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Two new nitrogen-fixing bacteria from the rhizosphere of mangrove trees: Their isolation, identification and in vitro interaction with rhizosphere Staphylococcus sp.

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1. SUMMARY

Two new diazotrophic bacteria, *Listonella anguillarum* and *Vibrio campbellii*, and one non-nitrogen-fixing bacterium, *Staphylococcus sp.*, were isolated from the rhizosphere of mangrove trees. Strains of these newly-defined diazotrophs are known as pathogenic bacteria in fish and shellfish. During the purification of diazotrophic species from the entire rhizosphere population, N_2 -fixation of the bacterial mixtures decreased. When grown in vitro in mixed cultures, the non-fixing bacterium *Staphylococcus* sp. increased the nitrogen-fixing capacity of *L. anguillarum* by 17% over the pure culture; the nitrogen-fixing capacity per bacterial cell increased 22%. This interaction was not due to a change in O_2 concentration. *Staphylococcus* sp. decreased the nitrogen-fixing capacity of *V. campbellii* by 15%.

These findings indicate that (i) other species of rhizosphere bacteria, apart from the common diazotrophic species, should be evaluated for their contribution to the nitrogen-fixation process in mangrove communities; and (ii) the nitrogen-fixing activity detected in. the rhizosphere of mangrove plants is probably not the result of individual nitrogen-fixing strains, but the sum of interactions between members of the rhizosphere community.

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2. INTRODUCTION

Mangroves are trees and shrubs from different botanical families which grow in the tidal zone of tropical and sub-tropical seas [1,2]. Mangrove communities are often located in estuaries which are semi-closed coastal bodies of water receiving fresh water from rivers or streams. These communities are considered highly productive; fallen mangrove leaves, after autolysis and microbial breakdown, produce detritus (plant material converted to dead organic

matter) which is the most important source of energy for the estuarine food chain. Thus mangroves, by introducing sizable quantities of organic material to the community, play an important and essential role in supporting a wide range of offshore marine organisms in the early stages of development, thereby sustaining coastal fisheries [1].

Many marsh systems and probably mangroves, are nitrogen deficient [3]. The decomposition of organic material originating from mangrove foliage, as well as terrestrial and marine animals residing in the community, can be considered a possible source of nitrogen. However, anaerobic decomposition processes in mangroves are very slow and probably contribute little to nitrogen recycling

