *S. alterniflora* and *J. roemerianus* plants were collected in June 1995. All the plants were from our field study site on Goat Island in the North Inlet salt marsh, near Georgetown, S.C. (de-

scribed previously [16, 31]). In the laboratory, roots were shaken and rinsed with deionized water to remove loosely associated sediment, stab inoculated into culture tubes of nitrogen-free semisolid media, and loosely capped. Root cultures were incubated in the dark at 30°C until clonal outgrowths from the roots were observed. Each clonal outgrowth was subcultured by stab inoculation into fresh nitrogen-free semisolid medium having the appropriate pH and carbon source and incubated at 30°C. Pure cultures were isolated by streak plating on nitrogen-free media and maintained on Bacto Marine Agar during characterization. Cultures were suspended in 5.0% dimethyl sulfoxide and 5.0% (final concentration) glycerol and stored frozen at -70°C.

Culture characterization. Gram-staining characteristics and cell morphologies were determined by standard methods (18). Motility was observed in wet mounts. The oxygen requirements were determined with Bacto Marine Agar semisolid medium supplemented with sodium thioglycolate as specified for the thioglycolate medium in the USP recipe (2). Preliminary physiological characterization and grouping of strains were based on results of API 20E testing (bioMérieux Vitek, Inc.). Test results were obtained as specified by the manufacturer. API NFT strips were also used for organisms giving few positive results or inconclusive results in the API 20E tests. API 20E strip test descriptions are as follows: ONPG (β -galactosidase), ADH (arginine dihydrolase), LDC (lysine decarboxylase), ODC (ornithine decarboxylase), CIT (citrate utilization), H₂S (sulfide production), URE (urease), TDA (tryptophane deaminase), IND (indole production), VP (Voges-Proskauer reaction), GEL (gelatin liquefaction), GLU (glucose fermentation), MAN (mannitol fermentation), INO (inositol fermentation), SOR (sorbitol fermentation), RHA (rhamnose fermentation), SAC (sucrose fermentation), MEL (melibiose fermentation), AMY (amylidase fermentation), ARA (arabinose fermentation), and NO₃ (nitrate reduction). API NFT strip test descriptions are as follows: NO₃ (nitrate reduction), TRP (indole production from tryptophane), GLU1 (glucose fermentation), ADH (arginine dihydrolase), URE (urease), ESC (esculin hydrolysis), GEL (gelatinase), PNPG (β -galactosidase), GLU2 (D -glucose assimilation), ARA (arabinose assimilation), MNE (mannose assimilation), MAN (mannitol assimilation), NAG (N -acetyl- D -glucosamine assimilation), MAL (maltose assimilation), GNT (D -gluconic acid assimilation), CAP (capric acid assimilation), ADI (adipic acid assimilation), MLT (L -malic acid assimilation), CIT (citric acid assimilation), and PAC (phenylacetic acid assimilation). Strains that differed by three or fewer test results were grouped. Cytochrome oxidase tests (bioMérieux Vitek, Inc.) were conducted separately to supplement these API data.

