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PURE CULTURE STUDIES OF SEAGRASS RHIZOPLANE ISOLATES

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ABSTRACT

Rhizosphere bacterial isolates from the seagrasses, *Halodule wrightii, Syringodium filiforme* and *Thalassia testudinum* were exposed to 95 different potential carbon sources on Biolog TM plates. Metabolic profiles of each isolate were determined and compared with a data base composed of 434 known species and groups. Metabolic community profiles were also compiled, based on the percentage of isolates utilizing each carbon source. Comparisons among the bacterial communities from each seagrass species were made and community similarity indices calculated. Eleven substrates could be used as a sole carbon source by over 90 percent of the overall population. These were: sucrose; methyl pyruvate; L-alanylglycine (96.4%); D-alanine (96.3%); dextrin; N-acetyl-p- glucosamine; cellobiose; D-mannose; D, L-lactic acid; L-alanine (92.9%); and L- proline (92.6%). Six substrates were used by less than ten percent of the overall population. These were: D, L-carnitine (7.2%), phenylethylamine; 2, 3- butanediol (7.1%); itaconic acid, i-erythritol (3.6%) and alpha-cyclodextrin (0%). Community similarity indices indicated that the microbial populations from *Thalassia* and *Syringodium* were most similar (Jaccard coefficient = .904), followed by *Halodule* and *Thalassia* (JC=.903), then *Halodule* and *Syringodium* (JC=.892). These results indicate that community metabolic profiles may be useful as a sensitive tool for monitoring microbial populations, as well as yielding useful biochemical information about individual isolates.

INTRODUCTION

The importance of bacteria associated with roots and rhizomes of seagrasses was reported by a number of studies (Smith, 1987). Many of these studies indicate a close nutritional interrelationship between the microflora and seagrasses. Nitrogen fixation was measured in the rhizosphere of a number of seagrass species (Capone, 1982; Capone, 1983; Patriquin & Knowles, 1972; Smith & Hayasaka, 1982a & b) and from seagrass rhizoplane isolates (Shieh, Simidu & Maruyama, 1987 & 1989). Other reported nitrogen transformations include nitrification (Boon, Moriarty & Saffigna, 1986a; Iizumi, Hattori & McRoy, 1980) and ammonium generation (Boon, Moriarty & Saffigna, 1986b; Iizumi, Hattori & McRoy, 1982; Smith, Hayasaka & Thayer, 1984). In addition, rhizoplane isolates were shown to be able to solubilise mineral phosphates (Craven & Hayasaka, 1982). Despite the nutritional importance of the seagrass rhizosphere microflora, this population has not been well characterized. Microbial activity measurements, however, have indicated relatively high rates of replication (Moriarty & Pollard, 1981 & 1982) and overall metabolism (Smith & Hayasaka, 1986); and at least some of the rhizoplane isolates show a positive chemotactic response toward amino acids formed in seagrass root exudate (Wood & Hayasaka, 1981. Smith, *et al.* (1982) reported that rhizoplane isolates were either aerobic or facultatively anaerobic and exhibited heavy metal sensitivity patterns more similar to water column isolates than to sediment isolates.

Positive identification of these isolates, even to the level of genus, remains unknown with few exceptions. Smith and Hayasaka (1982b) reported the isolation of a nitrogen-fixing *Klebsiella* sp. from the endorhizosphere of *Halodule wrighti* which was confirmed by fluorescent antibody staining (Schmidt & Hayasaka (1985). Shieh, *et al.* (1987 & 1989) identified nitrogen-fixers in the *Zostera marina* rhizosphere as belonging to the genera *Vibrio* and *Photobacterium*.

This communication reports results of the first in a series of studies designed to describe spatial and temporal variations in the seagrass rhizoplane microflora based on the ability of isolated strains to use 95 different compounds as sole carbon sources. These metabolic profiles were compared with a data base of known strains and community indices compared among the microflora of different seagrass species.