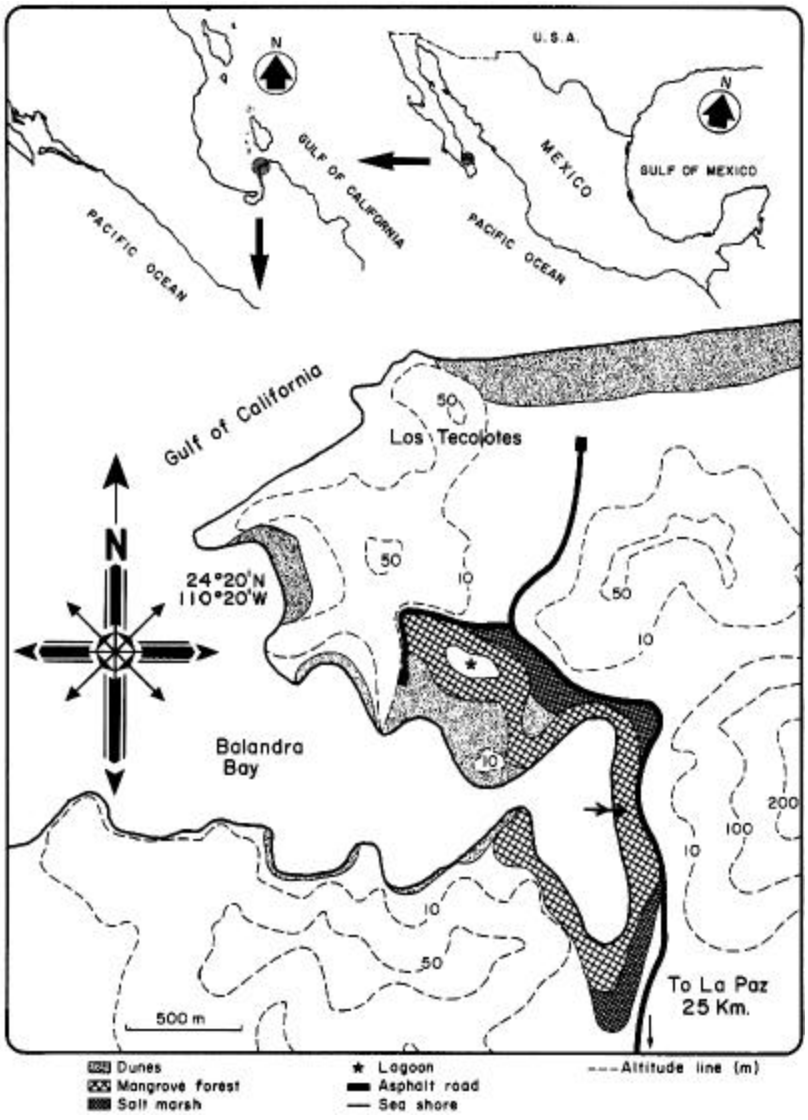

Fig. 1. Location of the mangrove community in Balandra Bay. Double arrows indicate sampling site.

[4]. Although the aerobic decomposition of leaves is quite rapid, and there are many local aerobic decomposition areas in a mangrove community, the dynamic tidal effects cause the leaves, as well as those dissolved nutrients from the soil, to drift from the system. Yet, despite this apparent nitrogen deficiency, mangrove communities appear as 'jungle-like' forests with an abundance of green healthy leaves. Nitrogen-fixation studies of mangrove ecosystems are scarce. However, this activity has been assumed to be widespread in mangrove communities [2]. Bacterial surveys of mangrove sediments have revealed several unidentified diazotrophic bacteria such as: sulfatereducing bacteria (numerically dominant), purple photosynthetic bacteria, blue-green bacteria, and aerobic or facultatively anaerobic heterotrophs. In South Florida mangroves, the rate of root-associated nitrogen-fixation was sufficient to supply much of the nitrogen requirement for plant growth [2]. However, the genera of the nitrogenfixing microbial community associated with mangrove roots have not been defined.

The objectives of this study were: (i) to demonstrate the presence of root-associated diazotrophs in two mangrove species: *Rhizophora mangle* and *Avicennia germinans*, present in a healthy, nonpolluted mangrove community at Balandra Bay, South Baja California, Mexico; (ii) to isolate and identify these diazotrophs; and (iii) to make preliminary, in vitro determinations of existing interactions between diazotrophic and non-diazotrophic rhizosphere bacteria isolated from mangrove roots.

3. **MATERIAL AND METHODS**

3.1. Study site

The mangrove system from which the samples were taken is located in Balandra Bay (Fig. 1). This mangrove community receives no fresh water and was chosen because it is the least disturbed system of La Paz Bay [5]. A community established in the intertidal zone was chosen as the sampling site. Three mangrove species were present in all stages of development: *Rhizophora mangle* L., red mangrove; *Avicennia germinans* (L.) Stern, black mangrove; and *Laguncularia racemosa* Gaertn., white mangrove.

3.2. Isolation of bacteria

Young mangrove plants of two species, *R. mangle* and *A. germinans* were harvested from the study site (Fig. 1, arrows) with the root-sediment ball intact. These were returned to the laboratory, and the adherent sediment was removed from the roots by rinsing the root systems in serial baths of natural seawater. The washed roots were cut into 3-cm segments, washed again in serial baths of sterile natural seawater, and suspended in 0.08 M phosphate buffer solution, supplemented with 0.05 M NaCl, pH 7.2 (phosphate buffer saline solution, PBS) for 3 min. These pieces were then cut into even shorter segments (10 mm) and placed in serum bottles (60 ml) containing 20 ml of modified OAB semisolid (0.05% agar) nitrogen-free medium [6]. OAB medium was further modified and was used as an enrichment and growth medium (HGB). This consisted of three components: (i) (g/890 ml of distilled water, to prepare 1 liter of medium) NaCl, 20.0; $MgS0_4 \cdot 7H_20$, 3.0; $CaCl_2$, 0.02; DL-malic acid, 5.0; NaOH, 3.0; yeast extract, 0.1; (ii) 10 ml of the following stock solution was added to solution (i) and autoclaved: (g/500 ml of distilled water) FeC13, 0.5; $NaMoO_4 \cdot 2H2O$, 0.1; $MnS0_4$, 0.105; H_3BO_3, 0.14; $CuC1_2 \cdot 2H_2O$, 0.0014; $ZnSO_4$, 0.012; (iii) 100 ml of PBS 0.39 M, pH 7.6, autoclaved separately and added to the previous mixture after cooling. The final pH of the HGB medium was 7.2. The inoculated bottles were incubated for 5 days at 25 ± 2°C and tested for nitrogenase activity by the acetylene reduction assay [7].

