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Enumeration, Isolation, and Characterization of N_2-Fixing Bacteria from Seawater

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Marine pelagic N_2-fixing bacteria have not, in general, been identified or quantified, since low or negligible rates of N_2 fixation have been recorded for seawater when blue-green algae (cyanobacteria) are absent. In the study reported here, marine N_2-fixing bacteria were found in all samples of seawater collected and were analyzed by using a most-probable-number (MPN) method. Two different media were used which allowed growth of microaerophiles, as well as that of aerobes and facultative anaerobes. MPN values obtained for N_2-fixing bacteria ranged from 0.4 to 1×10^3 per liter for water collected off the coast of Puerto Rico and from 2 to 5.5×10^2 per liter for Chesapeake Bay water. Over 100 strains of N_2-fixing bacteria were isolated from the MPN tubes and classified, yielding four major groups of NaCl-requiring bacteria based on biochemical characteristics. Results of differential filtration studies indicate that N_2-fixing bacteria may be associated with phytoplankton. In addition, when N_2-fixing bacteria were inoculated into unfiltered seawater and incubated in situ, nitrogenase activity could be detected within 1 h. However, no nitrogenase activity was detected in uninoculated seawater or when bacteria were incubated in 0.2-μm-filtered (phytoplankton-free) seawater. The ability of these isolates to fix N_2 at ambient conditions in seawater and the large variety of N_2-fixing bacteria isolated and identified lead to the conclusion that N_2 fixation in the ocean may occur to a greater degree than previously believed.

Marine environments are often described as nitrogen limited. As the concentration of dissolved nitrogen gas is approximately two orders of magnitude greater than other forms of inorganic N commonly found in marine waters (24), it was puzzling why N_2-fixing organisms, capable of converting nitrogen gas to ammonia, were not more prevalent. Calculations from the limited amount of data available in the literature suggested nitrogen fixation to be unimportant in the overall nitrogen economy of the ocean (4); however, this may reflect merely an inability to quantify the process with methods currently used in biological and chemical oceanography rather than an absence of the process itself.

For many years, the filamentous blue-green algae (cyanobacteria) were believed to be primarily responsible for N_2 fixation in oceanic waters because low or negligible in situ rates were observed in their absence and there was a correlation of in situ N_2 fixation with light intensity (31). However, evidence has been accumulating which documents the importance of bacterial N_2 fixation in many and diverse marine habitats. Brooks et al. (3) provided the first strong evidence of in situ bacterial N_2 fixation occurring in estuarine sediments. Since that time, the presence of heterotrophic nitrogen fixation in the absence of light has been demonstrated for a variety of marine and estuarine sediments (12, 14, 15, 20, 25, 28, 33). Bacteria-plant-associated N_2-fixing symbioses have been proposed for the marsh grass *Spartina alterniflora* (27), the seagrasses *Thalassia testudinum* and *Zostera marina* (5, 28), and the macroalga *Codium fragile* (13). Bacterial N_2 fixation is, at least in part, responsible for N_2 fixation observed for coral reef and mangrove communities (34, 36), as well as for intertidal communities (2, 7).

Another marine community which may be actively fixing N_2 is that associated with the deep ocean hydrothermal vents. The nitrogen isotope ratios in low-trophic-level animals from these deep sea vents more closely resemble those of deep sea N_2 and of marine organisms associated with N_2 fixation than those of organic nitrogen compounds in deep ocean water (30). This suggests that N_2 fixation may be the source of organic nitrogen for these vent communities. The N_2-fixing microorganisms may be free-living or may be intimately associated with vent animals. There are precedents for the direct association of N_2-fixing bacteria with marine animals. Nitrogenase activity associated with the gastrointestinal tracts of sea urchins has been documented (8–10, 26) and studies with $^{15}N_2$ have shown the incorporation of microbially fixed N into sea urchin tissues, demonstrating that N_2 fixation can be a source of N for the sea urchin (10). Most recently, cellulolytic nitrogen-fixing bacteria have been isolated in large numbers, apparently as a pure culture, from a specialized gland found in shipworms (32). These bacteria may be participants in a unique symbiotic relationship with shipworms which allows these shipworms to use wood as their principal food source.

