

Bacteriology of Ferromanganese Nodules from the Indian Ocean

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Abstract Bacteriological investigations of the ferromanganese nodules collected from the Indian Ocean indicate that the microbial populations associated with the nodules are, in general, comparable to those of the Pacific and Atlantic Oceans. Both Mn(II)-oxidizing and MnO₂-reducing psychrotrophic bacteria were present in the nodules. The maximum percentage of oxidizers was noticed on the surface of the nodules. The sediments harbored more inactive forms. Members of *Vibrio*, Enterobacteriaceae, *Bacillus*, *Micrococcus*, *Staphylococcus*, *Arthrobacter*, and coryneforms were encountered in the present study. Most of the isolates were able to grow on a wide range of sodium chloride concentrations (0 to 10%). The isolates elaborated a number of hydrolytic enzymes, namely, amylase, gelatinase, lipase, and phosphatase. The dominance of the gram-positive group is attributed to terrigenous influences. The present study clearly indicates that Indian Ocean nodules also harbor a variety of heterotrophic bacteria capable of mobilizing and immobilizing manganese.

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Introduction

Ferromanganese in the form of nodules and crusts occurs to varying extent on the seabed of the world's oceans. The nodules are reported to contain Mn (9.5–42.3%), Fe (2.47–19.64%), Ni (0.13–1.57%), Cu (0.07–1.28%) and Co (0.16–0.48%) (Anon., 1982). Detailed investigations carried out for the last two decades clearly indicate that ferromanganese nodules and deep sea sediments harbor bacteria. Some are able to catalyze Mn(II) oxidation and others are able to catalyze Mn(IV) reduction (Ehrlich, 1975). The Mn(II)-oxidizing bacteria, in the process of generating Mn(IV) oxides in the form of nodules, promote the removal of other cations of elements such as Ni, Cu, and Co from sea water and deposit them in the nodule. The Mn(IV)-reducing bacteria reverse this process. Some recent studies including electron microscopy and radiotracer experiments prove that bacteria play a major role in binding Mn(II) to particulates (Emerson et al., 1982). The enzymatic nature of these reactions has been demonstrated (Ehrlich, 1966, 1968; Trimble and Ehrlich, 1970). The biochemistry of the enzymatic interactions with manganese has also been investigated in bacteria isolated from nodules, and characterization of the enzymes involved has been attempted (Ehrlich, 1972, 1974; Arcuri and Ehrlich, 1979, 1980). The microbiological studies carried out so far have been restricted mainly to ferromanganese nodules collected from the Atlantic and Pacific Oceans. This paper describes the results of preliminary studies carried out with nodules and sediments collected from the Indian Ocean.

Materials and Methods

The samples used in the present study were collected on board R.V. *Gaveshani*, the research vessel of the National Institute of Oceanography, during cruises No. 86 and 87 (Jan. to Feb. 1981). Nodules and associated sediments were collected at 2°52.4'N and 58°8.9'E at a depth of 5400 m.

The nodule samples were collected with the help of "Preussag" freefall grab samplers, and the sediments were obtained

with a Peterson grab sampler. When these specimens were brought to the surface, no special precautions were taken to avoid surface contamination. Both nodules and central portions of sediment samples were aseptically removed, placed in sterile plastic containers, and immediately stored in a deep freeze. All analyses were carried out in the shore laboratory at Goa after a minimum storage period of two weeks.