High turbidity areas in bottles showing positive acetylene reduction activity (ARA) were sampled and the bacteria were spread on HGB medium and incubated at 25 ± 2°C for 3 days. Thirteen different colonial morphotypes were detected. To determine whether these colonial morphotypes were truly nitrogen-fixers, they were further tested for ARA. Plates were spread from bottles which showed high ARA until pure cultures were obtained. Two colonial morphotypes were verified as diazotrophs: The

conditions of growth and incubation were the same throughout the purification procedure. A non-fixing bacterium which was originally growing together with the nitrogen-fixers was purified and grown in nutrient agar (Merck) supplemented with 2% NaCl. All isolates, including diazotrophs, were stored in slants at 4°C for further characterization and identification.

3.3. Characterization and identification of the diazotrophic isolates

The cellular morphology of the pure isolates was determined with light microscopy (Zeiss). The shape, dimensions and motility of the bacteria, in addition to their being Gram-negative, oxidase-positive and capable of fermenting glucose, led to the assumption that the two diazotrophs probably belonged to the genus *Vibrio*. Therefore, standard biochemical tests for this genus were performed as follows: (1) catalase; (2) gas from D-glucose; (3) acid from: (i) glucose, (ii) sucrose, (iii) lactose, (iv) rhamnose, and (v) raffinose; (4) growth in 0%, 2% and 3% NaCl; (5) reduction of NO_3 to NO_2 ; (6) Voges-Proskauer test; (7) hydrolysis of gelatin; and (8) production of lipase [8-12]. Species identification was done by FAME analysis through gas chromatography of cell fatty acid methyl esters that have a chain length between 9 and 18 carbons long [13]. FAME analysis was carried out as a commercial service by Dr. J.W. Kloepper's laboratory, Auburn University, Alabama, USA.

3.4. Determination of the type of interaction existing between the nitrogen fixing bacteria and the non-fixing bacteria

The nitrogen-fixing isolates were grown in liquid HGB medium for 12 h in 125-ml Erlenmeyer flasks under agitation of 150 rpm at 25°C. One ml of culture was inoculated in 60-ml serum bottles sealed with cotton stoppers containing 13 or 15 ml of semi-solid (0.05% agar) HGB medium and incubated at 25 ± 2°C for 48 h. The non-fixing isolate was cultured separately in nutrient broth supplemented with 2% NaCI (Merck). After 96 h, the culture was washed three times at 4°C, 1700 x *g* for 12 min each time with PBS. The optical density of the bacterial culture was adjusted to 1.8 at 540 nm with PBS, and the concentration of bacteria was determined by the dilution plate count method on nutrient agar (6 x 10^8/ml). Two ml of the bacterial suspension were added to the bottles containing 14 ml of the 48-hour-old culture of the nitrogen-fixing isolates. Bottles containing 16 ml of pure culture of diazotrophs were used as control. All the bottles were incubated for an additional 72 h at the same temperature but without movement, after which ARA was performed.

After the acetylene reduction assay, duplicate viable plate counts of pure and mixed cultures were made: on solid HGB medium for pure cultures, and on nutrient agar supplemented with 2% NaCl for mixed cultures.

3.5. Acetylene reduction assay

The cotton stoppers of the serum bottles were replaced with rubber stoppers, and 1 ml of air was removed from the bottles with a syringe. One ml of acetylene (0.1 atm) was injected into the bottles which were then incubated for 3 or 24 h. One ml samples were injected into the gas chromatograph.

Ethylene analysis was accomplished by gas chromatography using a Varian 6000 gas chromatograph (Varian Instrument Group, USA) equipped with a hydrogen flame ionization detector (FID). Instrument operating conditions were as follows: a stainless-steel column 150 x 0.2 cm packed with Porapak N, 80/100, a column temperature of 60°C, an injector temperature of 50°C, a detector temperature of 200°C, N_2 carrier gas and H_2 at a flow rate of 25 ml/min, and air flow rate of 300 ml/min. The amount of ethylene was expressed as nmol ethylene per time unit. In the case of *L. anguillarum*, the amount of ethylene produced by a single cell was calculated by dividing the total amount of ethylene produced per 16 ml of culture, by the number of cells present in the culture (this was calculated by the viable plate count method).