Whereas many of the environments in which bacterial N_2 fixation is found are benthic, there is a report of nitrogen fixation associated with a bacteria-diatom symbiosis in the pelagic zone which indicates that these systems may contribute a significant amount of new nitrogen to oligotrophic waters (22). These results also indicate that standard shipboard techniques may significantly underestimate N_2 fixation occurring in oceanic waters.

Given the potential of N_2-fixing bacteria to alter significantly the nitrogen budgets of their surroundings, disappointingly few attempts have been made to quantify or identify marine pelagic, N_2-fixing bacteria (23, 33, 35). As a result, little is known about the distribution, activity, or classification of such organisms. In this study, most-prob-

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able-number (MPN) enrichment tubes were used to enumerate and isolate N_2-fixing bacteria. Differential filtration studies were used to determine whether N_2-fixing bacteria are associated with phytoplankton, and experiments with marine N_2-fixing bacteria isolated as a result of the studies reported here were conducted to assess the capability of the bacteria to fix N_2 in situ.

MATERIALS AND METHODS

Sampling locations. Samples of surface water were collected in the area of the Puerto Rican trench during two cruises aboard the R/V *Eastward* in December 1979 and February 1980. Transects were followed which ran north from Arecibo, Puerto Rico. In November 1980, sample collection was accomplished during a cruise of the R/V *Gyre*, on a transect running from Key West, Florida, to Puerto Rico. In June 1981, samples were collected during a cruise aboard the R/V *Endeavor* on a transect running from Barbados to Bermuda. Samples of water from Chesapeake Bay were obtained during cruises aboard the R/V *Ridgley Warfield* in March, May, August, September, and November of 1980 and in April 1981.

Sample collection. Surface-water samples (at depths of 1 to 5 m) were collected aseptically, using sterile Niskin bags (General Oceanics, Miami, Fla.), and were processed on board ship within 15 min of collection. Samples of bottom water were also collected with sterile Niskin bags at a depth of 1 m from the bottom from various locations in Chesapeake Bay.

Physical and chemical parameters of seawater. Water samples were collected on all cruises and analyzed for ammonia, nitrate, nitrite, orthophosphate, dissolved organic phosphate, dissolved organic nitrogen, and total particulate matter. Water temperature and salinity were determined at each station. Samples were also collected at each station for chlorophyll *a* determinations.

Media. The liquid N-deficient medium used in the studies consisted of three components: (i) triple-strength artificial seawater (NaCl, 0.9 M; $MgSO_4$, 0.15 M; $CaCl_2$, 0.03 M; KCl, 0.03 M; Tris, 0.15 M; adjusted to pH 7.5), 333 ml; yeast extract (Difco Laboratories, Detroit, Mich.), 100 mg; (ii) distilled water, 500 ml; mannitol, 4 g; $FeSO_4 \cdot 7H_2O$, 15 mg; $NaMoO_4 \cdot 2H_2$, 5 mg; and (iii) distilled water, 167 ml; K_2HPO_4, 0.8 g; KH_2PO_4, 0.2 g. These were mixed after each had been individually autoclaved (final pH 7.2), and 1 ml of filter-sterilized vitamin solution was added (riboflavin, 50 mg; *p*-aminobenzoic acid, 8 mg; nicotinic acid, 50 mg; biotin, 12 mg; thiamine-HCl, 80 mg; calcium panthothenate, 80 mg; inositol, 48 mg; cobalamine, 0.1 mg, distilled water, 100 ml). The medium included yeast extract and vitamins, after it had been observed that enrichment cultures in N-deficient liquid medium prepared with yeast extract and vitamins yielded the highest rates of nitrogenase activity, when compared with cultures grown in N-deficient liquid medium prepared with either no yeast extract or no vitamins or neither. The semisolid N-deficient medium used in this study consisted of the following (per liter of distilled water): KH_2PO_4, 4.23 g; K_2HPO_4, 5.41 g; glucose, 5.0 g; DL-malic acid, 3.0 g; yeast extract, 0.1 g; NaCl, 30 or 15 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.02 g; $FeCl_3$, 0.01 g; $NaMoO_4 \cdot 2H_2O$, 0.0005 g; NaOH, 1.5 g. The pH of the medium was adjusted to 7.0 with NaOH, and 1.75 g of agar (Difco) was added. The marine agar was composed of the following: Bacto-Peptone (Difco), 2.5 g; yeast extract, 0.5 g; seawater, 1 liter; $FePO_4$, 0.05 g; agar, 15 g. Upper bay yeast extract medium had the following composition (per liter of distilled water): yeast extract, 1.0 g; Difco Proteose Peptone, 1.0 g; KCl, 0.29 g; $MgSO_4 \cdot 7H_2O$, 2.7 g; NaCl, 9.06 g; agar, 20 g.