MATERIALS AND METHODS

Bacteria were aseptically isolated from root-rhizome systems of seagrasses growing in Graham's Harbor, San Salvador Island, Bahamas (Smith, 1987). A glycerol artifitual sea water (GASW) medium was used for initial isolation and maintenance of the cultures (Smith & Hayasaka, 1982b). This medium had been previously found to be optimal for seagrass rhizoplane isolates by reciprocal plating. Isolates were then characterized for Gram's stain, motility in deep stabs, and colony morphology (Gerhardt, 1981). Cell morphometrics were determined using microscopy and image analysis (Cue 3 System, Olympus Corp.). Only strains differing in one or more of these characteristics were used for biochemical screening on Biolog (TM) GN Plates. These plates contained 95 different carbon sources in microwells (along with a water control) and a tetrazolium dye to indicate oxidative activity. Individual strain profiles were entered in a computer containing the Microlog (TM) 1, release 2.00, version DE software, and matched with the data base containing 434 species and groups of Gram-negative bacteria (Marello & Bochner, 1989). The percentage of the bacterial population from each seagrass species, able to use each carbon source was then calculated and community similarity indices determined using Ecological Analysis (TM) software (Oakleaf Systems).

Rates of glutamate deamination were determined for selected isolates, some of which could use glutamate as a sole carbon source and some which could not. Deamination rates were determined for washed cultures by the method described by Smith (1988) and reported as the difference in ammonium ion evolution between glutamate amended and unamended serum bottles. All determinations were performed in triplicate.

RESULTS AND DISCUSSION

Most of the seagrass rhizoplane isolates did not match very well with the data base (Table 1-3). This was not surprising for two reasons. First, the isolates were obtained from a unique environment. There were no profiles of isolates from this environment in the data base, although there is a version of the software that is expandable. Second, the data base was "young". As more environmental isolates and particularly, seagrass rhizoplane isolates, are added to the data base, the more likely close profile matches will be made. Nevertheless, one excellent match was obtained (*Pseudomonas diminuta* from *Thalassia*) and two good matches observed (*Klebsiella pneumoniae* from *Thalassia* and *Citrobacter freundii* from *Syringodium*).

Among the carbon sources listed in Table 4, over 90 per cent of the overall population could utilize sucrose, D-mannose, cellobiose, N-acetyl-D-glucosamine and dextrin and less than 20 percent of the overall population could use psicose, L-fucose, xylitol and i-erythritol. The overall distribution was somewhat more characteristic of *Halodule* isolates than those from other seagrasses.

The most frequently metabolized organic acid was methylpyruvate followed by D, L-lactic acid, then succinic acid (Table 5). Less than 15 percent of the isolates could use D-glucosaminic acid or sebacic acid, and only *Syringodium* isolates (12 per cent) could use itaconic acid. Phosphylated substrates (Table 7) were only used by 25 percent of the *Syringodium* and *Thalassia* isolates, but about half of the *Halodule* isolates utilized these compounds. About 25 percent of the isolates used the nucleotides uridine and thymidine as carbon sources, which may lead to questions concerning their utility as indicators of replication. Among the

Table 1. Identification and Similarity Index of *Halodule* Isolates.