The nodules were dark brown in color and porous (Mn, 20.3%; Fe, 13.3%). According to Meyer's (1973) classification, they belong to Kg-type of nodules: ellipsoidal to irregular, spheroidal in shape, and intergrown with a smooth surface. The sediments were mainly oozes of calcareous and siliceous nature. The surface of each nodule was scraped with a sterile scalpel and pulverized in a sterile porcelain mortar and pestle. The bacteria in the material were enumerated by plating. After surface sampling, each nodule was broken aseptically, its core was taken and pulverized as mentioned above, and the bacteria in the material were counted by plating. The sediment samples were tested without any pretreatment. All samples were diluted serially before plating by the pour-plate method. In addition, enumeration was also attempted by the capping agar method (Ehrlich et al., 1972) wherein the inoculum was added to about 5 mL of melted medium (nutrient agar), mixed thoroughly, and poured onto the surface of solidified agar in Petri dishes. For the present investigation five different media were employed: (1) Nutrient agar prepared with sea water (for total heterotrophs), (2) Kuster's agar (actinomycetes), (3) Thiosulfate-citrate-bile salt-sucrose (TCBS) agar (*Vibrio*), (4) nitrogen-free medium (nitrogen fixers) and (5) sodium thioglycollate agar (anaerobes). Duplicate sets of the plates were incubated at two different temperatures—one set at room temperature ($27 \pm 2^\circ\text{C}$) and the other at 5°C . The colonies appearing on the plates incubated at room temperature were counted after 5 days, and those on plates incubated at 5°C were counted after 2 to 3 weeks of incubation, when the maximum number of colonies had appeared. The populations were calculated on a dry weight basis.

In order to study the characteristics of bacteria that developed from nodules and sediments, about 200 colonies were isolated at

random from all media. However, only 120 of these were used for testing manganese-oxidizing and -reducing activities. After their purity was checked, they were tested by conventional methods for amylolytic, gelatinolytic, lipolytic, phosphate-solubilizing, nitrate-reducing, and sugar-fermenting (sucrose and lactose) activities. These cultures were also examined for their sodium chloride requirement and tolerance by being grown in peptone broth supplemented with different concentrations of salt. The isolates were identified following the scheme suggested by Schewan et al. (1969) for marine bacteria and according to Bergey's *Manual of Determinative Bacteriology* (Buchanan and Gibbons, 1974).

Representative cultures from sediment and nodule were tested for the ability to oxidize Mn^{2+} (accretion) and reduce $Mn(IV)$ (dissolution) under laboratory conditions following methods described by Ehrlich (1966). All measurements of manganese concentration in test solutions were made by using an atomic absorption spectrophotometer (Perkin-Elmer AAS, Model 5000).

Results

Bacterial Populations Associated with the Nodule and Sediments

Plates incubated at room temperature ($28 \pm 2^\circ C$) always showed higher populations than those kept at low temperature ($5^\circ C$), irrespective of the media used (Table 1). It was also observed that distinct differences in population density existed between the two nodules tested. However, the surface of the nodules exhibited higher populations when compared with the core region. In the case of low-temperature incubation, the core population was almost nil on pour plates. However, when the capping-agar technique was used, significant populations were recorded from the core region. Since sufficient care was taken to avoid contact of core samples with the other portions of the broken nodule, the observed population in the core might have been an autochthonous one. In the case of the sediments, the capping-agar technique always yielded higher population when compared with

Table 1
Bacterial Population Associated with Nodule and Sediment

Sample	Area	Bacterial Population (no./g)					
		Nutrient Agar		Thiosulfate-Citrate-Bile-Salt-Agar		N ₂ -free Medium	
		28 ± 2°C	5°C	CA-28 ± 2°C ^a	28 ± 2°C	5°C	28 ± 2°C
Nodule-A	Surface	13.2 × 10 ⁵	70.2 × 10 ⁴	43.9 × 10 ⁴	15.8 × 10 ⁴	NC	NC
	Core	10.0 × 10 ⁴	NC ^b	15.6 × 10 ⁴	12.5 × 10 ⁴	NC	NC
Nodule-B	Surface	22.1 × 10 ⁶	39.2 × 10 ⁵	84.6 × 10 ⁵	0.8 × 10 ⁴	3.0 × 10 ⁴	10.9 × 10 ⁶
	Core	71.4 × 10 ⁴	NC ^c	14.3 × 10 ⁵	NE ^c	NE	7.1 × 10 ⁴
Sediment	Nodule area	19.4 × 10 ⁵	12.1 × 10 ⁵	34.6 × 10 ⁵	1.6 × 10 ⁴	NC	5.1 × 10 ⁴
Sediment	Non-nodule area	84.0 × 10 ⁴	21.8 × 10 ⁴	95.0 × 10 ⁴	0.6 × 10 ⁴	NC	6.3 × 10 ⁴

^aCA-28 ± 2°C = Capping-agar plates incubated at 28 ± 2°C.