3.6. Determination of oxygen concentration in the medium

The oxygen concentration was measured in pure and mixed cultures immediately after measuring the ARA

Table 1

Production of ethylene per type of culture

Mangrove species	Enrichment culture (nmol ethylene 3 h^{-1} 16 ml^{-1} culture)	Bacterial species	Pure culture (nmol ethylene 24 h^{-1} 16 ml^{-1} culture) [a]
R. mangle roots	6048 ± 456	*Listonella anguillarum*	3626 ± 72.5
A. germinans roots	6512 ± 330	*Vibrio campbellii*	1441 ± 28.3

[a] 24-h incubation period for ARA determination was required because there was no measurable ARA after 3 h.

with an oxygen meter, nodel 54 ARC (Yellow Springs Instruments, USA) equipped with an oxygen probe, model 5775. As the probe did not fit into the serum bottles, the bacterial suspension was carefully transferred to 20-ml vials. No air was allowed between probe and the suspension. The amount of oxygen incorporated into the suspension after transferring, was found to be unsignificant (data not presented).

3.7. Determination of soluble organic nitrogen

Samples were taken of mangrove seawater and of the sediment surrounding the roots. Ten ml of sediment were diluted in 100 ml of seawater, agitated for 5 min and allowed to settle. The supernatant was decanted. Both the seawater and the supernatant obtained after washing the sediment were filtered separately until the water appeared transparent. Ammonia and soluble organic nitrogen in mangrove sediment and seawater, were determined as described by Strickland and Parsons [14].

Table 2

Biochemical and physiological characteristics of N_2-fixing bacteria isolated from mangrove roots

Test	Bacterial species	
	Listonella anguillarum	*Vibrio campbellii*
Gram stain	−	−
Oxidase	+	+
Catalase	+	+
Gas from glucose	−	−
Acid from:		
Glucose	+	+
Sucrose	+	+
Lactose	−	+
Raffinose	−	−
Rhamnose	+	−
Growth in % NaCl		
0%	+	+
2%	+	+
3%	+	+
Reduction of NO_3^-	+	+
Voges-Proskauer test	−	−
Hydrolysis of gelatin	−	−
Production of lipase	−	−
Aggregates in liquid N_2 free medium	−	+

3.8. Experimental design and statistical analysis

Treatments were replicated three times and experiments were carried out at least twice. Results presented are the means of all replicates. Significance between treatments was determined by Student's t-test analysis at $P \leq 0.05$. The acetylene reduction assay for the enrichment culture was performed once with 5 replicates and the analysis is presented as standard error.

4. RESULTS

4.1. Isolation *of nitrogen fixing bacteria from mangrove roots*

The assay showed high acetylene reduction activity in all the enrichment cultures (Table 1.) After spreading on agar plates, seven different colony types were obtained from *A. germinans* and six from *R. mangle*. Of all the colonies tested, two exhibited ARA (one from each mangrove species), although the values were lower than for the original enrichment cultures (Table 1). These two putative N_2-fixers were plated again and subsequently purified. They were named BALRHI 9010 (isolated from *Rhizophora* roots) and BALAVI9010 (from *Avicennia* roots). During these purification processes the cultures gave rise to a

different colony type which showed no nitrogenase activity. This isolate, BALRA9010, was isolated from the roots of both mangrove species. The pure nitrogen-fixing isolates exhibited no ARA after 3 h and were therefore incubated another 24 h until ARA was detected (Table 1). The soluble organic nitrogen in mangrove seawater and washed sediment was found to be 6.3 µg N/1 and 6.7 µg N/1, respectively. The ammonia was found to be 3.8 µg/l in mangrove seawater and 5 µg/1 in the sediment.

4.2. Characterization and identification of the bacterial isolates

BALAV19010 and BALRH19010 were characterized by standard biochemical tests (Table 2). The cellular morphology and colonial morphotype of the two diazotrophic isolates and the non-fixing isolate were as follows: cells of the isolate BALAV19010 were straight rods, motile, 1-2 µm in length X 0.5 ± 0.2 µm in diameter, grouped in pairs but mostly occurring singly. The colonies grown on solid HGB medium were transparent, 1-2 mm in diameter, irregular and glistening. It formed aggregates in liquid nitrogen-free medium, and was identified as *Vibrio campbellii* in FAME analysis with a match of 0.4 and a difference from the second choice of more that 0.1; these results mean the identification can be accepted as accurate with reasonable assuredness.