Bacterial enumeration. Numbers of culturable, aerobic, heterotrophic bacteria present in water samples were determined by plating on marine agar. Water samples were filtered in triplicate through sterile 0.2-μm nitrocellulose membrane filters (Gelman Sciences, Inc., Ann Arbor, Mich.), and the filters were then placed on marine agar plates and incubated for 7 days at 25°C before counting. Total bacterial numbers were determined by acridine orange staining and epifluorescent microscopy (AODC) (29). For MPN determinations, water samples were filtered in triplicate through sterile 0.2-μm nitrocellulose membrane filters. The filters were rolled onto sterile forceps and placed in tubes containing 8 ml of liquid mannitol broth or semisolid malate-glucose medium. All enrichment tubes were incubated in the dark at 25°C. Nitrogenase activity was determined by the acetylene (C_2H_2) reduction assay as follows: when visible turbidity developed, the tubes were closed with serum stoppers and injected with C_2H_2 (pC_2H_2 = 1 kPa). Ethylene (C_2H_4) production was analyzed, after incubation for 7 days at 25°C, by gas chromatography (11). The criterion used to record an MPN tube as positive was production of C_2H_4. The MPN of N_2-fixing bacteria was estimated from inoculated series of three tubes per dilution, using the tables of Cochran (6).

Isolation of N_2-fixing bacteria. MPN tubes which exhibited nitrogenase activity after inoculation, as described above, were subcultured into fresh medium and nitrogenase activity was determined as described above. The transfer process was repeated three times and all tubes demonstrating nitrogenase activity after the third transfer were plated onto marine agar. Individual colonies were picked, purified, and assayed as pure cultures for nitrogenase activity, using N-deficient medium. Pure cultures of N_2-fixing isolates were readily obtained by repeated subculturing.

Identification and classification of isolates. The methods of MacFaddin (18) were used to identify strains, with exceptions and additional tests as cited below. Gram-staining reactions were recorded from heat-fixed smears of fresh cultures after staining with the Hucker modification of the Gram stain. Motility and cell shape were determined by direct observations of wet mounts of fresh broth cultures, using phase-contrast microscopy. All strains were tested for ability to grow at different concentrations of NaCl in 1% tryptone broth at 25°C. Growth was assessed visually 1 and 5 days after inoculation. Ability to grow at various temperatures was tested by inoculating strains into 1% tryptone broth containing the optimum amount of NaCl for each strain as determined by salt tolerance tests and incubating inoculated tubes in constant-temperature water baths. Growth was assessed visually 1 and 5 days after inoculation. NaCl was added to all test media to 0.5%, if at least 0.5% NaCl was not already present. Sensitivity to 2,4-diamino-6,7-diisopropyl pteridine (o/129) was determined on plates of heart infusion agar (Difco) containing 10 or 140 μg of 0/129. Ability to hydrolyze DNA, gelatin, and starch and ability to swarm on a solid medium were tested according to Lee et al. (17). Luminescence was determined after growth for 24 h on the following agar medium: Oxoid nutrient broth no. 2, 25 g; NaCl, 5 g; $MgCl_2$, 4 g; KCl, 1 g; agar, 20 g; distilled water, 1 liter. Luminescence was recorded after the plates had been observed for 5 min in the dark.

