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Enumeration and Identification of Nitrogen-Fixing Bacteria from Forage Grass Roots

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Root-soil cores were collected from forage grasses growing in a subtropical region of Texas and tested for acetylene reduction activity. The population density of nitrogen-fixing bacteria was measured, using various media and incubation conditions. Bacteria were confirmed as nitrogen fixing, using the acetylene reduction assay, and were classified according to standard biochemical and cultural methods. The majority of the nitrogen-fixing bacteria isolated from roots were *Enterobacter cloacae* or *Klebsiella pneumoniae*. Root-associated, nitrogen-fixing bacteria were isolated from 21 of 24 root-soil cores. The population densities of nitrogen-fixing bacteria ranged from approximately 10^4 to 3×10^7 per g of root. Population density on roots was significantly correlated with the rate of acetylene reduction but the relationship was not linear.

A survey of forage grasses for associative N_2 fixation in Texas demonstrated that many soil-root cores actively reduced acetylene to ethylene (19). The present investigation extended the previous study to determine the population size and to classify the diazotrophic bacteria associated with grass roots in a subtropical region. *Azospirillum*, previously referred to as *Spirillum*, was the principal diazotroph isolated from roots of grasses in Brazil (5) and has been isolated from grasses in Florida (16). In temperate regions of the United States, the principal diazotrophic bacteria isolated from grass roots are *Bacillus* spp. and members of the family *Enterobacteriaceae* (10, 12, 14).

Presumably, a large population of diazotrophic bacteria on plant roots would result in higher rates of acetylene reduction (AR) activity. Published information on this topic is very limited. Correlations between AR activity and numbers of diazotrophic bacteria in soil (9) and on rice root segments (17) are poor. Close correlation between population size and N_2 fixation is sporadic in occurrence because N_2 fixation is growth linked and dependent upon cell proliferation (4). Nevertheless, there must be a critical population size to provide the needed biomass and nitrogenase for significant rates of N_2 fixation.

MATERIALS AND METHODS

Plant roots. Bacteria were cultured from plant roots in core (8 cm in diameter by 11 cm in length) samples collected from native and introduced forage grasses in unfertilized pastures. Samples were collected from eight species growing at seven sites (Table 1). Sites were moist at the time of sampling. Sampling sites were within a triangular area of Texas extending from College Station on the north (30.5°N, 96°W) to Rockport on the south (28°N, 97.5°W) to Beaumont on the east (30°N, 94.5°W).

All cores were tested for AR activity for 24 h, by the method of Weaver et al. (19), immediately before they were processed for bacterial population counts. Some soil-plant cores were placed in a greenhouse for several weeks after sampling to allow for regrowth of shoots and to reestablish an equilibrium between roots and soil before measurements of AR activity and bacterial population (Table 1). Other cores were processed for culturing within 36 h after removal from the field.

To culture N_2-fixing bacteria, plant roots were separated from the soil and processed by using aseptic technique. All roots which could be separated from the soil were cut from shoots, rinsed five times by swirling in 250 ml of distilled water, and transferred to a mortar containing 10 ml of 0.1 M phosphate buffer (pH 6.5). They were ground with a pestle for 30 s, and 1 ml of supernatant was transferred and serially diluted. Two serial dilutions were made of each sample. Dilutions of 10^{-3} and 10^{-4} were plated from plants 1 to 22, and from plants 23 and 24, the 10^{-2} and 10^{-3} dilutions were plated. Duplicate spread plates of the various media were inoculated. Lower dilutions were used for later samplings because experience indicated that they would not be overgrown. Roots were transferred to weighing dishes, dried at 65°C, and weighed.

Media and incubation conditions. Modified ($CaCl_2$) nitrogen-free Hino and Wilson medium (H & W, pH 7.7) (8) was used. This medium was modified further by adjusting the phosphate buffer to a lower pH (H & W, pH 6.7). Nitrogen-free malate medium (2) was modified by using 0.8 g of K_2HPO_4, 0.8 g of KH_2PO_4, and 5 g of malic acid per liter. Solid media were prepared with 15 g of agar per liter. Semisolid media were prepared with 5 g of agar per liter. MacConkey agar (Difco Laboratories, Detroit, Mich.)