Identification of *nifH*-containing strains. Dot blot hybridizations were next used to confirm the potential of each API strain group to fix N₂. DNA was extracted from one member of each group by using a modified Marmur (29) procedure including a cetyltrimethylammonium bromide purification step (32). DNA quality (i.e., relative molecular weight) and quantity were assessed by gel electrophoresis and fluorometry, respectively. Dot blots were prepared with 1 μ g of DNA/dot from each isolate by methods described previously (27) and with positively charged nylon membranes (Tropix, Inc., Bedford, Mass.). The blots were UV cross-linked, baked for 30 min at 80°C under vacuum, and stored at 4°C until used. Prehybridization and hybridization solutions consisted of 5 \times SSC (1 \times SSC is 150 mM NaCl and 15 mM sodium citrate), 2 \times Denhardt's solution (1 \times Denhardt's solution is 0.02% bovine serum albumin, 0.02% Ficoll, and 0.02% polyvinylpyrrolidone), 0.1% *N*-lauroylsarcosine, and 0.02% sodium dodecyl sulfate (SDS) and contained 100 pmol of biotinylated *nifH*-specific probe, RL-43R (5'-ATGCCGATCCGCGAAAACAAAGCCAGGAGATCTACATCGTC). Prehybridization (2 h) and hybridization (2 h) mixtures were incubated at 40°C. The blots were then washed twice in 2 \times SSC-0.1% SDS for 5 min each and twice in 0.5 \times SSC-0.1% SDS at 45°C for 15 min each. Probe detection used the Southern-Light chemiluminescence detection system (Tropix, Inc.). After a 72-h exposure, hybridization signal intensities were scored against positive and negative controls. *Azospirillum lipoferum* was used as the positive control, and *Bacillus subtilis* and *Staphylococcus aureus* served as the negative controls. These results were verified by PCR amplification with *nifH*-specific primers. PCR amplification was carried out in a 25- μ l reaction volume. The amplification mixture included template DNA (25 ng), reaction buffer, 1.5 mM MgCl₂, 0.2 mM each deoxynucleoside triphosphate, 10 pmol of primers RL-25 (5'-CAGATCAG[C/A/G]CCGCC[C/G]AG[A/G]CG[A/C]AC) and RL-28 (5'-GGTAT[C/T]GG[C/T]AA[A/G]T[C/G]C[AC]G[C]AC) per μ l, and 0.1 U of *Taq* DNA polymerase (Perkin-Elmer, Norwalk, Conn.). DNA amplification was performed with the following temperature profile: initial denaturation (94°C for 30 s); 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a 2-min extension at 72°C. Amplimers were scored after electrophoresis in 1.5% agarose gels.

Diazotroph characterization. Strains that hybridized with the *nifH* probe and showed a PCR amplimer of the appropriate size were examined further by using the BIOLOG system. Each group representative was grown overnight at 30°C on Bacto Marine Agar. Colonies were collected with sterile cotton swabs and resuspended in 25 ml of sterile saline (0.85% NaCl [pH 7.0]). Inoculum turbidity was measured spectrophotometrically (λ = 590 nm) and ranged from 53 to 59% transmittance. GN Microplate test panels were inoculated with these suspensions and incubated for 24 h at 30°C. The Microlog 2 automated system and accompanying software was used for data collection and to standardize optical density values.

Analysis of physiological data. BIOLOG optical density values were analyzed in SYSTAT 7.0 (42) by cluster analysis with Euclidean distances, and a corre-

TABLE 1. Strain yields from each plant source obtained by using the root stab culture technique and the four carbon sources listed

Carbon source	No. of strains from ^a :		
	SS	TS	J
Citrate	24	35	55
Malate	31	35	55
Glucose	6	19	4
Sucrose	6	14	55
Total	67	103	169

^a SS and TS, short- and tall-form *S. alterniflora*, respectively; J, *J. roemerianus*.

sponding dendrogram was constructed with average linkages. Principal-components analysis was conducted in SAS (38).

RESULTS AND DISCUSSION

Root inoculation into semisolid media was used for recovery of rhizosphere and rhizoplane diazotrophs. Clonal outgrowths from root surfaces were observed after incubation for 24 to 48 h at 30°C. These outgrowths either extended directly from the root surface or were observed as dense bands, most of which were located below the medium surface. A total of 67 strains were recovered from short *Spartina* plants, 103 were recovered from tall *Spartina*, and 169 were recovered from *Juncus*. Most of these organisms were facultative anaerobes with a preference for microaerophilic growth conditions, although a few obligate aerobes and microaerophiles were also isolated. Rhizosphere O₂ supply reflects a balance between lacunal transport- (45) and mass transport (9)-derived inputs and consumption driven by the demands of root and microbial metabolism and abiotic chemically reduced species. Facultative anaerobes probably have a selective advantage in the rhizosphere, with regularly fluctuating oxic (during periods of active photosynthesis) and anoxic conditions. Obligate anaerobes were not examined in this study.

All 339 strains stained gram negative. The majority were motile rods, although eight *Juncus* strains and four tall-form and two short-form *Spartina* strains were nonmotile cocci. Pleomorphism was common in week-old cultures, and pigmentation was observed in some cases. Pigmented strains were isolated from all three plant types, with most being isolated from tall *Spartina* and *Juncus*. Fourteen red, yellow, or orange strains were isolated.