STRAIN	DATE	BEST MATCH	SIM	ID LEVEL
SSH2A1	7/90	*Klebsiella pneumoniae*	.690	GOOD
8210	7/89	*Pseudomonas glycinea*	.492	POOR
SSHC1516	12/89	*Klingella kingae*	.483	POOR
8233	7/89	*Xanthomonas maltophilia*	.424	POOR
8111	7/89	*Klebsiella terrigena*	.413	POOR
SSH11B3	7/90	*Vibrio pelagius*	.404	POOR
SSH12B3	7/90	*Vibrio carchariae*	.399	POOR
8232	7/89	*Xanthomonas maltophilia*	.370	POOR
SSH1B4	7/90	*Klebsiella terrigena*	.355	POOR
SSHC3	12/89	*Clavibacter michiganense*	.344	POOR
SSH21B2	7/90	*Vibrio parahaemolyticus*	.342	POOR
8411	7/89	*Enterobacter agglomerans*	.328	POOR
SSHC7	12/89	*Vibrio parahaemolyticus*	.326	POOR
8112	7/89	*Xanthomonas maltophilia*	.286	POOR
SSHC9	12/89	*Vibrio harveyi*	.283	POOR
SSHC10	12/89	*Salmonella sp.*	.279	POOR
824	7/89	*Pseudomonas glycinea*	.269	POOR
8231	7/89	*Sphingobacterium multivorum*	.224	POOR
SSHC14	12/89	*Vibrio vulnificus*	.218	NONE
SSH3A1	7/90	*Haemophilus aphrophilus*	.211	NONE
SSH3B1	7/90	*Vibrio harveyi*	.202	NONE
SSH4B2	7/90	*Vibrio harveyi*	.173	NONE
SSH13B1	7/90	*Salmonella sp.*	.171	NONE
SSHC5P	12/90	*Vibrio vulnificus*	.135	NONE
SSH12B2	7/90	*Actinobacillus sp.*	.130	NONE
SSH1B2	7/90	*Vibrio harveyi*	.104	NONE
SSHC2	12/90	*Haemophilus haemolyticus*	.100	NONE
SSHC4	12/89	*Vibrio mimicus*	.098	NONE
8412	7/89	*Yersinia enterocolitica*	.087	NONE
SSHC8	12/89	*Vibrio vulnificus*	.085	NONE
SSHC12	12/89	*Vibrio anguillarum*	.075	NONE
SSHC6	12/89	*Vibrio vulnificus*	.039	NONE
SSH4B1	7/90	*Vibrio anguillarum*	.047	NONE
SSH4A1	7/90	*Vibrio splendidus*	.044	NONE
SSH2B2	7/90	*Vibrio mimicus*	.032	NONE
SSH1A1	7/90	*Enterobacter taylorae*	.022	NONE
8221	7/89	*Aeromonas hydrophila*	.003	NONE
SSH12B1	7/90	*Pseudomonas vesicularis*	.003	NONE
8222	7/89	*Salmonella sp.*	.002	NONE
SSH1A2	7/90	*Enterobacter agglomerans*	.001	NONE

Table 3. Identification and Index of *Thalassia* Isolates.

STRAIN	DATE	BEST MATCH	SIM	ID LEVEL
20621	7/89	*Pseudomonas diminuta*	.780	EXCELLENT
SST2A2	7/90	*Klebsiella pneumoniae*	.764	EXCELLENT
SST32A	7/90	*Clavibacter michiganense*	.647	GOOD
SST1B2	7/90	*Clavibacter michiganense*	.595	GOOD
SSTFB5	12/89	*Aeromonas hydrophila*	.537	GOOD
2042	7/89	*Klebsiella pneumoniae*	.509	GOOD
SSTFB3	12/89	*Alcaligenes faecalis*	.459	POOR
20632	7/89	*Xanthomonas maltophilia*	.457	POOR
SSTFB4	12/89	*Alcaligenes faecalis*	.379	POOR
SSTFB8	12/89	*Vibrio mediteranei*	.371	POOR
SSTFB7	12/89	*Vibrio harveyi*	.283	POOR
2043	7/89	*Xanthomonas maltophilia*	.279	POOR
SSTFB10	12/89	*Vibrio alginolyticus*	.273	POOR
2041	7/89	*Enterobacter agglomerans*	.219	NONE
SST3B1	7/90	*Vibrio carchariae*	.217	NONE
SST32B3	7/90	*Vibrio carchariae*	.193	NONE
SST1B1	7/90	*Clavibacter michiganense*	.187	NONE
SST11A4	7/90	*Vibrio parahaemolyticus*	.174	NONE
SST4A2	7/90	*Enterobacter agglomerans*	.136	NONE
SST11A3	7/90	*Aeromonas hydrophila*	.131	NONE
SST21B2	7/90	*Vibrio mimicus*	.113	NONE
2021	7/89	*Xanthomonas maltophilia*	.096	NONE
SST2B2	7/90	*Vibrio harveyi*	.091	NONE
SST23B	7/90	*Vibrio harveyi*	.072	NONE
SST4B1	7/90	*Vibrio parahaemolyticus*	.069	NONE
SST4A1	7/90	*Salmonella sp.*	.052	NONE
20631	7/89	*Sphingobacterium multivorum*	.027	NONE
SST4B2	7/90	*Vibrio metschnikovi*	.007	NONE
SST1A2	7/90	*Enterobacter aerogenes*	.002	NONE

Table 2. Identification and Similarity Index of *Syringodium* Isolates.