Differential filtrations. On several occasions, seawater was filtered through nitrocellulose filters of various pore sizes

TABLE 1. Enumeration of N_2-fixing bacteria in marine and estuarine water

Sampling location	Date	Depth (m)	Water temp (°C)	Salinity (‰)	CFU (per liter)[a]	AODC (per liter)	MPN of N-fixing bacteria (organisms per liter)[b]
Puerto Rico, 32 km from shore	Dec. 1979	1	26.5	35.1	3.6×10^3	2.74×10^8	2.3
Puerto Rico, 64 km from shore		1	27.1	35.6	5.4×10^3	2.84×10^8	0.4
Puerto Rico, 112 km from shore		1	26.7	36.0	2.3×10^4	2.11×10^8	0.4
Puerto Rico, 6 km from shore	Feb. 1980	1	26.5	ND[c]	3.5×10^3	ND	1.0×10^1
Puerto Rico, 16 km from shore		1	26.3	ND	1.7×10^3	3.57×10^8	1.81
Puerto Rico, 48 km from shore		1	25.9	ND	4.1×10^3	4.0×10^8	6.35
Puerto Rico, 68 km from shore		1	25.8	ND	2.4×10^3	3.35×10^8	1.35
Puerto Rico, 77 km from shore		1	25.8	ND	4.4×10^3	2.78×10^8	2.41
Puerto Rico, 97 km from shore		1	25.7	ND	3.5×10^4	2.64×10^8	6.35
Puerto Rico, 129 km from shore		1	25.7	ND	1.2×10^4	2.95×10^8	6.35
Puerto Rico, 145 km from shore		1	25.5	ND	2.0×10^3	2.62×10^9	6.51
Chesapeake Bay	Mar. 1980	1	5.4	8.8	2.6×10^7	2.30×10^9	1.05×10^2
Chesapeake Bay		30	4.7	16.12	2.0×10^7	2.48×10^9	2
Chesapeake Bay		1	6.3	10.72	1.9×10^7	2.62×10^9	4.65×10^1
Chesapeake Bay		11.8	5.3	14.41	3.0×10^7	1.49×10^9	2.15×10^1
Chesapeake Bay		1	6.5	14.13	2.7×10^7	2.18×10^9	7.5×10^1
Chesapeake Bay		13.0	5.9	14.58	2.5×10^7	2.44×10^9	1.15×10^1
Chesapeake Bay		1	6.5	17.71	5.1×10^7	2.52×10^9	4.5
Chesapeake Bay		30.0	5.1	16.72	5.7×10^7	2.39×10^9	4.5
Mouth of Chesapeake Bay	May 1980	1	19.2	30.52	2.1×10^3	6.1×10^8	4.5
Mouth of Chesapeake Bay		19.1	13.1	32.81	ND	ND	1.35
Chesapeake Bay-Eastern Bay	Aug. 1980	1	28.9	12.00	3.3×10^6	1.3×10^{10}	2.1×10^2
Chesapeake Bay	Sept. 1980	1	21.9	14.9	ND	3.97×10^9	4.3×10^1
Chesapeake Bay		11.2	22.7	16.4	ND	2.1×10^9	7.5×10^1
Chesapeake Bay-Eastern Bay	Sept. 1980	1	21.9	15.4	ND	ND	9.3×10^1
Chesapeake Bay-Eastern Bay		11.8	23.4	17.8	ND	ND	9.3×10^1
Chesapeake Bay	Nov. 1980	1	9.9	16.55	ND	3.54×10^9	9
		11.2	9.5	16.84	ND	3.31×10^9	1.5×10^2
Chesapeake Bay	Apr. 1981	1	10.0	14.4	ND	ND	2.3×10^1
		13.6	10.6	15.8	ND	ND	2.3×10^1

[a] CFU on marine agar for water samples from Puerto Rico and the mouth of the Chesapeake Bay. For samples in Chesapeake Bay, upper bay yeast extract medium was used.
[b] MPN in mannitol medium.
[c] ND, Not done.

(100, 50, 8, 1.2, 0.8, 0.2 μm) and the filters were placed in tubes of media for MPN determination, as described above.

In situ incubations. To determine whether N_2-fixing bacteria previously isolated from seawater samples were capable of fixing N_2 when inoculated into seawater, in situ incubations were done as follows. Bacteria were grown for 12 h in marine broth at 25°C with shaking. Cells were harvested by centrifugation and washed with sterile three-salts solution (KCl, 0.01 M; $MgSO_4 \cdot 7H_2O$, 0.028 M; NaCl, 0.4 M). After harvesting and washing, the cells were suspended in sterile three-salts solution and an aliquot of each strain was counted by AODC. Ca. 10^6 cells per ml were added to aliquots of either unfiltered seawater or seawater filtered through either 8.0- or 0.2-μm nitrocellulose filters. Uninoculated water samples were used as controls. The sample to be incubated, followed by 5.0 ml of freshly generated C_2H_2, was drawn into a 50-ml syringe. The syringe needle was plugged with a rubber stopper. The samples were suspended at a depth of 1 m at a station in Chesapeake Bay for up to 4 h. At the end of the incubation period, the gas phase was injected into a sterile Vacutainer (BBL Microbiology Systems, Cockeysville, Md.). Ethylene production was determined by gas chromatography. An aliquot of water was also processed for AODC.