The carbon sources we used are important constituents of grass root exudates (carboxylic acids) and plant tissues (carbohydrates) (7, 43). Citrate- or malate-supplemented media yielded the majority of strains from all three plant types, with relatively few isolates being recovered from glucose- or sucrose-supplemented *Spartina* root cultures (Table 1). In contrast, sucrose-supplemented root cultures yielded a substantial number of strains from *Juncus* roots. Recovery of a large and diverse array of diazotrophs on the carboxylic acids (Table 1; also see Table 6) may reflect a true preference for these substrates by rhizosphere diazotrophs (26).

A total of 243 of 339 strains gave adequate responses (at least three positive test results) on the API 20E test strips. However, 63 of 339 strains performed poorly on the API 20E test strips and were characterized with the API NFT strips. The remaining 33 strains were uncharacterized due to insufficient data from both test strips.

The results of the API 20E tests are summarized in Fig. 1. Overall, the test result profiles for the tall-*Spartina* and *Juncus*

strain collections appear quite similar, differing substantially from these for the short *Spartina* strains. Strains from tall *Spartina* and *Juncus* frequently ($\geq 50\%$ of strains) showed positive results in the CIT, IND, GEL, GLU, MAN, and AMY API 20E tests. The result profiles of the API NFT tests for tall-*Spartina* and *Juncus* strains were also similar (Fig. 2). Strains from tall *Spartina* and *Juncus* were typically positive for the ESC, GEL, PNP, GLU2, ARA, MAN, MAG, MAL, GNT, and MLT API NFT tests. These API results indicate striking physiological similarities between tall-*Spartina* and *Juncus* strain collections. However, the API test strips provided only a limited data set, and conclusions from such data should be drawn with caution. When strains were compared further by using the BIOLOG system, there was no strong similarity between tall-*Spartina* and *Juncus* strain collections. Key results from the three strain collections are summarized below.

At least some short-*Spartina* strains were positive for all tests on both API 20E and API NFT test strips, but no clear patterns of test results were evident. More strains positive for several API 20E tests were recovered from short *Spartina* with citrate- or malate-supplemented media than with glucose- or sucrose-containing media (Table 2). Only three strains from glucose- or sucrose-supplemented root cultures could be characterized with the API 20E test strips, and very few positive results were obtained. Citrate medium strains (hereafter referred to as citrate strains) were more frequently positive for fermentation of several carbohydrates (MAN, INO, SOR, RHA, MEL, AMY, and ARA) than were the malate strains. Malate strains were more frequently positive than citrate strains for GEL and IND.

A total of 22 short-*Spartina* strains were characterized by the API NFT tests; the majority of these (13 strains) were from the malate-supplemented medium. This limited data set prevented detailed comparisons across carbon sources. Broadly, all API

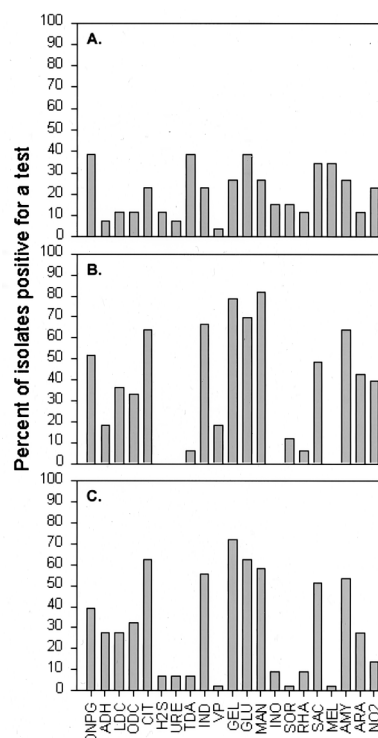


FIG. 1. Percentage of the total strain collection for each plant type that was positive for each API 20E test reaction. (A) Short-form *S. alterniflora*; (B) tall-form *S. alterniflora*; (C) *J. roemerianus*.