STRAIN	DATE	BEST MATCH	SIM	ID LEVEL
19222	7/89	*Citrobacter freundii*	.577	GOOD
SSSFB9	12/89	*Vibrio carchariae*	.544	GOOD
19212	7/89	*Capnocytophaga gingivalis*	.489	POOR
SSSFB3	12/89	*Enterobacter agglomerans*	.473	POOR
SS4A2	7/90	*Clavibacter michiganense*	.419	POOR
SSSFB7	12/90	*Vibrio alginolyticus*	.322	POOR
SSSFB5	12/89	*Vibrio carchariae*	.314	POOR
SSS4A1	7/90	*Haemophilus parainfluenzae*	.279	POOR
SSS62A4	7/90	*Vibrio vulnificus*	.266	POOR
SSSFB8	12/89	*Clavibacter michiganense*	.265	POOR
SS21A2	7/90	*Vibrio anguillarum*	.254	NONE
1942	7/89	*Pseudomonas glycinea*	.249	NONE
SSS5A1	7/90	*Enterobacter gergoviae*	.235	NONE
SSSFB4	12/89	*Salmonella sp.*	.230	NONE
SSS2A3	7/90	*Enterobacter aerogenes*	.228	NONE
SSS21A3	7/90	*Vibrio harveyi*	.218	NONE
19612	7/89	*Enterobacter gergoviae*	.203	NONE
SSS1B1	7/90	*Vibrio mimicus*	.198	NONE
SSS62A3	7/90	*Vibrio metschnikovii*	.181	NONE
SSSFB6	12/89	*Vibrio vulnificus*	.162	NONE
19611	7/89	*Xanthomonas campestris*	.156	NONE
SSS3A1	7/90	*Vibrio mimicus*	.131	NONE
SSS2B1	7/90	*Klebsiella ozaenae*	.125	NONE
19411	7/89	*Enterobacter gergoviae*	.114	NONE
SSS5A2	7/90	*Enterobacter gergoviae*	.107	NONE
SSSFB2	12/89	*Moraxella bovis*	.060	NONE
SSS1B2	7/90	*Klebsiella trevisanii*	.053	NONE
19211	7/89	*Sphingobacterium multivorum*	.046	NONE
SSS1A1	7/90	*Pantoea agglomerans*	.003	NONE
SSS2A2	7/90	*Vibrio metschnikovii*	.002	NONE
SSS2B2	7/90	*Hafnia alvei*	.002	NONE
SSS6A3	7/90	*Enterobacter agglomerans*	.002	NONE

Table 4. Percentage of seagrass rhizoplane isolates using various carbohydrates, sugars and alcohols as sole carbon sources.

CARBON SOURCE	*Halodule*			*Syringodium*			*Thalassia*		
	1	2	3	1	2	3	1	2	3
sucrose	100	83	88	100	78	94	88	50	94
D-mannose	100	67	53	88	78	41	88	67	82
cellobiose	100	83	59	88	78	47	88	50	71
N-acetyl-D-glucosamine	100	75	77	88	100	59	88	67	65
dextrin	100	67	82	88	78	71	88	67	82
Tween 40	91	42	88	88	67	59	88	50	82
D-fructose	91	92	94	88	100	82	88	100	82
Tween 80	82	42	82	88	22	71	100	50	65
D-trehalose	82	75	82	88	44	94	88	67	65
maltose	82	83	65	88	89	76	88	67	76
gentiobiose	82	33	71	88	56	59	88	17	71
alpha-D-glucose	82	83	82	88	100	100	88	67	88
beta-methyl glucoside	73	67	53	88	22	65	88	33	65
lactulose	64	0	47	88	22	59	88	0	47
alpha-lactose	73	83	47	88	22	77	75	0	47
D-melibiose	73	17	29	88	0	35	75	0	82
turanose	64	25	47	88	44	53	62	17	41
N-acetyl-D-galactosamine	64	0	35	88	44	41	62	17	24
D-galactose	45	50	65	62	56	71	38	33	82
D-mannitol	27	75	88	38	100	77	62	67	94
L-rhamnose	18	17	41	62	22	35	38	17	41
D-raffinose	27	17	35	38	22	29	38	0	29
L-arabinose	27	33	18	50	22	18	25	0	29
D-sorbitol	18	58	77	62	33	12	25	17	65
m-inositol	9	50	53	25	33	47	38	17	29
D-arabitol	9	0	18	25	11	12	38	0	18
adonitol	9	0	41	25	11	24	38	0	29
glycogen	9	67	82	50	89	82	12	67	94
psicose	27	67	59	12	44	47	12	50	65
L-fucose	9	0	53	38	22	18	0	0	24
xylitol	0	8	29	12	22	47	25	0	41
i-erythritol	0	8	6	0	0	18	12	0	18
alpha-cyclodextrin	0	42	71	0	67	59	0	33	56