RESULTS AND DISCUSSION

Bacteria capable of reducing acetylene (N_2-fixing bacteria) were found in all samples of seawater collected and analyzed by the MPN technique. MPN ranged from 0.4 to 1×10^3 per liter for water samples collected off the coast of Puerto Rico and from 2 to 5.5×10^2 per liter for Chesapeake Bay water samples (Table 1). Significant correlation ($P < 0.05$) between numbers of N_2-fixing bacteria present at any given location and physical and chemical parameters measured in the study (data not shown) was not observed.

Two different N-free media were routinely used to quantify and isolate N_2-fixing bacteria. MPN values for N_2-fixing bacteria determined in the two media were not significantly different (Table 2). However, by using two types of media, we were able to isolate a larger variety of bacteria, since certain groups of bacteria appeared in culture only in one of the two media.

A total of 118 different strains of N_2-fixing bacteria were isolated from all sources. All strains isolated were halotolerant, i.e., able to grow and fix N_2 in media containing 3% NaCl. However, 43% of the strains did not demonstrate an absolute requirement for NaCl. Of 92 strains isolated from seawater (salinity, approximately 35‰), 68% demonstrated an absolute requirement for NaCl for growth. In contrast, of 26 strains collected in estuarine waters (salinity, approximately 15‰), only 19% demonstrated an absolute requirement for NaCl for growth. The difference, if any, between marine and freshwater bacteria is not yet fully understood. MacLeod (19) noted that marine bacteria have special requirements for inorganic ions, partly to supply the needs of the organism for growth and metabolism and partly to

TABLE 2. Comparison of mannitol medium and malate-glucose medium for determining the MPN of N_2-fixing bacteria in seawater samples

Sampling location	Date	CFU (per liter)[a]	AODC (per liter)	MPN of N_2-fixing bacteria (organisms per liter)[b]	
				Mannitol medium	Malate-glucose medium
Chesapeake Bay	Mar. 1980	2.6×10^7	2.30×10^9	1.05×10^2	5.5×10^2
Chesapeake Bay	Mar. 1980	2.0×10^7	2.48×10^9	2	3.5
Chesapeake Bay	Mar. 1980	1.9×10^7	2.62×10^9	4.65×10^1	1.2×10^2
Chesapeake Bay	Mar. 1980	3.0×10^7	1.49×10^9	2.15×10^1	4.5
Chesapeake Bay	Mar. 1980	2.7×10^7	2.18×10^9	7.5×10^1	1.2×10
Chesapeake Bay	Mar. 1980	2.5×10^7	2.44×10^9	1.15×10^1	2
Chesapeake Bay	Mar. 1980	5.1×10^7	2.52×10^9	4.5	2
Chesapeake Bay	Mar. 1980	5.7×10^7	2.39×10^9	4.5	0
Mouth of the Chesapeake Bay	May 1980	2.1×10^3	6.1×10^8	4.5	8
Mouth of the Chesapeake Bay	May 1980	ND[c]	ND	1.35	1
Chesapeake Bay-Eastern Bay	May 1980	3.3×10^6	1.3×10^{10}	2.1×10^2	7.5×10^1

[a] CFU on upper bay yeast extract medium for samples collected in March and August. For samples collected in May, 2216 agar (Difco) was used.
[b] The MPN of N_2-fixing bacteria was not significantly different for the two types of media for any given samples of water.
[c] ND, Not done.