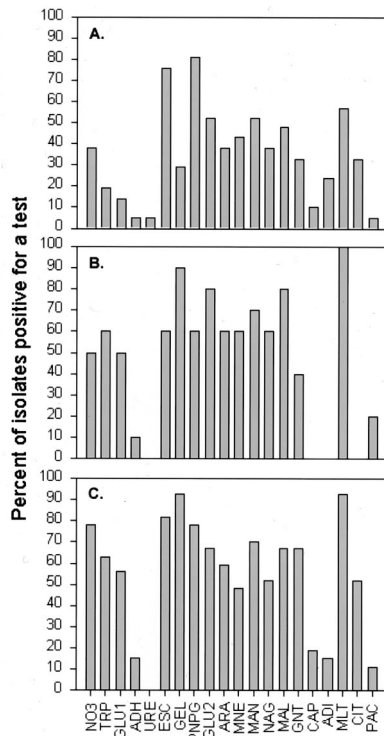


FIG. 2. Percentage of the total strain collection for each plant type that was positive for each API NFT test reaction. (A) Short-form *S. alterniflora*; (B) tall-form *S. alterniflora*; (C) *J. roemerianus*.

NFT tests except URE were positive for at least some strains, but very few tests could be considered diagnostic (positive for $\geq 50\%$ of isolates) for these short-*Spartina* strains. The most useful tests were PNPG and MLT.

Tall-*Spartina* strains grew well on all four carbon sources, although more strains were recovered from citrate- or malate-supplemented media (Table 3). None of these strains gave positive results for the H₂S, URE, INO, or MEL tests on the API 20E strips, and very few gave positive results for ADH ($n = 8$), TDA ($n = 4$), VP ($n = 8$), or SOR ($n = 4$). Overall, citrate and malate strains had similar result profiles. These strains were often positive for LDC, ODC, and GLU, while the sucrose and glucose strains yielded very few positive results for these tests (two, two, and eight, respectively). Sucrose and glucose strains were differentiated by their results in the ONPG and ARA tests. AMY and GEL were frequently positive for strains recovered on all carbon sources.

TABLE 2. API 20E test summary for short-form *S. alterniflora* strains

Test(s) positive for strains from ^a :							
C ($n = 18$)		M ($n = 18$)		S ($n = 2$)		G ($n = 1$)	
$\geq 50\%$	0%	$\geq 50\%$	0%	$\geq 50\%$	0%	$\geq 50\%$	0%
MEL	H ₂ S	GEL	URE	H ₂ S	All other tests	TDA	All other tests
	URE		ARA	GEL		IND	
	VP			GLU			
				SAC			

^a Tests that were positive for $\geq 50\%$ of the strain collection or for no strains (0%) are listed for each carbon source (C = citrate; M = malate; S = sucrose; G, glucose) used in strain isolation. Sample sizes (n) are given in parentheses.

TABLE 3. API 20E test summary for tall-form *S. alterniflora* strains

Tests positive for strains from ^a :							
C ($n = 31$)		M ($n = 25$)		S ($n = 11$)		G ($n = 17$)	
$\geq 50\%$	0%	$\geq 50\%$	0%	$\geq 50\%$	0%	$\geq 50\%$	0%
LDC	H ₂ S	ONPG	H ₂ S	ONPG	H ₂ S	ONPG	ADH
ODC	URE	LDC	URE	CIT	URE	IND	H ₂ S
CIT	INO	ODC	TDA	MAN	VP	GEL	URE
IND	SOR	CIT	INO	AMY	INO		TDA
GEL	RHA	IND	MEL	ARA	SOR		INO
GLU	MEL	GEL			MEL		RHA
MAN		GLU					MEL
SAC		MAN					
AMY		AMY					

^a Tests that were positive for $\geq 50\%$ of the strain collection or for no strains (0%) are listed for each carbon source (C, citrate; M, malate; S, sucrose; G, glucose) used in strain isolation. Sample sizes (n) are given in parentheses.

Fourteen strains from tall *Spartina* were examined with the API NFT strips. None of these strains were positive for CIT or CAP tests. Not surprisingly, glucose ($n = 4$) and sucrose ($n = 5$) strains showed overall similar test result profiles. Interestingly, all glucose strains were positive for TRP but none of the sucrose strains were. Four of the five sucrose strains were positive for ARA, MNE, and NAG, while only a single glucose strain was positive for MNE. Malate strains ($n = 4$) were positive for all tests except ADH, URE, ADI, and PAL.

Many strains were recovered from *Juncus* roots for all carbon sources except glucose (Table 4). Citrate strains were differentiated from the others by giving positive results in the H₂S, TDA, GLU, SAC, AMY, and ARA tests. Malate strains were frequently positive for GEL (96%), ONPG (65%), IND (50%), and MAN (50%). Less than 10% of the citrate, malate, and sucrose strain collections gave positive results in the VP, INO, SOR, RHA, and MEL tests.