sample times, 1=7/89, 2=12/89, 3=7/90.

Table 5. Percentage of rhizoplane isolates using various organic acids as sole carbon sources.

CARBON SOURCE	*Halodule* 1	2	3	*Syringodium* 1	2	3	*Thalassia* 1	2	3
methyl pyruvate	100	25	41	88	67	59	100	67	65
D,L-lactic acid	100	50	82	88	67	94	88	33	88
succinic acid	91	92	59	88	89	47	88	33	88
citric acid	91	83	71	88	100	59	75	83	71
mono-methyl succinate	73	17	18	88	0	18	100	33	24
cis-aconitic acid	82	83	77	88	100	29	75	67	71
alpha-ketobutyric acid	45	8	41	100	11	59	75	33	29
alpha-hydroxybutyric acid	64	8	18	88	22	18	50	33	29
propionic acid	64	50	59	75	56	29	62	50	47
bromosuccinic acid	55	50	77	38	100	59	88	83	71
formic acid	45	8	18	88	11	82	38	17	18
beta-hydroxybutyric acid	36	0	12	62	0	24	50	33	24
D-gluconic acid	55	67	82	38	78	88	38	83	82
D-saccharic acid	45	0	24	62	11	29	25	17	29
D-galacturonic acid	45	59	41	38	44	59	25	17	35
succinamic acid	36	33	24	25	0	47	50	33	24
D-glucuronic acid	45	75	65	25	44	53	25	17	41
malonic acid	27	33	29	38	11	35	25	33	24
alpha-ketoglutaric acid	45	33	71	88	56	53	38	67	69
alpha-ketovaleric acid	9	0	12	62	11	6	25	0	12
acetic acid	27	58	82	0	44	65	38	67	82
D-galactonic acid lactone	36	8	24	12	0	18	12	17	24
gamma-hydroxybutyric acid	9	0	12	38	0	12	12	0	35
p-hydroxyphenylacetic acid	36	0	12	0	0	0	12	0	6
D-glucosaminic acid	9	8	41	25	22	41	12	0	41
quinic acid	9	0	29	25	22	24	12	0	24
sebacic acid	0	0	0	25	0	0	12	0	0
itaconic acid	0	8	12	12	11	18	0	83	12

sample times, 1=7/89, 2=12/89, 3=7/90.

Table 6. Percentage of seagrass isolates using nitrogenous organic compounds as sole carbon sources.

CARBON SOURCE	*Halodule* 1	2	3	*Syringodium* 1	2	3	*Thalassia* 1	2	3
L-alanylglycine	100	42	82	88	67	77	100	83	77
D-alanine	91	25	88	100	89	47	100	83	77
L-alanine	100	67	77	88	100	71	88	100	77
L-proline	100	58	77	75	89	71	100	83	71
glycyl-L-glutamic acid	82	33	77	88	67	41	75	50	77
L-serine	91	83	82	88	89	82	62	67	71
alaninamide	64	0	18	88	0	29	75	0	18
L-threonine	73	58	77	75	67	77	75	83	65
L-glutamic acid	36	58	59	75	100	77	50	100	65
glycyl-L-aspartic acid	36	42	53	75	56	41	50	50	59
L-asparagine	36	75	65	62	89	82	62	83	71
L-leucine	27	0	6	88	0	29	38	33	29
L-aspartic acid	27	58	77	62	78	65	50	67	53
L-histidine	36	42	41	50	22	65	38	33	41
D-serine	55	8	53	38	44	71	12	17	59
glucuronamide	55	42	47	0	33	47	25	33	65
L-ornithine	9	8	41	50	11	53	25	50	35
hydroxy-L-proline	9	17	65	25	67	71	25	33	71
L-phenylalanine	9	0	12	38	0	29	12	17	12
gamma-aminobutyric acid	9	17	12	38	22	18	12	0	12
L-pyroglutamic acid	0	17	18	38	11	35	12	33	12
D,L-carnitine	9	0	18	0	0	6	12	0	6

