J. Exp. Mar. Biol. Ecol., 163 (1992) 31–41

JEMBE 01837

Nitrogen fixation (acetylene reduction) associated with the zoanthid *Palythoa tuberculosa* Esper

W. Y. Shieh and Y. M. Lin

Institute of Oceanography, National Taiwan University, Taipei, Taiwan, ROC

(Received 16 December 1991; revision received 5 June 1992; accepted 11 June 1992)

Abstract: Nitrogen fixation by the zoanthid *Palythoa tuberculosa* Esper was determined by the acetylene reduction technique. The samples were collected from Nanwan Bay of Kenting National Park in Taiwan during the period of September 1990 to January 1991. More than 70% of zoanthid samples (10 of 14) showed detectable levels of nitrogenase activity within 16 h of incubation under either aerobic or anaerobic conditions. Most of the zoanthid-associated nitrogen-fixing bacteria were probably distributed on the surface rather than inside the zoanthid tissues. Sixty-one strains in total of marine nitrogen-fixing bacteria belonging to the family Vibrionaceae were isolated from selected zoanthid samples that showed nitrogenase activity. All isolates utilized the combined nitrogen sources of NH_4Cl and yeast extract aerobically and anaerobically, but they fixed N_2 as the sole nitrogen source only under anaerobic conditions. The greatest levels of nitrogenase activity of these isolates were detected at 1–3% NaCl and at 0.5–2% glucose (as a sole source of carbon and energy).

Key words: N fixation; N-fixing bacterium; *Palythoa tuberculosa*

Introduction

Heterotrophic nitrogen-fixing bacteria are taxonomically diverse, including obligate aerobes, obligate anaerobes, facultative anaerobes and microaerophiles (Shieh, 1988). They require organic compounds in large amounts as energy sources for enhancement of nitrogen fixation. It is estimated that these bacteria require at least 1 g of glucose to fix 10–20 mg of molecular nitrogen (Postgate, 1982). For this reason, heterotrophic nitrogen-fixing bacteria inhabiting marine environments would not be active under free-living conditions because of the limited organic carbon content. The organic carbon content including the dissolved and suspended forms in seawater is not more than 1.3 $mg \cdot l^{-1}$ in offshore areas such as in Sagami Bay, Japan and the Kuroshio region of the Pacific Ocean (Kogure et al., 1980). It is rarely greater than 6 $mg \cdot l^{-1}$ even in the eutrophic area like Tokyo Bay of Japan (Kogure et al., 1980). According to several reports, the restriction of nitrogen fixation in sea water to sediments of various sources is due to insufficient organic substrate (Maruyama et al., 1974; Herbert, 1975; Zuberer & Silver 1978; Shieh et al., 1989a). Development of associative relationships with

Correspondence address: W. Y. Shieh, Institute of Oceanography, National Taiwan University, PO Box 23–13, Taipei, Taiwan, ROC.

marine animals or plants would be favorable for marine nitrogen-fixing bacteria to express nitrogenase activity. As a consequence of this association, nitrogen-fixing bacteria can directly utilize exudates and/or cell debris of the associated partners. Indeed, various plants living in estuarine and coastal regions have been demonstrated to have nitrogenase activities which originated from associated heterotrophic bacteria. These plants include several species of seagrass (Patriquin & Knowles, 1972; Capone & Budin, 1982; Shieh et al., 1989a) and mangrove (Zuberer & Silver, 1978; Hicks & Silvester, 1985), the marsh grass *Spartina alterniflora* (Patriquin & Denike, 1978; Patriquin & McClung, 1978), and the macroalga *Codium fragile* (Head & Carpenter, 1975). In marine animals, direct association of nitrogen-fixing bacteria with the gastrointestinal tracts of sea urchins was reported and it was demonstrated that microbially fixed nitrogen can be a source of nitrogen for the sea urchins (Guerinot & Patriquin, 1981). An unidentified bacterial species capable of both nitrogen fixation and cellulose degradation under microaerophilic conditions has been isolated from a specialized gland found in shipworms. The symbiosis between this nitrogen-fixing bacterium and the shipworms may allow the shipworms to use wood as their principal food source (Waterbury et al., 1983). Species of marine animals are abundant. Little is known, however, about the association of heterotrophic nitrogen-fixing bacteria with marine animals except the sea urchin and the shipworm. Nitrogenase activities detected from the other marine animals including sea squirts (Paerl, 1984), corals (Williams et al., 1987), and sponges (Wilkinson & Fay, 1979) have been attributed to autotrophic nitrogen-fixing microorganisms (cyanobacteria or photosynthetic bacteria).