Cells of the isolate BALRH19010 were: straight or curved rods, motile, 1-2 µm in length x 0.6 ± 0.2 µm in diameter, grouped in pairs but mostly occurring singly. The colonies grown in HGB medium were transparent, 1-2 mm in diameter, irregular and glistening. It was identified as Lis*tonella anguillarum* in FAME analysis with a match of 0.55 and a difference from the second choice of more than 0.1; these results make the identification accurate with reasonable assuredness.

The two species of diazotrophs are, in fact, closely related, even if they are currently considered to belong to two different genera (see DISCUSSION).

Cells of BALRA9010 were Gram-positive cocci occurring singly, in pairs, in tetrads and in clusters. Colonies grown on nutrient agar + 2% NaCl were cream colored, 0.5-1.0 mm in diameter, circular, entire and glistening. BALRA9010 showed no ARA and was identified as a species belonging to the genus *Staphylococcus* in FAME analysis. The match obtained (0.3 with a difference of less than 0.1 from

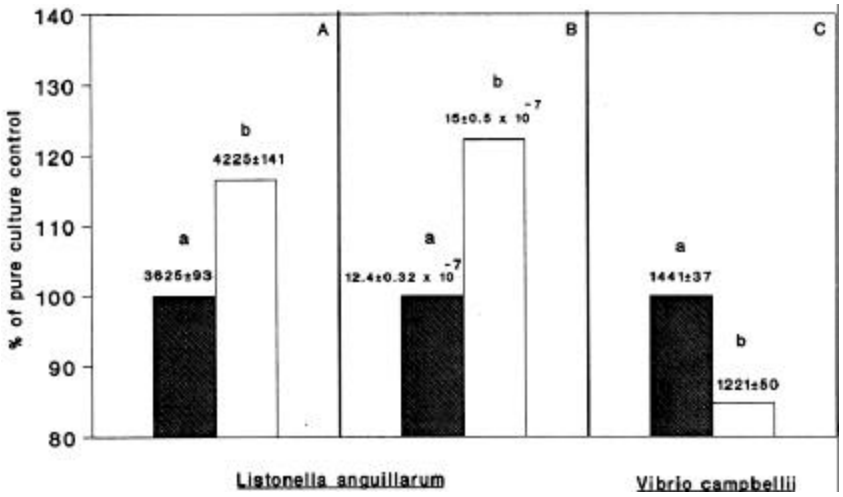

Fig. 2. (A) Percentage of ethylene produced per 16 ml of culture of *L. anguillarum* in 24 h. Numbers above the bars represent the amount of ethylene produced by the corresponding culture expressed in nanomoles per 24 h (**A**), and per cell (**B**). (**C**) Percentage of ethylene produced per 16 ml of culture of *V. campbellii* in 24 h. ! , pure cultures; ' , mixed cultures with the non-N_2-fixing bacteria. The amount of ethylene produced by the control was considered to be 100%. Results are the means of three identical experiments. Bars denoted by a different letter differ significantly at a confidence interval of 95% (Student's *t*-test).

the second choice) was not sufficient for identification at the species level.

4.3. Determination of the type of interaction existing between the nitrogen fixing species and Staphylococcus sp.

When *Staphylococcus sp.* was grown in mixed culture with *Listonella anguillarum,* the ARA increased 17% over the control (Fig. 2A). ARA per cell in mixed culture was calculated based on viable counts and showed an increase of 22% over control (Fig. 2B). The bacterial counts showed the population of *L. anguillarum* to be similar in both mixed (1.83 X 10^8/ml) and pure cultures (1.75 X 10^8/ml). When *Staphylococcus* sp. was grown in mixed culture with V. *campbellii,* the ARA of the mixed culture was lower than that of the control (Fig. 2C). It was not possible to make bacterial counts of *V. campbellii* because it forms massive aggregates when grown in a N-free medium. Mild sonication to disrupt the aggregates was not tested because it is not known how such procedures affect cell viability. Thus, the amount of ethylene produced per bacterial cell was not calculated.

When *Staphylococcus sp.* was grown in a mixed culture with *V. campbellii* the decrease in oxygen concentration was significant. However, when it was grown in a mixed culture with *L. anguillarum,* the decrease in oxygen was not significant (Fig. 3). No growth of *Staphylococcus sp.* was detected in nitrogen-free medium or in nutrient agar supplemented with 2% NaCl when the mixed cultures were streaked on the agar plates for performing bacterial counts. However, it was possible to count it on nutrient agar (2% NaCl) when grown in pure culture.