TABLE 3. Biochemical and physiological characteristics of marine N_2-fixing bacteria isolated in this study

Characteristic	Result[a] in group:			
	I	II	III	IV
Oxidation/fermentation	F	F	F	O
Gas from glucose	−	−	−	−
Catalase	+	+	−	+
Oxidase	+	−	−	−
Indole	V	−	−	−
Methyl red	+	+	+	−
Voges-Proskauer	−	+	+	−
Growth in:				
0% NaCl	−	−	−	−
3% NaCl	+	+	+	+
7% NaCl	+	V	+	−
10% NaCl	V	V	V	−
Growth at:				
4°C	+	+	+	+
20°C	+	+	+	+
37°C	+	+	+	+
Acid production from:				
Arabinose	+	+	+	−
Cellobiose	+	+	+	−
Galactose	+	+	+	−
Glucose	+	+	+	−
Lactose	V	+	+	−
Mannitol	+	+	+	−
Mannose	+	+	+	−
Rhamnose	V	V	V	−
Salicin	V	V	+	−
Sucrose	+	+	+	−
Xylose	+	+	+	−
Moeller's medium				
Arginine	V	−	−	−
Lysine	−	−	−	−
Ornithine	−	−	−	−
Nitrogenase	+	+	+	+
Sensitivity to o/129	−	−	−	−
Hydrolysis of:				
Gelatin	V	V	+	−
Starch	V	V	V	V
Swarming on solid media	−	−	−	−
H_2 production	−	−	−	−

[a] F, Fermentative; +, positive; −, negative; V, variable; O, oxidative.

maintain the structural integrity of the cell. The isolates which displayed a requirement for NaCl for growth were classified to genus or species or both. All of the NaCl-requiring strains are gram-negative, motile rods and can be roughly divided into four clusters of strains, based on biochemical characterization (Table 3). Group I and II organisms have been assigned to the genus *Vibrio*. Although the current definition of *Vibrio* states that no species fixes molecular nitrogen (1), a nitrogen-fixing species, *Vibrio diazotrophicus*, has recently been described (11). Group I strains have been assigned to this species on the basis of biochemical and morphological characteristics. In addition, a representative of this group, designated CB42, has also been shown to belong to this species on the basis of DNA/DNA hybridization (11). Group II organisms, which are oxidase negative, have not been assigned to a species. At present, there are only two oxidase-negative species of *Vibrio* listed in *Bergey's Manual of Systematic Bacteriology* (1), neither of which has been reported to fix N_2. The assignment of strains in group II to species in *Vibrio* must await further characterization, including DNA/DNA hybridization studies.

Group III strains appear to represent a new genus of marine bacteria, distinguishable from other genera on the basis of oxidase and catalase reactions, both of which are negative. Group IV strains may be members of the genus *Oceanospirillum*. They are oxidative in metabolism, unlike strains of groups I, II, and III which demonstrate ability to ferment carbohydrates. Group IV strains are unable to utilize carbohydrates even oxidatively, resembling members of the genus *Oceanospirillum*.

Clearly, marine N_2-fixing bacteria ubiquitous to marine waters can be recovered in culture, although the numbers of diazotrophs detected by the MPN technique in this study were small (Table 1). The results possibly reflect an inability to culture many of the marine, N_2-fixing bacteria on the types of media used in this study (for a discussion of the cultivation of marine bacteria, see Martin and MacLeod [21]). Previous attempts to identify N_2-fixing bacteria isolated from the marine environment have used several methods, including plating on nitrogen-free media (23, 35) and plating on media designed to be selective for certain genera of bacteria, including *Clostridium* (3) and *Desulfovibrio* (15).

Although bacteria capable of growth on an agar medium without added nitrogen have been isolated, many strains so isolated proved to be nitrogen-scavenging bacteria and were not capable of fixing N_2 when assayed by the acetylene reduction method (16). Bacteria proven to be capable of fixing nitrogen have been isolated by use of selective media; however, the choice of selective media precludes isolating other N_2-fixing forms that may be present yet are incapable of growth on the media used. The strategy we followed allowed for growth of a relatively wide variety of aerobes, facultative anaerobes, and microaerophiles. By isolation and purification of cultures consistent in the ability to fix N_2, a variety of microorganisms representing a diversity of physiological types were obtained.

Since N_2-fixing bacteria were found in water samples collected at all locations sampled, experiments were carried out to determine whether the N_2-fixing bacteria were associated with a given class of particulate matter, with specific focus on phytoplankton. Significantly fewer N_2-fixing bacteria were found to be associated wtih 100- and 50-μm size fractions compared with 8.0-, 1.2-, 0.8-, and 0.2-μm size fractions (Table 4).