Palythoa tuberculosa Esper, a colonial zoanthid species found in warm temperate zone waters, is known to be one of the most toxic marine organisms producing palytoxin (Hirata et al., 1979). Nitrogen fixation by heterotrophic bacteria associated with this invertebrate is a common phenomenon. We here present evidence of this phenomenon, and also describe the isolation and characterization of the zoanthid-associated nitrogen-fixing bacteria.

Materials and Methods

Collection and Preparation of Samples

Nanwan of Kenting National Park is a bay located on the south coast of Taiwan, in which fringing reefs are widely distributed and abundant species of marine invertebrates including the zoanthid *Palythoa tuberculosa* are found. From the shallow water regions of the bay, the colonies of *P. tuberculosa* were collected in September, October and November 1990, in January 1991, and in April 1992. Assays for nitrogenase activity were set up within a few hours of sample collection. Either the zoanthid colonies were used directly after washing gently in sterile sea water for 1 min or they were surface-sterilized in 70% ethanol for 20 s, followed by rinsing in 3 changes of sterile sea water. Approximately 2 g wet mass of each zoanthid sample was placed in a 25-ml

flask; to each flask was added 2.5 ml of seawater buffer (20 mM Tris in 80% sea water, pH 8.0) or the same buffer containing glucose at a concentration of 0.5%. All flasks were sealed with rubber stoppers.

A 5 g wet mass of fresh zoanthids, after being gently rinsed in sterile sea water, was shaken vigorously in 50 ml of sterile NaCl-Tris buffer (30 g NaCl and 0.24 g Tris in 1 l of deionized water; pH 8.0) containing Tween 80 (2 ppm). This shaken solution (zoanthid-surface washed water) was used for bacterial enumeration. Similar shaking treatment was also performed in nitrogen-free liquid (NFL) medium containing neither glucose nor dulcitol (see BACTERIAL MEDIA). This zoanthid-surface washed NFL medium was filtered through a 0.2-μm membrane filter (Schleicher & Schuell, Germany) to remove bacteria. The filtrate was used to determine the effects of zoanthid-derived organic matter on growth and nitrogenase activity of nitrogen-fixing isolates.

MEASUREMENT OF NITROGENASE ACTIVITY

Assays of nitrogenase (acetylene reduction) activity associated with the zoanthid samples were performed under either aerobic or anaerobic conditions. Anaerobic conditions were established by flushing the flasks for 5 min with argon gas. Time courses were initiated after acetylene (2.0 ml; ca. 0.088 mmol) was injected into each flask using a gas-tight syringe. Both aerobic and anaerobic samples were incubated at 25 °C in the dark. Gas samples (0.2 ml) were withdrawn at 4-h intervals up to 16 h with a gas-tight syringe. They were analyzed for ethylene and acetylene using a gas chromatograph (Shimadzu GC-14A) equipped with a flame ionization detector (FID) and with connected columns of Porapak Q and N (both 3 mm × 2 m) at 70 °C. Helium was used as the carrier gas at a flow rate 40 ml·min^{-1}. Control samples incubated under air or Ar in the absence of acetylene produced no ethylene after incubation for 1 day. To measure the nitrogenase activity of the nitrogen-fixing isolates, cells cultivated in glucose NFL medium (see BACTERIAL MEDIA) under N_2 were harvested in the early stationary phase of growth. The cells were washed with NaCl–Tris buffer and then resuspended in NFL medium (5 ml) in 25-ml test tubes. Turbidity of the cell suspensions was adjusted to absorbance 0.2–0.3 (at 600 nm). After sealing with rubber stoppers, all air in each tube was replaced by Ar; then acetylene (2.0 ml) was injected. Incubation was performed at 25 °C in the dark; gas samples (0.2 ml) were removed from the tubes every 4 h and analyzed for the amount of ethylene produced. After the measurement of acetylene reduction, both the zoanthid samples and cells of nitrogen-fixing isolates were dried in an oven at 90 °C for 2–3 days and weighed. Specific nitrogenase activity was expressed as nmol C_2H_4·g dry wt^{-1}·h^{-1} for the zoanthid samples and nmol C_2H_4·mg dry wt. cells^{-1}·h^{-1} for the nitrogen-fixing isolates, respectively.