5. DISCUSSION

The high ARA shown by the enrichment cultures of roots showed that there were root-associated, nitrogen-fixing bacteria in mangrove roots. Zuberer [2] found 5-20 µg NH_4 /1 in mangrove seawater and traces of nitrate and nitrite. Balandra Bay seawater and sediments had similar values, far below the 40-160 µg NH_4/g reported as inhibitory to nitrogenase activity. Where mangroves grow, the availability of nitrogen in seawater and sediment is apparently poor. In our study, the presence of root-associated, nitrogen-fixing bacteria in mangrove roots may give a clue to the well-being of the tested community. N_2-fixation in mangrove communities has been suggested to be significant to the well-being of a mangrove system, especially in areas of limited nitrogen [2].

N_2 fixation has been reported in vibrios [12] as well as in some marine vibrios [15,16]. However

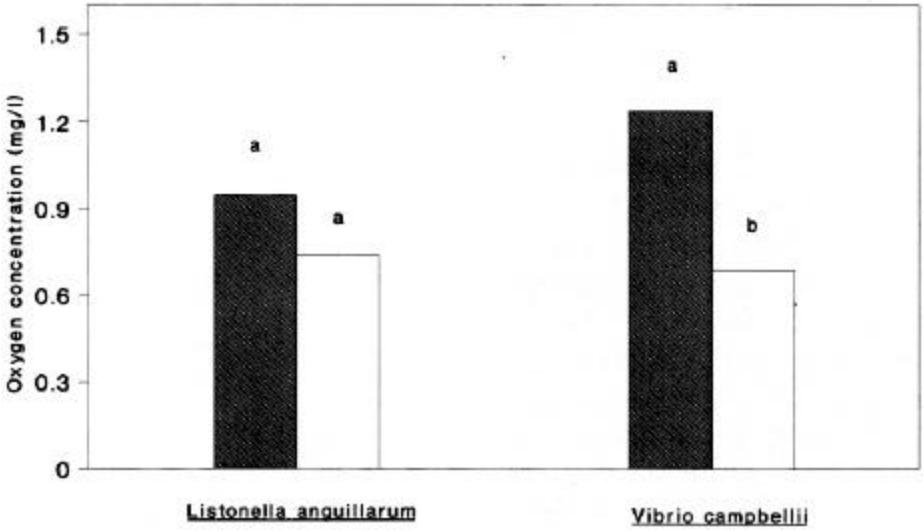

Fig. 3. Oxygen Concentration (mg/ml) in ! pure and ' mixed cultures. Bars denoted by a different letter differ significantly at $P \leq 0.05$ (Student's *t*-test).

this ability has not been reported for *V. campbel*lii or *L. anguillarum* [17]. *Listonella anguillarum* appears as *Vibrio anguillarun* in Bergey's Manual of Systematic Bacteriology *(1984)* [17]. Later, Nearhos and Fuerst [18] proposed the incorporation of V. *anguillarum* into the new genus *Listonella.*

V. anguillarum is a pathogen of marine fishes and eels and a major cause of disease in fish culture [17]. *V. campbellii is* better known as a pathogen for shellfish. Its presence in high numbers is considered a potential public health hazard in Hong Kong and other sub-tropical Asian countries [19]. This study shows these species as also having the ability to fix nitrogen under aerobic conditions. While the strains of *V. campbellii* and *L. anguillarum* obtained from the rhizosphere of mangroves appear to be beneficial to the mangroves of our study, it is still unclear whether they are pathogenic to the fish and shellfish.

Bacteria belonging to the genus *Staphylococ*cus have been found attached to leaves, rhizomes and roots of the seagrass *Halophila ovalis* [20]. Members of the same bacterial genera have been isolated from marine and estuarine surface water samples [21] and cause disease in cultured fish. It has also been shown that these isolates are different from human isolates [22]. *Staphylococcus sp.* has been isolated from shrimp as well [23].