sample times, 1=7/89, 2=12/89, 3=7/90.

Table 7. Percentage of seagrass rhizoplane isolates using various substrates as sole carbon sources.

CARBON SOURCE	*Halodule* 1	2	3	*Syringodium* 1	2	3	*Thalassia* 1	2	3
glycerol	45	58	77	62	67	88	38	83	71
glucose-1-phosphate	55	42	65	25	56	50	25	67	71
inosine	36	42	94	50	67	94	25	67	88
urocanic acid	36	17	29	38	33	53	25	17	35
D,L-alpha-glycerol-P	45	25	71	25	67	77	25	33	65
glucose-6-phosphate	45	42	77	25	56	71	25	67	77
uridine	18	42	82	25	67	65	38	50	82
thymidine	18	25	65	12	67	88	38	33	77
2-aminoethanol	9	0	12	38	0	24	0	0	12
putrescine	27	17	12	12	11	24	0	17	6
phenylethylamine	0	0	12	12	0	12	12	0	12
2,3-butanediol	0	0	18	12	0	18	12	0	29

sample times, 1=7/89, 2=12/89, 3=7/90.

carbon sources listed in Table 7, the least used were; 2-aminoethanol, putrescine, phenylethylamine and 2, 3-butanediol (less than 20 percent).

The uptake of labeled amino acids has often been used as a measure of overall microbial metabolic activity. In addition, deamination has been proposed as an activity measurement for natural microbial populations (Smith, 1988). Table 6 shows the percentage of isolates which used various amino acids and nitrogenous compounds as sole carbon sources. The most frequently used was L-alanylglycine (96.4 percent), D-alanine (76.3), L-alanine (92.9) and L-proline (92.6), but the specific frequencies varied among seagrass sources. Glutamic acid was used as a sole carbon source by about half the overall population, as was the deamination product, alpha-ketoglutaric acid (Table 6), but not necessarily by the same strains. The ability of selected strains to use glutamate and alpha-ketoglutarate as a sole carbon source and the deamination rate of glutamate associated with these strains was shown by Morgan and Smith (this volume). Strains that could use glutamate as a sole carbon source had relatively low deamination rates. The highest deamination rates were observed with strains that could not use glutamate but could use alpha-ketoglutarate (strains 8210 and 1942). One strain (8231) could use neither glutamate nor alpha-ketoglutarate as a sole carbon source, but had a relatively high deamination rate (402 umoles h^{-1}), indicating that with some isolates, deamination may only be one step in a pathway for taking up glutamate from the environment.

Community metabolic profiles, calculated on the basis of percentage of the microbial population utilizing each carbon source, were most similar between *Thalassia* and *Syringodium* (Jaccard Coefficient=.904) and *Thalassia* and *Halodule* (JC= .903). The greatest difference was between *Syringodium* and *Halodule* (JC=.892), which was not a large difference at all.

In summary, obtaining metabolic profiles of environmental isolates from techniques described in this paper can yield a number of interesting results. First, the identity of isolates can be obtained by extending the data base of tested isolates from a particular environment. It is likely that new species will be discovered, as indicated by the "no identification" matches from Table 1-3. Also, metabolic pathways (some probably novel pathways) may be indicated as with strain 8231. These pathways may play a major role in carbon cycling in the environment. Finally, community metabolic profiles can be determined, saved in a data base, and compared with other microbial communities. It is possible that changes profiles may indicate changes in the overall environment (for example, pollution, sedimentation or other factors which may require monitoring).

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