BACTERIAL MEDIA

Nitrogen-free liquid (NFL) media used for the enrichment of nitrogen-fixing bacteria consisted of the following 4 parts: (i) basal medium (NaCl, 25 g; $MgSO_4 \cdot 7H_2O$, 5 g;

$CaCl_2$, 10 mg; $Na_2MoO_4 \cdot 2H_2O$, 7.3 mg; Tris, 6.1 g; deionized water, 800 ml) adjusted to pH 8.0; (ii) glucose or dulcitol (5 g) dissolved in deionized water (100 ml); (iii) K_2HPO_4 (0.2 g) dissolved in deionized water (50 ml); (iv) $FeCl_3 \cdot 6H_2O$ (13.5 mg) dissolved in deionized water (50 ml). The four parts were autoclaved separately and combined after cooling to room temperature. Polypepton-yeast (PY) broth consisted of Polypepton (Daigo, Tokyo, Japan, 2 g) and Bacto-yeast extract (Difco, 0.5 g) in 80% sea water (1 l). The pH was adjusted to 7.6. Bacto-agar (Difco) was added to this medium for the preparation of semisolid (3 $g \cdot l^{-1}$) and agar (15 $g \cdot l^{-1}$) media.

ISOLATION AND CHARACTERIZATION OF NITROGEN-FIXING BACTERIA

Zoanthid samples that showed nitrogenase activities under either aerobic or anaerobic conditions were selected for the isolation of nitrogen-fixing bacteria. Each sample, together with the supplemented solution, was put into sterile NaCl-Tris buffer (17.5 ml) containing Tween 80 (2 ppm) and then shaken vigorously for 3 min. This shaken solution (zoanthid-surface washed water; 1 ml) was serially diluted with sterile NaCl-Tris buffer (9 ml). A volume (1 ml) of each dilution (10^4–$10^7 \times$) was transferred to a rimless tube (18 x 150 mm) containing glucose or dulcitol NFL medium (5 ml). All culture tubes were set in anaerobic jars (Difco) and incubated at 25 °C in the dark for 5 days. The air in each anaerobic jar was replaced by N_2 prior to incubation. Aerobic enrichment cultivation was not performed, since preliminary experiments have indicated that nitrogen-fixing bacteria were not significantly enriched in the cultures incubated under air. One loopful of the anaerobic cultures that developed marked turbidity were transferred to fresh (glucose or dulcitol) NFL medium (5 ml) for a second anaerobic enrichment cultivation. For the isolation of nitrogen-fixing bacteria, one loopful of the cultures with positive growth in the second enrichment were smeared onto PY agar plates, and the plates were incubated aerobically at 25 °C for 2–3 days. One to two predominant types of colonies appearing on the plates were plucked off and purified by successive streaking on PY agar plates. The isolates that again showed significant growth in glucose NFL medium under N_2 were maintained in PY semisolid medium and stored at 20 °C.

Characterization of the nitrogen-fixing isolates was performed according to the methods previously described by Shieh et al. (1988a).

GROWTH AND NITROGENASE ACTIVITY OF NITROGEN-FIXING ISOLATES IN ZOANTHID-SURFACE WASHED MEDIUM

The nitrogenase activity of nitrogen-fixing isolates suspended in the 0.2-μm-filtered zoanthid-surface washed NFL medium (see COLLECTION AND PREPARATION OF SAMPLES) was measured by the method described in MEASUREMENT OF NITROGENASE ACTIVITY. The cell suspensions of nitrogen-fixing bacteria in the same filtrate were also used for growth studies. Their turbidity, however, was adjusted to absorbance 0.02–0.03 (at 600 nm) instead of 0.2–0.3 for measurement of nitrogenase activity.