The presence of N_2-fixing bacteria in the 8- and 1.2-μm fractions can result either from the association of bacteria with plankton or particulate material retained on the filters or by entrapment of free-living bacteria. We therefore enumerated bacteria in the filtrate after each filtration step and found that passage through 100-, 50-, 8-, and 1.2-μm filters did not alter the AODC numbers significantly. Therefore, it is concluded that some of the N_2-fixing bacteria retained by 8- and 1.2-μm filters are not free-living bacteria entrapped on the filters, but instead are associated with plankton or with particulate matter.

A question which should be addressed is whether the N_2-fixing bacteria isolated in this study are actually fixing N_2 in situ. Obviously, enumeration and isolation of many bacteria capable of fixing N_2 in vitro does not necessarily prove the ability to fix N_2 in the natural environment. However, the laboratory results did indicate that the capability exists and fixation of N_2 can occur when environmental conditions are suitable for nitrogen fixation. In general, N_2-fixing bacteria require an adequate supply of carbon for energy, a low pO_2 environment to protect the oxygen-labile nitrogenase enzyme complex, and a low concentration of fixed N in the environment to prevent repression of nitrogenase activity. In 1971, Maruyama et al. (23) suggested that an associative relationship with plankton could be favorable for N_2-fixing bacteria because the process of N_2 fixation requires a large amount of energy. However, the oxygen evolved during photosynthesis by the phytoplankton might inhibit the oxygen-sensitive nitrogenase complex. Dark enhancement of N_2 fixation has been observed for a bacteria-diatom symbiosis (22), which is suggestive of a temporal separation of oxygen-evolving photosynthesis and oxygen-inhibited N_2 fixation. To determine whether N_2-fixing bacteria could fix N_2 in the presence of phytoplankton which were actively photosynthesizing, previously isolated N_2-fixing bacteria were incubated in situ in the light in natural seawater in the presence and absence of phytoplankton. No nitrogenase activity was detected when bacteria were incubated in 0.2-μm-filtered (phytoplankton-free) seawater or for uninoculated seawater controls (Table 5). Nitrogenase activity, however, was detectable when bacteria were incubated in unfiltered or 8-μm-filtered seawater (Table 5). The results of this experiment demonstrate that N_2 fixation can occur in the light in natural seawater at ambient pO_2 levels. That pure cultures of N_2-fixing bacteria were capable of fixing N_2 when incubated in situ in the presence of, but not the absence of, plankton could explain the dependence on light for N_2 fixation as reported by other investigators and suggests that the phytoplankton provide bacteria with a necessary factor (s), most probably an exudate acting as carbon source. In turn, the N_2-fixing bacteria may serve as a source of fixed N for phytoplankton, via either cell autolysis or direct export of fixed N. As the in situ incubations were not done in the dark, it is not known whether this system is light dependent. That there are many different species of N_2-fixing bacteria present in the ocean lends credence to the belief that more N_2 fixation occurs in the ocean than previously believed, and efforts to determine in situ rates of bacterial N_2 fixation in the ocean should be renewed.

TABLE 4. MPN of N_2-fixing bacteria in samples of seawater after differential filtration[a]

Filter pore size (μm)	MPN of N_2-fixing bacteria (organisms per liter)
100	0[b]
50	0
8	0.9×10^1
1.2	2.3×10^1
0.8	2.3×10^1
0.2	4.3×10^1

[a] The MPN value for a sample of water collected at the same time as the water used in the differential filtration and passed through only a 0.2-μm filter was 2.1×10^2.

[b] There were significantly less N_2-fixing bacteria associated with 100- and 50-μm size fraction.

TABLE 5. Nitrogenase activity of strain CB42 after inoculation into seawater

Treatment of seawater sample	AODC ($\times 10^7$ per ml)	N_2ase activity (nmol of N fixed h^{-1})[a]	
		Expt 1	Expt 2
Unfiltered, before addition of N_2-fixing bacteria	1.30	0	0
Unfiltered, after addition of N_2-fixing bacteria	1.30	0	0
Unfiltered, after addition and incubation	3.27	25.6	26.2
8-μm filtered, after addition and incubation	6.27	23.0	34.2
0.2-μm filtered, after addition and incubation	0.84	0	0

[a] This assumes a 3:1 ratio of C_2H_4 reduced/N_2 fixed. Inoculum size for each tube was 2.2×10^6 cells.

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