Twenty-seven *Juncus* strains were characterized with the API NFT strips. Only a single glucose strain gave some positive results on these strips. Strains from sucrose, malate, and citrate were positive for most tests, with URE being the notable exception. Citrate ($n = 13$) and malate ($n = 7$) strains showed very similar utilization profiles and differed markedly only in

TABLE 4. API 20E test summary for *Juncus* strains

Tests positive for strains from ^a :							
C ($n = 36$)		M ($n = 26$)		S ($n = 55$)		G ($n = 3$)	
$\geq 50\%$	0%	$\geq 50\%$	0%	$\geq 50\%$	0%	$\geq 50\%$	0%
CIT	VP	ONPG	H ₂ S	None	H ₂ S	CIT	ADH
GEL	SOR	IND	URE		INO	GEL	LDC
GLU		GEL	TDA		SOR		ODC
AMY		MAN	VP		MEL		H ₂ S
			RHA				URE
			MEL				IND
							VP
							GLU
							MAN
							INO
							SOR
							RHA
							MEL
							NO ₃

^a Tests that were positive for $\geq 50\%$ of the strain collection or for no strains (0%) are listed for each carbon source (C = citrate, M = malate, S = sucrose, and G = glucose) used in strain isolation. Sample sizes (n) are given in parentheses.

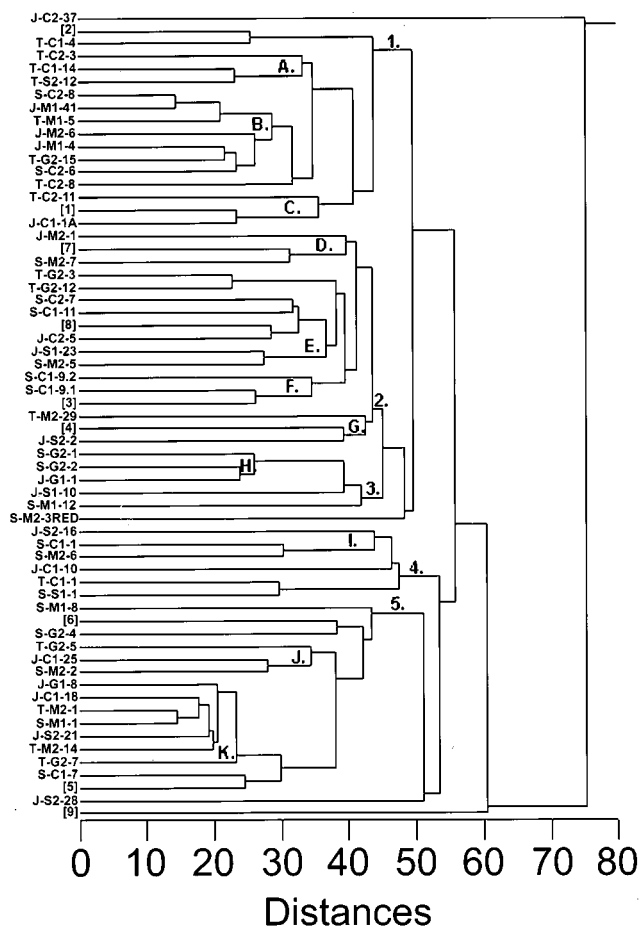


FIG. 3. Dendrogram constructed from Euclidean distances of BIOLOG test results for unknown diazotroph strains and known diazotroph species. Strains are designated by zone (S, short-form *S. alterniflora*; T, tall-form *S. alterniflora*; J, *J. roemerianus*), carbon source (C, citrate; M, malate; G, glucose; S, sucrose), pH (1, pH 7.0; 2, pH 7.5), and strain number. Known diazotrophs are as follows: [1] *Vibrio diazotrophicus*, [2] *Azospirillum brasilense*, [3] *Azotobacter chroococcum*, [4] *Klebsiella pneumoniae*, [5] *Azospirillum lipoferum*, [6] *Azotobacter diazotrophicus*, [7] *Rhizobium meliloti*, [8] *Rhizobium leguminosarum*, and [9] *Azotobacter vinlandii*. Strain groupings and clusters are illustrated numerically and by letter at the appropriate node, respectively.

the frequencies of positive results for a few key tests (MNE, ARA, NAG, and CIT). In addition, neither citrate or malate strains assimilated ADI, although sucrose strains were frequently (67%) positive for this test.