BACTERIAL COUNTS

The shaken solution (see COLLECTION AND PREPARATION OF SAMPLES) was decimally diluted with NaCl-Tris buffer containing Tween 80 (2 ppm). A volume (1 ml) of each dilution was transferred to a rimless tube (18 x 150 mm) containing either glucose NFL or PY broth medium (5 ml). The glucose NFL and PY broth media were used for the enumeration of nitrogen-fixing bacteria and heterotrophic bacteria, respectively. All cultures were then incubated at 25 °C in the dark for 7 days either under air (for the enumeration of heterotrophic bacteria) or under N_2 (using anaerobic jars; for the enumeration of nitrogen-fixing bacteria) conditions. The criterion used to distinguish a positive tube for estimation of most-probable-number (MPN) was the development of visible turbidity. The MPN of the nitrogen-fixing bacteria (facultative anaerobes only) and heterotrophic bacteria (both aerobes and facultative anaerobes), which were associated with the zoanthid surface was estimated by three-tube inoculation method described elsewhere (Shieh et al., 1989b).

RESULTS AND DISCUSSION

Table I shows nitrogen fixation by the zoanthid samples without glucose supplement. Most of these samples (8 of 14 at 8–12 h and 10 of 14 at 12–16 h) exhibited detectable levels of nitrogenase activity under either aerobic or anaerobic conditions. The

TABLE I

Nitrogen fixation (acetylene reduction) by samples of *Palythoa tuberculosa* without glucose supplement.

Sample no.	Sampling date	nmol ethylene·g dry $wt^{-1}·h^{-1}$ [a]		
		0–8 h	8–12 h	12–16 h
Aerobic				
1	Sep. 1990	0	0.63	0.57
2	Sep. 1990	0.58	0.90	0.95
3	Oct. 1990	0	1.28	1.56
4	Nov. 1990	0	0	0
5	Nov. 1990	0	0	0.81
6	Jan. 1991	0	0	0.78
7	Jan. 1991	0	0.65	1.30
Anaerobic				
8	Sep. 1990	0	1.23	0.85
9	Sep. 1990	0	0.73	1.40
10	Oct. 1990	0	0	0
11	Nov. 1990	0	1.05	1.42
12	Nov. 1990	0	0.75	0.90
13	Jan. 1991	0	0	0
14	Jan. 1991	0	0	0

[a] Data were calculated from ethylene produced during the indicated periods.

results indicated that nitrogen-fixing bacteria were generally associated with the zoanthid *Palythoa tuberculosa*. They also indicated that the associated nitrogen-fixing bacteria might have utilized the zoanthid-derived organic matter (exudates and/or cell debris) as an energy source for nitrogen fixation in the unsupplemented medium. The zoanthid-derived organic matter, however, might not be an effective energy source, since glucose addition significantly enhanced the zoanthid-associated nitrogenase activity (Table II). On the other hand, the concentration of glucose supplemented in the present study may not be comparable to that of zoanthid-derived organic matter in nature. The difference in the amount of the energy sources also would make it difficult to evaluate the efficiency of the two. More samples demonstrated nitrogenase activity in aerobic incubation than that in anaerobic incubation (7/7 vs. 4/7 for glucose-supplemented samples and 6/7 vs. 4/7 for samples without glucose supplement, Tables I and II). This difference, if significant, remains to be elucidated.

Extended lag periods from several hours to more than 1 day before the detection of nitrogenase activity have been reported for sediments and excised roots of various plants (Patriquin & Knowles, 1972; Patriquin & Denike, 1978; Zuberer & Silver, 1978; Dicker & Smith, 1980; Shieh et al., 1989a). This phenomenon has been variously attributed to substrate limitation (Day et al., 1975) and inactivation of nitrogenase by O_2 (Patriquin, 1978). In the present experimental system, a lag period of 8–12 h also occurred before the expression of nitrogenase activity (Table I). This lag might partially

TABLE II

Nitrogen fixation (acetylene reduction) by the glucose-supplemented samples of *Palythoa tuberculosa*.

Sample No.	Sampling date	nmol ethylene·g dry $wt^{-1}·h^{-1}$ [a]		
		4–8 h	8–12 h	12–16 h
Aerobic				
15	Sep. 1990	1.79	2.10	1.75
16	Sep. 1990	2.80	3.45	3.33
17	Oct. 1990	0	2.38	1.50
18	Nov. 1990	0	0.56	1.00
19	Nov. 1990	0	0	0.85
20	Jan. 1991	0	1.75	2.83
21	Jan. 1991	0.50	1.05	1.85
Anaerobic				
22	Sep. 1990	1.60	2.50	1.38
23	Sep. 1990	0.80	0.88	1.23
24	Oct. 1990	0	0	0
25	Nov. 1990	0	0	0
26	Nov. 1990	0	0	0
27	Jan. 1991	1.03	5.20	12.05
28	Jan. 1991	0.89	4.65	4.03

[a] Data were calculated from ethylene produced during the indicated periods. Nitrogenase activity in all cases was undetectable within 4 h of incubation.

result from insufficient carbon sources (from the zoanthid), because the lag period significantly decreased to 4–8 h when glucose was added to the zoanthid samples (Table II). The use of a liquid phase in our work might also have delayed the appearance of nitrogenase activity; several hours may have been required for diffusion and equilibration of acetylene to the active sites under our conditions.