During the purification procedure, we observed a decrease in ARA. This phenomenon has already been reported for terrestrial diazotrophs [24-27]. In some cases, ARA was completely lost. A possible explanation may be the need for different bacteria to associate in order to create suitable conditions for N_2 fixation [25]. It has been speculated that dinitrogen-fixers grow best in the presence of other heterotrophic bacteria which may stimulate the nitrogen-fixers by physical and/or biochemical activities, thus receiving the required N compounds from the dinitrogen-fixers [28]. Alternatively, this association may reduce the oxygen tension in the medium, thus facilitating N_2 -fixation. With *Azospirillum*, N_2 fixation has been found to be highest at low oxygen concentrations [29]. We demonstrated that the non-fixing bacteria increased the nitrogen-fixing
capacity of *L. anguillarum* when grown in mixed culture. However, we obtained this increase in N_2-fixation in spite of the non-significant decrease in oxygen tension of the mixed culture.

N_2-fixation by *V. campbellii,* was reduced in the presence of the non-fixer. The lowering of the oxygen tension in the mixed culture did not result in an increase in the nitrogen-fixing efficiency as expected. The aggregative nature of *V. campbellii* when grown in N-free medium restricted us from making direct bacterial counts; consequently, we were unable to determine if the reduction in the ARA values was an outcome of a decrease in the concentration of either fixing or non-fixing bacteria in the mixed culture. This reduction was not apparent in the ARA values obtained for the enrichment culture. There are infinitely more bacterial species for the diazotrophs to interact with, therefore it is possible to hypothesize the existence of both positive and negative interactions for N_2-fixation in the mangrove rhizosphere.

This study was restricted to diazotrophs capable of utilizing malate as sole carbon source. By diversifying the carbon source, or by using a combined carbon source medium [30], other diazotrophs may be discovered and their potential interaction with each other should be studied.

We conclude that (i) other rhizosphere bacterial species, apart from the common diazotrophic species, should be evaluated for their contribution to the nitrogen-fixation process in mangrove communities; and (ii) the nitrogen-fixing activity that takes place in the rhizosphere of mangrove plants is probably not the result of individual nitrogen-fixing strains, but the sum of the interactions between members of the rhizosphere community.

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REFERENCES

[l] Clough, B.F. (1982) Preface. In: Mangrove Ecosystems in Australia: structure, function and management, (Clough, B.F., Ed.), p. xv. Colorcraft Ltd, Hong Kong.

[2] Zuberer, D. (1976) Biological dinitrogen-fixation associated with Florida mangroves. Ph.D Thesis. University of South Florida. pp. 172.

[3] Zuberer, D. and Silver, W.S. (1978) Biological dinitrogen fixation associated with Florida mangroves. Appl. Environ. Microbiol. 35, 567-575.

[4] Boto, K.G. (1982) Nutrient and organic fluxes in mangroves. In Mangrove ecosystems in Australia: structure, function and management, (Clough, B.F., Ed.), pp. 239 246. Colorcraft, Hong Kong.

[5] Gallo, J.P., Maeda, A., and Maravilla, O. (1980). Mangrove systems of The Bay of La Paz as exploitable resources. Memories of the second international workshop on biosalinity, La Paz, B.C.S. Mexico. Plenum Press.

[6] Okon, Y., Albrecht, S.L., and Burris, R.H. (1977) Methods for growing *Spirillum Lipoferum* and for counting it in pure culture and in association with plants. Appl. Environ. Microbiol. 33, 85-88.

[7] Hardy, R.W., Holsten, R.D., Jackson, E.K. and Burns, R.C. (1968) The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. Plant Physiol. 43, 1185-1207.

[8] Baumann, P. and Baumann, L. (1981) The marine Gram-negative eubacteria: genera *Photobacterium, Beneckea, Alteromonas, Pseudomonas* and *Alcaligenes.* In: The prokaryotes: a handbook on habitats, isolation and identification of bacteria. (Starr, M.P. and Stolp, H. Eds.), pp. 1302-1331. Springer Verlag, New York.

[9] Guerinot, M.L. and Colwell, R.R (1985) Enumeration, isolation and characterization of N_2 -fixing bacteria from seawater. Appl. Environ. Microbiol. 50, 350-355.

[10] Lee, J.V., Hendrie, M. and Shewan, J.M. (1979) Identification of *Aeromonas, Vibrio* and related organisms. In: Identification methods for microbiologists (Skenner, F.A. and Lovelock, D.W., Eds.), pp. 151-165. Academic Press, London.

[11] Wung-Yang, S., and Maruyama, Y. (1989) Nitrogen-fixation by marine agar-degrading bacteria. J. Gen. Microbiol. 134, 1821-1825.

[12] Urdaci, M.C., Stal, L.J., and Marchand, M. (1988) Occurrence of nitrogen-fixation among *Vibrio spp.* Arch. Microbiol. 150, 224-229.