The two pH values used did not appear to impose any significant selective pressure in these isolations. We routinely record rhizosphere porewater pH values between 7.0 and 7.5 at

our study site (37), and this difference is not likely to be significant to rhizosphere microbiota in the salt marsh.

API 20E and API NFT profiles were useful for eliminating redundancies in the culture collection, both within and across plant types. Strains differing by no more than three test results were grouped, yielding a total of 86 groups. The groups ranged in size from 30 strains to 1 strain. Four API groups were arbitrarily chosen, and the strains within them were tested by the BIOLOG system. Each of these groups had effectively homogeneous BIOLOG substrate utilization profiles, differing by no more than three test results among member strains. This validates the API grouping approach.

All *nifH* hybridization results, positive or negative, were verified by PCR amplification. Of the 86 API strain groups, 54 (63%) had *nifH* sequences and were presumably capable of nitrogen fixation. The fixed-nitrogen-free media efficiently selected for diazotrophs, approximately 72% of the total strains recovered. However, nitrogen-efficient but nondiazotrophic microbes were also recovered. Persistence of these organisms may have been due to nitrogen inputs from the root tissues during the original enrichments and to trace nitrogen levels in the media during isolation. Presumably these organisms are adapted to scavenging low levels of nitrogen from the environment (23, 53).

Standardized BIOLOG OD values were used to construct a dendrogram in order to facilitate identification of physiological groups within the total culture collection (Fig. 3). Major branch points were examined by principal-components analysis to determine the key substrate classes (i.e., carbohydrates, carboxylic acids, and amino acids) responsible for major strain subdivisions (Table 5). Carbohydrate, carboxylic acid, and amino acid utilization accounted for 100% of the variability in the diazotroph strain data sets. Groups 1 and 5 preferentially utilized carboxylic acids, and group 4 preferentially used carbohydrates. Groups 2 and 3 preferred both carbohydrates and amino acids to carboxylic acids. Organisms falling outside these five groups utilized either very few of the BIOLOG substrates or all of them. A more detailed examination was conducted on clusters containing three or more API groups to again identify specific substrate class preferences (Table 6). Clusters A, B, C, and D preferentially utilized amino acids, and clusters E, F, and H preferentially utilized carbohydrates. Clusters G, I, J, and K showed no clear substrate class preferences. Overall, clear differences do exist among clusters based on substrate utilization profiles, although some overlap in these profiles is apparent. Signature substrates were identified for several clusters and are as follows: cluster A, glucuronamide; cluster C, alaninamide and glycyl-L-aspartic acid; cluster F, α -lactose, α -D-lactose lactulose, and 2-aminoethanol; cluster H, L-rhamnose; and cluster I, γ -aminobutyric acid.

These data also permitted us to perform a preliminary evaluation of the taxonomy of some diazotroph clusters (Fig. 3).

TABLE 5. Principal-components analysis of normalized BIOLOG optical density values for major dendrogram branches, represented by major strain groups^a

Substrate class	Result for strain group:														
	1			2			3			4			5		
	PC1	PC2	PC3	PC1	PC2	PC3	PC1	PC2	PC3	PC1	PC2	PC3	PC1	PC2	PC3
Carbohydrates	-0.68	0.33	0.65	0.72	0.17	-0.67	0.64	0.18	0.75	0.71	0.00	0.71	-0.68	0.33	0.65
Carboxylic acids	0.72	0.13	0.68	0.11	0.93	0.35	-0.49	0.84	0.22	-0.71	0.00	0.71	0.72	0.13	0.68
Amino acids	0.14	0.93	-0.33	0.69	-0.33	0.65	0.59	0.51	-0.63	0.00	1.00	0.00	0.14	0.93	-0.33

^a BIOLOG tests results for three major substrate groups, the carbohydrates, carboxylic acids, and amino acids, accounted for 100% of the variability in the data sets.