Some bacteria in tissue/body wall folds and all of those inside the zoanthids would survive after surface sterilization in 70% ethanol for 20 s. None or negligible nitrogenase activity, however, was detected from the surface-sterilized, glucose-supplemented zoanthid samples within 16 h of incubation (data not shown). This result would imply that most associated nitrogen-fixing bacteria were attached to the external surface of the zoanthid and that few, if any, of these bacteria were internal or had invaded the zoanthid tissues.

Sixty-one strains in total of nitrogen-fixing bacteria were isolated from selected zoanthid samples that showed nitrogenase activity under either aerobic or anaerobic conditions. Only facultatively anaerobic nitrogen-fixing heterotrophs would have been significantly enriched in the second anaerobic enrichment cultures in the present anaerobic enrichment procedure (Shieh et al., 1987, 1989b). The isolation of these bacteria from the enrichment cultures was subsequently performed aerobically on PY agar plates. Facultatively anaerobic nitrogen-fixing bacteria can grow both aerobically and anaerobically by utilizing a variety of nitrogen compounds such as those in PY agar as the nitrogen sources, although they fix N_2 as the sole nitrogen source for growth only under no or low oxygen conditions (Shieh et al., 1987, 1988b). Thus anaerobic incubation was not necessary at this step. All the isolated bacteria proved to be typical facultatively anaerobic nitrogen fixers. They showed significant growth in glucose NFL medium under N_2 within 1–3 days of incubation (absorbance at 600 nm increased from less than 0.01 to greater than 0.2), indicating that they utilized N_2 as the sole nitrogen source for growth under anaerobic conditions. No growth, however, was observed in this medium under air. In contrast, NH_4Cl and yeast extract served as nitrogen sources for cells growing both under air and under Ar in the glucose NFL medium (data not shown). Anaerobically, all isolates expressed detectable levels of nitrogenase activity in the glucose NFL medium within 8–12 h of incubation; the activity in most cases was greater than 10 nmol $C_2H_4 \cdot$mg dry wt. cells$^{-1} \cdot h^{-1}$. All isolates were halophilic heterotrophs and required NaCl (1–3%) and glucose (0.5–2%; as a source of carbon and energy) for the expression of high levels of nitrogenase activity (for examples see Figs. 1 and 2).

The dissolved organic matter released from the zoanthids proved to be only slightly effective for enhancement of bacterial nitrogen fixation; only low nitrogenase activity was detected from cell suspensions of strain PTN14 in the 0.2-μm-filtered zoanthid-surface washed NFL medium (0.83 ± 0.80 nmol $C_2H_4 \cdot$mg dry wt. cells$^{-1} \cdot h^{-1}$ during 8–12 h of incubation). Plate counts on PY agar medium indicated that strain PTN14 grew significantly in the same filtrate under both aerobic and anaerobic conditions (3.8×10^6 cells$\cdot$ml^{-1} at 0 h to 1.3×10^8 cells$\cdot$ml^{-1} at 12 h under air, and 5.3×10^6