TABLE 1. *Characteristics of soil-root cores collected from grasses for isolation of nitrogen-fixing bacteria*

Plant no.	Site no.	Grass	Soil properties		AR[a] (μmol of C_2H_4 $core^{-1}$ 24 h^{-1})
			Texture	pH	
1	1	*Paspalum notatum* Flugge	silt loam	8.1	2.4
2	1	*Cynodon dactylon* (L.) Pers.	clay loam	7.8	3.3
3	1	*Cynodon dactylon* (L.) Pers.	sandy loam	7.0	1.9
4	2	*Paspalum notatum* Flugge	silt loam	7.4	1.7
5	2	*Paspalum dilatatum* Poir.	loam	6.4	2.0
6	2	*Axonopus affinis* Chase	sandy loam	5.8	2.0
7	2	*Paspalum notatum* Flugge	sandy loam	6.0	1.4
8	2	*Axonopus affinis* Chase	sandy clay loam	6.9	0
9	2	*Paspalum notatum* Flugge	sandy loam	6.6	0
10	2	*Paspalum* sp.	sandy clay loam	7.3	0
11	2	*Paspalum* sp.	sandy loam	7.0	0
12	3	*Andropogon* sp.	silt loam	7.8	2.9
13	4	*Paspalum dilatatum* Poir.	silt loam	6.4	1.9
14	4	*Paspalum urvillei* Steud.	sandy loam	8.6	2.1
15[b]	4	*Paspalum dilatatum* Poir.	loam	6.7	0
16[b]	4	*Paspalum urvillei* Steud.	sandy clay loam	7.6	5.5
17[b]	4	*Paspalum urvillei* Steud.	sandy loam	7.6	4.8
18	4	*Paspalum dilatatum* Poir.	loam	7.1	0
19	5	*Paspalum dilatatum* Poir.	sandy loam	6.7	1.4
20	5	*Cynodon dactylon* (L.) Pers.	sandy loam	6.4	0
21	6	*Paspalum dilatatum* Poir.	clay loam	6.0	0
22	6	*Paspalum plicatum* Michx.	clay loam	6.3	0
23[b]	7	*Cynodon dactylon* (L.) Pers.	loamy sand	5.8	0.8
24[b]	7	*Cynodon dactylon* (L.) Pers.	loamy sand	5.1	1.6

[a] To convert to kilograms of N fixed per hectare over the growing season, multiply by 2. See reference 19 for assumption in converting AR to N fixed.

[b] Plant was cultured immediately after removal from the field.

was used. Incubation time was for 72 h, except for MacConkey agar which was incubated for 48 h. Incubation temperatures were 30°C for anaerobic and MacConkey plates and 22°C for candle jar plates. Three O_2 concentrations were utilized to provide conditions best suited for culture of N_2-fixing microorganisms (Table 2). Anaerobags (Cedanco, Division of H.P.B., Inc., Hudson, Mass.) were used as incubation chambers for anaerobic cultures. An anaerobic atmosphere was provided by the method of Attebery and Finegold (1). Microaerophilic conditions were provided by candle jars for H & W and malate medium. MacConkey plates were incubated aerobically.

Enumeration and classification of bacteria. The total number of diazotrophic colonies formed on plates during incubation was counted. A representative colony of each colonial type was used for qualitative measurement of AR activity and for classification of the bacteria. Colonies were tested for AR activity after stab subculturing in semisolid H & W (pH 7.7) and malate medium. Cultures were incubated aerobically for 48 h at 30°C, tube caps were replaced with serum stoppers, and 10% of the atmosphere was replaced with C_2H_2. The tubes were then incubated for 24 h at 30°C, and the quantity of C_2H_4 produced was measured by using flame ionization gas chromatography. Cultures demonstrating AR activity were streaked onto nutrient agar to check for purity and were identified by standard biochemical tests (6, 18).