[l3] Sasser, M. (1990) Identification of bacteria through fatty acid analysis. In: Methods in Phytobacteriology (Klement, Z., Rudolph, K. and Sands, D.C., Eds.), pp. 199204. Akademiai Kiado, Budapest.

[14] Strickland, J., and Parsons, T.R. (1977) Determination of soluble organic nitrogen. In: A practical handbook of seawater analysis. (Stevenson, J.C. and Watson, J., Eds.), pp. 82-85, 143-147. Alger Press, Canada.

[15] Shieh, W.Y., Simidu, U., and Maruyama, Y. (1987) Isolation of a nitrogen-fixing *Vibrio* species from the roots of eelgrass *(Zostera marina).* J. Gen. Appl. Microbiol. 33, 321-330.

[16] Guerinot, M.L., West, P.A., Lee, J.V., and Colwell, R.R. (1982) *Vibrio diazotrophicus sp.* nov., a marine nitrogenfixing bacterium. Int. J. Syst. Bacteriol. 32, 350-357.

[17] Baumann, P., Furniss, A.L. and Lee, J.V. (1984) Genus Vibrio. In: Bergey's manual of systematic bacteriology, Vol. 1. (Krieg, N.R and Holt, J., Eds.). Williams and Wilkins, Baltimore, MD.

[18] Nearhos, S.P., and Fuerst, J.A. (1987) Reanalysis of SS r RNA sequence data for the Vibrionaceae with the CLUSTAN program suite. Curr. Microbiol. 15, 329-335.

[19] Chan-Kwong, Y., Woo, M.L., Lam, L.Y. and French, G.L. (1989) *Vibrio parahaemolyticus* and other halophilic Vibrios associated with seafood in Hong Kong. J. Appl. Bacteriol. 66, 57-64.

[20] Wahbeh, M.L, and Mahasneh, A.M. (1984) Heterotrophic bacteria attached to leaves, rhizomes and roots of three seagrass species from Aqaba (Jordan). Aquat. Bot. 20, 87-96.

[21] Gunn, B.A., and Colwell, R.R. (1983) Numerical taxonomy of *Staphylococci* isolated from the marine environment. Int. J. Syst. Bacteriol. 33, 751-759.

[22] Sugiyama, A., and Kusuda, R. (1981) Studies on the characters of *Staphylococcus epidermis* isolated from diseased fishes. 3. comparative studies of the serological properties between the isolates and the strains of human origin. Fish Pathol. 16, 43-49.

[23] Beckers, H.J., Van Leusden, F.M. and Tips, P.D. (1985) Growth and enterotoxin production of *Staphylococcus aureus* in shrimp. J. Hyg. 95, 685-693.

[24] Balandreau, J. (1986) Ecological factors and adaptive processes in N_2 fixing bacterial populations of the plant environment. Plant Soil. 90, 73-92.

[25] Lindberg, T., and Granhall, U. (1984) Isolation and characterization of dinitrogen-fixing bacteria from the rhizosphere of temperate cereals and forage grasses. Appl. Environ. Microbiol. 48, 683-689.

[26] Eskew, D.L., Focht, D.D., and Ting, LP. (1977) Nitrogen fixation, denitrification, and pleomorphic growth in a highly pigmented Spirillum lipoferum. Appl. Environ. Microbiol. 34, 582-585.

[27] Lethbridge, G., Davidson, M.S., and Spading, G.P. (1982) Critical evaluation of the acetylene reduction test for estimating the activity of nitrogen-fixing bacteria associated with the roots of wheat and barley. Soil Biol. Biochem. 14, 27-35.

[28] Tyler, M.E., Milam, J.R., Smith, R.L., Schank, S.C., and

Zuberer, D.A. (1979) Isolation of *Azospirillum* from diverse geographic regions. Can. J. Microbiol. 25, 693-697.

[29] Hurek, T., Reinhold, B., Niemann, E.G., and Fendrik, I. (1988) N_2-dependent growth of *Azospirillum* spp. in batch cultures at low concentrations of oxygen. *Azospirillum* 4: Genet. Physiol. Ecol. (Klingmüller, W., Ed.) pp. 114-121. Springer-Verlag Berlin.

[30] Rennie, R.J. (1981) A single medium for the isolation of acetylene reducing (dinitrogen-fixing) bacteria from soils. Can. J. Microbiol. 27, 8-14.