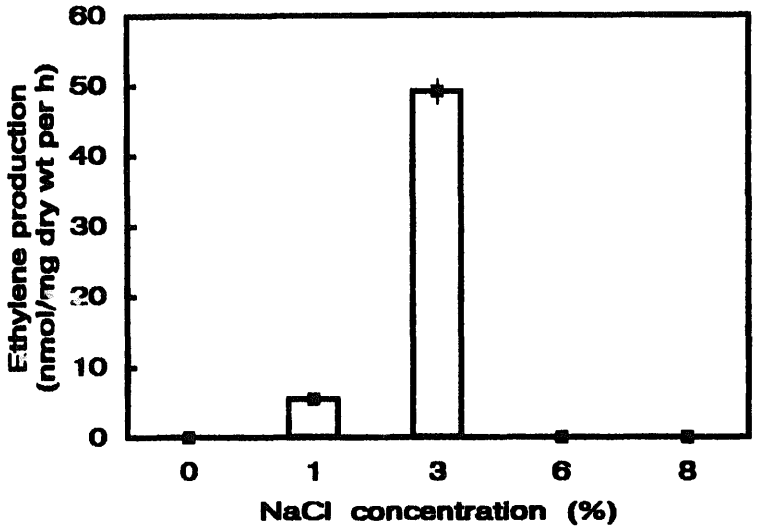

Fig. 1. Effect of NaCl on nitrogenase activity of strain PTN14 under anaerobic conditions. Mean values of duplicate experiments with range are shown.

cells·ml^{-1} at 0 h to 1.1×10^8 cells·ml^{-1} at 12 h under Ar). These results, however, confirmed that the zoanthid-derived organic matter could be a good nutrient for growth of the associated nitrogen-fixing bacteria.

All nitrogen-fixing isolates were Gram-negative rods that were motile in PY broth medium. They fermented glucose and required NaCl for growth. These characteristics clearly indicate that all isolates belong to the family Vibrionaceae. However, they were

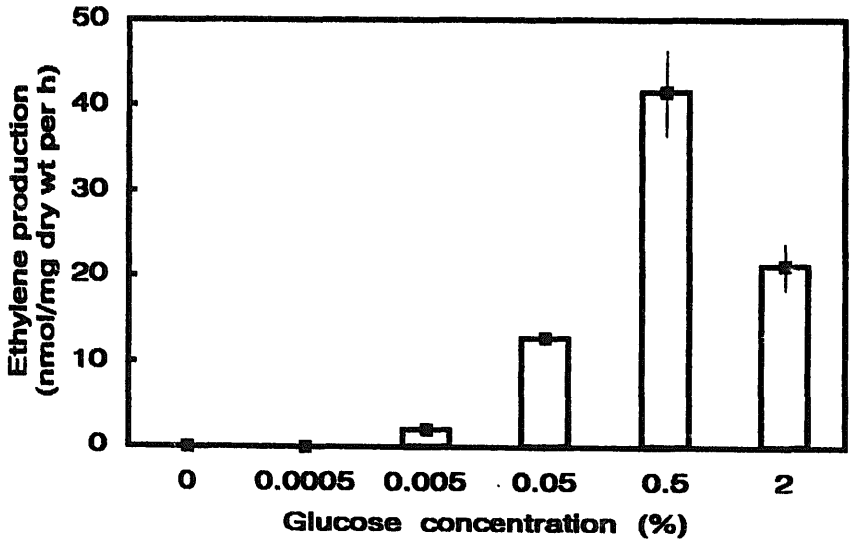

Fig. 2. Effect of glucose on nitrogenase activity of strain PTN14 under anaerobic conditions. Mean values of duplicate experiments with range are shown.

TABLE III

Characteristics of isolated nitrogen-fixing bacteria.

Characteristic	Type of nitrogen-fixing isolates[a]					
	I	II	III	IV	V	VI
Oxidase	–[b]	+	+	+	+	+
Agarase	–	+	–	–	–	–
Fermentation of:						
Dulcitol	–	–	+	+	–	–
Inositol	–	–	+	–	+	–
Growth in:						
0% NaCl	–	–	–	–	–	–
3% NaCl	+	+	+	+	+	+
6% NaCl	+	+	+	+	+	+
8% NaCl	v	–	+	v	+	v
10% NaCl	–	–	–	–	–	–

[a] All strains were motile Gram-negative rods that were positive for glucose fermentation and catalase but negative for swarming, pigmentation and gas from glucose fermentation.
[b] +, positive; –, negative; v, variable between strains.

roughly divided into six types according to the results of the following tests: oxidase, agarase, and fermentation of dulcitol and inositol (Table III). Halophilic, facultatively anaerobic, nitrogen-fixing bacteria including the present six types have been isolated from various marine sources (Guerinot & Colwell, 1985; Shieh et al., 1988b, 1990). Nitrogen-fixing bacteria (type II) capable of agar liquefaction, however, have not previously been isolated from marine organisms.