Statistical analysis. Variances in plate counts were tested against a chi-square distribution to determine whether bacteria were evenly dispersed in the diluent after maceration of the roots (7, 20). The counts on duplicate plates from both serial dilutions were used to provide four counts on each sample. Only samples having between 30 and 300 colonies per plate on all four plates were included in this analysis. Correlation analyses of the number of diazotrophic bacteria per gram of dry root and the AR activity of soil-root cores were made, using linear regression analysis and the Spearman rank correlation test (3).

RESULTS AND DISCUSSION

Textural classes of soils from which plants were collected ranged from loamy sand to clay loam (Table 1). Soil pH ranged from moderately acid to alkaline, and AR rates ranged from 0 to 5.5 μmol of C_2H_4 24 h^{-1} $core^{-1}$. The higher rates are somewhat higher than those reported for grasses in temperate regions (2, 10, 15). No strong correlation is apparent between soil texture, species of grass, or collection site and the AR rate. The six highest rates of AR activity were for the six samples collected from soil more alkaline than pH 7.4, but there was not a statistical correlation between soil pH and AR activity.

Media incubated anaerobically (<1.0% O_2)

TABLE 2. *Numbers of N_2-fixing bacteria on selective media under different incubation conditions*

Plant no.	Dominant bacterial species	Log_{10} count per g of dry root for:					
		Anaerobic H & W		Candle jar			Aerobic Mac-Conkey
				H & W		Malate (pH 6.7)	
		pH 6.7	pH 7.7	pH 6.7	pH 7.7		
1	*E. cloacae*	6.16	6.16	—[a]	—	6.08	5.40
2	*K. pneumoniae*	—	5.64	—	—	—	5.83
	E. cloacae	7.36	7.34 (2)[b]	7.32	7.39	7.33	7.30 (2)
3	*K. pneumoniae*	4.54	—	—	—	—	—
	E. cloacae	6.04	6.04	7.63	6.11	—	—
4	*K. pneumoniae*	—	5.16	6.72	—	—	5.24
5	*E. cloacae*	5.41	5.40	—	6.38	—	—
6	*E. cloacae*	6.58 (2)	6.57 (2)	6.44 (2)	6.35	6.54	—
7	*K. pneumoniae*	—	—	—	—	—	4.48
	E. cloacae	6.61 (2)	6.50 (2)	6.50 (2)	6.31	6.76 (2)	7.50
8	*E. cloacae*	5.74 (2)	5.91 (2)	4.70 (2)	5.18	5.76 (2)	5.30
9	Total[c]	6.66 (2)	6.79	6.72	6.66	6.62	6.76
10	None[d]						
11	None[d]						
12	*K. pneumoniae*	6.08	6.00	6.80	—	—	6.49
13	Total[c]	5.72	5.76	—	—	—	—
14	*K. pneumoniae*	5.38	—	—	—	—	5.16
	E. cloacae	6.92	6.90 (2)	7.00 (3)	6.91 (2)	6.95 (3)	6.91
	Unidentified	—	—	—	—	6.23	—
15[e]	*K. pneumoniae*	5.54	—	—	—	—	—
	Bacillus sp.	—	—	—	5.54	—	—
16[e]	*E. cloacae*	6.65 (2)	4.45	6.63 (2)	6.67	6.48 (2)	6.42
17[e]	*K. pneumoniae*	5.54	5.66	4.93	—	—	—
	E. cloacae	6.37	6.37 (2)	6.47	6.71	6.71	—
18	None[d]						
19	*K. pneumoniae*	4.58	—	—	—	—	—
	E. cloacae	5.66	5.63	5.90	6.00	—	—
20	*E. cloacae*	5.53	5.00	—	6.32	—	5.23
21	*E. cloacae*	4.64	4.77	4.17	—	—	—
22	*K. pneumoniae*	—	—	—	—	—	5.46
	E. cloacae	4.62	4.62	4.62	—	—	4.62
23[e]	*E. cloacae*	X[f]	4.30	—	X	3.32	X
24[e]	*E. cloacae*	X	6.48	X	6.32	4.48	X

[a] —, Petri plates were overgrown with N_2-scavenging bacteria, and no diazotrophs were cultured.
[b] The number of different colonial types detected is given within parentheses.
[c] Unable to distinguish between bacterial species by colonial morphology. Both *E. cloacae* and *K. pneumoniae* were isolated.
[d] No diazotrophs were isolated at lowest dilution.
[e] Plant was cultured immediately after removal from the field.
[f] X, Medium not used.

generally contained fewer N-scavenging bacteria than media incubated in a candle jar. When N_2-fixing bacteria were able to proliferate without competition, they appeared as comparatively large colonies.