TABLE 6. BIOLOG substrate class (carbohydrate, carboxylic acid, amino acid) utilization by dendrogram clusters

Substrate class	Substrate class utilization ^a by cluster:										
	A (n = 3)	B (n = 7)	C (n = 3)	D (n = 3)	E (n = 6)	F (n = 3)	G (n = 3)	H (n = 3)	I (n = 3)	J (n = 3)	K (n = 7)
Carbohydrates	27/28	19/28	21/28	28/28	22/28	23/28	20/28	23/28	14/28	14/28	10/28
Carboxylic acids	23/26	12/26	14/26	13/26	19/26	17/26	13/26	11/26	19/26	7/26	11/26
Amino acids	18/20	16/20	16/20	16/20	17/20	13/20	12/20	7/20	17/20	2/20	5/20

^a Number of class substrates used/total number of class substrates. Clusters A, B, and C fall into group 1; clusters D, E, F, and G fall into group 2; cluster H is group 3; cluster I is group 4; and clusters J and K fall into group 5.

The majority of the clusters contained unidentified organisms that probably belong to the families *Vibrionaceae* and *Enterobacteriaceae*. However, these families are distinguished primarily on the basis of a small set of phenotypic traits, i.e., negative Gram reaction, bacillus cell shape, facultative oxygen requirement, and cytochrome oxidase production. Substantially more data are needed to extend these preliminary identifications. Fermentation and substrate utilization results for other clusters are consistent with their placement in the families *Rhizobiaceae*, *Spirillaceae*, *Azotobacteraceae*, and *Pseudomonadaceae*. Based on the results obtained in this study, clusters C, D, and F were the only taxonomically coherent clusters. Other clusters contained organisms apparently differing in taxonomic affiliations. Identification to species level was possible for several strains. Cluster B contained three strains that were identified as *Vibrio parahaemolyticus*, *Vibrio anguillarum*, and *Vibrio alginolyticus*. One strain in cluster K was identified as *Pseudomonas syringae*, and another from the same cluster was apparently a *Flavobacterium* species but did not correspond to any documented species in this genus. Similar major taxonomic groupings have been identified in other nonlegume diazotroph-plant systems (5, 50, 53). Overall, the clusters identified were diverse and contained representatives from several taxonomic groupings. The API and BIOLOG tests provided convenient tools for characterization, although additional physiological and molecular biological testing will be required for complete classification.

Known diazotrophs were also examined to evaluate the utility of BIOLOG results for identification of rhizosphere diazotrophs (data not shown). Most cultures of known diazotrophs showed fewer than three discrepancies between the BIOLOG test results and the known characteristics of the organisms (22). However, others (*Azotobacter chroococcum*, *Vibrio diazotrophicus*, and *Rhizobium meliloti*) produced BIOLOG test results differing substantially from their known physiological traits. All of our known and unknown cultures were grown on the same rich medium, Bacto Marine Agar, before inoculation of the BIOLOG test plates. This was done to promote consistency in test results, allowing comparisons among the strains to be made. However, several of our type strains are routinely cultivated on defined media containing low levels of NaCl, consistent with their terrestrial origins. Bacto Marine Agar, while supporting growth, was stressful for some of these organisms, perhaps resulting in atypical test results. Similar stress-driven atypical results could occur for some of the unknown strains, but in the interests of consistency and expediency, results from Bacto Marine Agar cultures were used throughout.

Primary productivity in the salt marsh requires substantial new nitrogen inputs from diazotrophy to offset the export of fixed nitrogen (6, 23). Clearly, diazotrophs and diazotrophic interactions are very important in this environment. It has been suggested for many ecosystems that taxonomically uncharacterized species are ubiquitous, and consequently our under-

standing of microbial diversity is presently quite poor (12, 17, 35, 41). It is clear that even well-studied functional groups, such as the diazotrophs, contain many unknown species. This study provides a substantial amount of new information on this important group of organisms and has identified several key features of diazotrophs from the *S. alterniflora* and *J. roemerianus* rhizospheres and rhizoplanes. Isolation on laboratory medium is known to bias microorganism recovery, with the potential for loss of important species. However, this technique does yield the pure cultures required for ecophysiological studies and permits some characterization of the physiological diversity of diazotrophs in the rhizospheres of salt marsh grasses. Future studies will quantitatively monitor the physiological groups of diazotrophs identified here to address diazotroph assemblage stability, interactions with host plants, and responses to geochemical stressors.

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