Zuberer & Silver (1978) suggested that when the nitrogenase activities of natural samples determined under aerobic and anaerobic conditions do not greatly differ, the responsible nitrogen-fixing microorganisms are probably facultatively anaerobic. The zoanthid samples tested in the present study might be the case, especially considering

TABLE IV

Most-probable-number (MPN) counts of selected bacterial groups associated with the surface of the zoanthid *Palythoa tuberculosa* (cells·g dry wt zoanthids^{-1}).

Expt. No.[a]	N_2-fixing heterotrophs[b]	Aerobic heterotrophs[c]
1	4.9×10^3	1.1×10^7
2	1.0×10^4	3.5×10^6
3	5.6×10^4	2.6×10^7
4	1.0×10^4	2.1×10^5

[a] All experiments were performed using the zoanthid samples collected in April 1992.
[b] Only facultatively anaerobic nitrogen-fixing bacteria would have been counted.
[c] The counting values could be expected to contain facultative anaerobes.

that a large number of facultatively anaerobic nitrogen-fixing bacteria (4.9×10^3 to 5.6×10^4 cells·g dry wt. zoanthids^{-1}) were generally associated with the zoanthid (Table IV). Facultatively anaerobic nitrogen-fixing bacteria fix nitrogen only under no or low oxygen conditions when cultivated in pure cultures (Shieh et al., 1987, 1988b). One might, therefore, argue that nitrogen fixation by these bacteria in the present study was only under anaerobic or low oxygen system. The argument is that oxygen-depleted microenvironments could occur in a variety of microbially colonizable, organic or inorganic surfaces (Paerl & Carlton, 1988). Our results may further indicate that zoanthid-associated nitrogen fixation may play a role in the nutrition of these animals and in providing inputs of ammonium nitrogen into the tropical coastal environments.

Acknowledgements

We are grateful to Dr. C.-F. Dai, Institute of Oceanography, National Taiwan University, for his help in identifying the zoanthid *Palythoa tuberculosa*. Our preparation of the manuscript benefited from comments by Dr. J.-S. Chen and two anonymous reviewers. This study was supported by the National Science Council, Republic of China (NSC79-0209-B002a-02 and NSC80-0209-B002a-02).

References

Capone, D.G. & J.M. Budin, 1982. Nitrogen fixation associated with rinsed roots and rhizomes of the eelgrass *Zostera marina*. *Plant Physiol.*, Vol. 70, pp. 1601–1604.

Day, J.M., M.C.P. Neves & J. Dobereiner, 1975. Nitrogenase activity on roots of tropical grasses. *Soil. Biol. Biochem.*, Vol. 7, pp. 107–112.

Dicker, H.J. & D.W. Smith, 1980. Acetylene reduction (nitrogen fixation) in a Delaware salt marsh. *Mar. Biol.*, Vol. 57, pp. 241–250.

Guerinot, M.L. & R.R. Colwell, 1985. Enumeration, isolation, and characterization of N_2-fixing bacteria from sea water. *Appl. Environ. Microbiol.*, Vol. 50, pp. 350–355.

Guerinot, M.L. & D.G. Patriquin, 1981. The association of N_2-fixing bacteria with sea urchins. *Mar. Biol.*, Vol. 62, pp. 197–207.

Head, W.D. & E.J. Carpenter, 1975. Nitrogen fixation associated with the marine macroalga *Codium fragile*. *Limnol. Oceanogr.*, Vol. 20, pp. 815–823.

Herbert, R.A., 1975. Heterotrophic nitrogen fixation in shallow estuarine sediments. *J. Exp. Mar. Biol. Ecol.*, Vol. 18, pp. 215–225.

Hicks, B.J. & W.B. Silvester, 1985. Nitrogen fixation associated with the New Zealand mangrove (*Avicennia marina* (Forsk.) Vierh. var. *resinifera* (Forst. f.) Bakh.). *Appl. Environ. Microbiol.*, Vol. 49, pp. 955–959.

Hirata, Y., D. Uemura, K. Ueda & S. Takano, 1979. Several compounds from *Palythoa tuberculosa* (coelenterata). *Pure Appl. Chem.*, Vol. 51, pp. 1875–1883.

Kogure, K., U. Simidu & N. Taga, 1980. Distribution of viable marine bacteria in neritic sea water around Japan. *Can. J. Microbiol.*, Vol. 26, pp. 318–323.