MacConkey was not selective for N_2-fixing bacteria, and all colonial types had to be tested for AR activity. Of the colonies tested for AR activity from MacConkey agar, 26% were positive, whereas 78% of the colonies from H & W (pH 7.7) incubated anaerobically were positive.

The population of N_2-fixing bacteria on roots of grasses was enumerated on 21 of 24 soil-root cores collected (Table 2), which indicates the nearly ubiquitous nature of N_2-fixing bacteria on grass roots. Population densities ranged from 1×10^4 to 1×10^7/g of root, with the majority of samples having populations in the range between approximately 1×10^5 and 1×10^6/g of root. Agreement between population sizes for the various media and incubation conditions was generally closer than an order of magnitude when counts were obtained. There were no great differences between populations on roots of plants cultured after incubation in the greenhouse and those cultured shortly after removal

from the field. This is evidence that populations were not increased by damage to roots from the coring device but is not evidence that changes in populations did not occur during a 24-h AR assay incubation time.

A comparison of counts obtained in this study with those obtained by other investigations is not fully acceptable because different techniques were utilized. However, the counts are within the range reported by others (2, 11, 17).

There was a significant Spearman rank correlation coefficient ($P = 0.01$) between AR activity of soil-root cores and the population of N_2-fixing bacteria on roots, but the relationship was not linear. Roots from four of nine plants which showed no AR activity had a population of greater than 1×10^5 N_2-fixing bacteria per g. Apparently, a sizeable population may be present without providing the enzyme activity needed for significant rates of N_2 fixation. These results are supportive of the idea that N_2 fixation is not directly related to population size (4). The method used in our investigation provides evidence against a close relationship between population size and AR activity because both were measured on the entire root system of the core. If bacteria were not evenly distributed in the diluting medium, the method could not accurately evaluate the relationship. Low variances (<10) of counts on quadruplicate plates indicated that the bacteria were evenly distributed (20).

Enterobacter cloacae and *Klebsiella pneumoniae* were most frequently isolated from the roots (Table 2). The frequency of isolation was similar for both the plants cultured directly from the field and those maintained in a greenhouse for several weeks before culturing (Table 2). *E. cloacae* was isolated from 16 cores, and *K. pneumoniae* was isolated from 10 cores. A gram-positive, sporeforming rod identified as a *Bacillus* sp. was isolated from plant 15, and an unidentified bacterium was isolated on malate medium from plant 14. There was no apparent relationship between sampling time, grass species, or soil and the type of N_2-fixing bacterium isolated (Tables 1 and 2).

Biochemical reactions of *K. pneumoniae* agreed well with those listed by Edwards and Ewing (6). Reactions to the Moeller test for ornithine decarboxylase were recorded but were not used in identification of *E. cloacae*. Pilsucki et al. (13) demonstrated that the Moeller test for ornithine decarboxylase is not dependable for *Enterobacter* identification. This became apparent during our study when isolates of bacteria from the same dilution but growing on different media showed identical biochemical reactions for all tests except ornithine decarboxylase. Another unusual result encountered in identification of *E. cloacae* was that 95% of the isolates were raffinose negative, but were expected to be positive (6).

Results of this experiment illustrate the almost ubiquitous association between diazotrophic bacteria and grass roots in a subtropical region. Populations of the bacteria on the roots seem potentially high enough for significant rates of N_2 fixation to occur in a relatively short time when the proper conditions for proliferation occur. Two species of bacteria, *K. pneumoniae* and *E. cloacae*, were the principal diazotrophs isolated from the roots. Grass species, soil pH, or soil texture did not seem to influence the population of diazotrophs. Additional research is needed to determine whether inoculation of grasses will result in the establishment of isolated diazotrophs on the roots and the conditions needed for expression of significant rates of N_2 fixation.

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