Maruyama, Y., T. Suzuki & K. Otobe, 1974. Nitrogen fixation in the marine environment: the effect of organic substrates on acetylene reduction. In, *Effect of the Environment on Microbial Activities*, edited by R.R. Colwell & R.Y. Morita, University Park Press, Baltimore, pp. 341–353.

Pearl, H.W., 1984. N_2 fixation (nitrogenase activity) attributable to a specific *Prochloron* (Prochlorophyta)-ascidian association in Palau, Micronesia. *Mar. Biol.*, Vol. 81, pp. 251–254.

Pearl, H.W. & R.G. Carlton, 1988. Control of nitrogen fixation by oxygen depletion in surface-associated microzones. *Nature*, Vol. 332, pp. 260–262.

Patriquin, D.G., 1978. Factors affecting nitrogenase activity (acetylene reduction activity) associated with excised roots of the emergent halophyte *Spartina alterniflora* Loisel. *Aquat. Bot.*, Vol. 4, pp. 193–210.

Patriquin, D.G. & D. Denike, 1978. In situ acetylene reduction assays of nitrogenase activity associated with the emergent halophyte *Spartina alterniflora* Loisel: methodological problems. *Aquat. Bot.*, Vol. 4, pp. 211–226.

Patriquin, D. & R. Knowles, 1972. Nitrogen fixation in the rhizosphere of marine angiosperms. *Mar. Biol.*, Vol. 16, pp. 49–58.

Patriquin, D.G. & C.R. McClung, 1978. Nitrogen accretion, and the nature and possible significance of N_2 fixation (acetylene reduction) in a Nova Scotian *Spartina alterniflora* stand. *Mar. Biol.*, Vol. 47, pp. 227–242.

Postgate, J.R., 1982. Enzymology. In, *The Fundamentals of Nitrogen Fixation*, Cambridge University Press, London, pp. 20–59.

Shieh, W.Y., 1988. *Marine nitrogen-fixing bacteria isolated from an eelgrass (Zostera marina) bed in Aburatsubo Inlet, Kanagawa, Japan.* Ph.D. dissertation, University of Tokyo, Tokyo, Japan, 159 pp.

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

Shieh, W.Y., U. Simidu & Y. Maruyama, 1988a. Nitrogen fixation by marine agar-degrading bacteria. *J. Gen. Microbiol.*, Vol. 134, pp. 1821–1825.

Shieh, W.Y., U. Simidu & Y. Maruyama, 1988b. New marine nitrogen-fixing bacteria isolated from an eelgrass (*Zostera marina*) bed. *Can. J. Microbiol.*, Vol. 34, pp. 886–890.

Shieh, W.Y., U. Simidu & Y. Maruyama, 1989a. Nitrogenase activity of heterotrophic bacteria associated with roots of eelgrass *Zostera marina*. *Nippon Suisan Gakkaishi*, Vol. 55, pp. 853–857.

Shieh, W.Y., U. Simidu & Y. Maruyama, 1989b. Enumeration and characterization of nitrogen-fixing bacteria in an eelgrass (*Zostera marina*) bed. *Microb. Ecol.*, Vol. 18, pp. 249–259.

Shieh, W.Y., U. Simidu & Y. Maruyama, 1990. A *Photobacterium*-like bacterium able to fix nitrogen. *Antonie van Leeuwenhoek*, Vol. 57, pp. 51–54.

Waterbury, J.B., C.B. Calloway & R.D. Turner, 1983. A cellulolytic nitrogen-fixing bacterium cultured from the gland of Deshayes in shipworms (Bivalvia:Teredinidae). *Science*, Vol. 221, pp. 1401–1403.

Wilkinson, C.R. & P. Fay, 1979. Nitrogen fixation in coral reef sponges with symbiotic cyanobacteria. *Nature*, Vol. 279, pp. 527–529.

Williams, W.M., A.B. Viner & W.J. Broughton, 1987. Nitrogen fixation (acetylene reduction) associated with the living coral *Acropora variabilis*. *Mar. Biol.*, Vol. 94, pp. 531–535.

Zuberer, D.A. & W.S. Silver, 1978. Biological dinitrogen fixation (acetylene reduction) associated with Florida mangroves. *Appl. Environ. Microbiol.*, Vol. 35, pp. 567–575.