^bNC = No colony growth.

^cNE = Not estimated.

pour plating. The results also show that more populations existed in the sediment sample collected from the nodule area than in that from the non-nodule area. *Vibrio*-like organisms (VLO) could be isolated readily from both nodule and sediment samples when TCBS medium was used. Generally, nodules exhibited more VLO than the sediments. As to bacteria capable of growing on nitrogen-free medium, only one nodule of the two tested showed the presence of this group, which accounted for 49.1% of the total surface population and 9.9% of the total core population. In this instance we also noted a greater population density at the higher incubation temperature than at 5°C. Both sediment samples harbored this group. No appreciable difference between the nodule and non-nodule area was noticed. No actinomycete or fungal colonies were recorded, even though a special medium (Kuster's agar) was employed for their cultivation. Similarly, no anaerobic forms were detected.

Bacterial Characteristics

Both gram-positive and gram-negative bacteria were detected in nodules and sediments (Table 2). However, most of the isolates were found to be gram-positive. They included sporing forms, nonsporing rods, and coccoid forms. Gram-positive nonsporing forms constituted as much as 62% of the nodule surface population, whereas spore formers accounted for only 17.6%. However, the sediment from the nodule area yielded smaller populations of these forms than those from sediment of the non-nodule area. Aerobic spore formers, *Bacillus* spp., were present in all the samples examined. The nodule surface harbored the greatest population. Cocci were found to be present only in the nodule core and the nodule area sediment. More gram-negative bacteria were recorded in the core of the nodule than from the nodule surface. Among the sediment samples, the nodule area harbored more of this form than the non-nodule area. Another interesting observation was that the greatest percentage (38.1%) of the motile forms was observed in the core of the nodule. Besides *Bacillus* spp. and *Vibrio* spp., members of *Staphylococcus*, *Micrococcus*, Enterobacteriaceae, and *Arthrobacter* (coccoid

Table 2
Percentage Occurrence of Various Morphological, Physiological,
and Biochemical Groups of Bacteria in Nodule and Sediment

Characteristics	Nodule		Sediment	
	Surface (34)*	Core (21)*	Nodule Area (21)*	Non-nodule Area (16)*
Gram-negative rods	14.7	28.6	23.8	12.5
Gram-positive sporing rods	16.7	9.5	4.8	6.3
Gram positive nonsporing rods/plcomorphic	64.7	42.6	52.4	68.8
Gram-positive cocci	0.0	14.3	14.3	0.0
Growth with NaCl				
0%	58.9	75.0	61.9	50.0
0.5%	91.2	90.0	100.0	100.0
1.5%	100.0	100.0	100.0	100.0
3.0%	100.0	100.0	100.0	100.0
7.0%	100.0	90.0	100.0	100.0
10.0%	94.1	85.0	100.0	100.0
Utilization of sugars				
Sucrose	50.0	57.1	76.2	87.5
Lactose	64.7	14.3	14.3	68.8
Nitrate reduction	76.4	66.7	42.8	25.0
Lipolytic	50.0	52.3	42.8	56.2
Gelatinolytic	61.7	42.8	47.6	68.7
Amylolytic	29.4	19.0	14.2	56.2
Phospholytic	44.1	42.8	61.9	81.2

*Total number of isolates tested.

forms) were found to be associated with the nodules. Out of the 92 strains examined, *Bacillus* constituted 10.87% and *Vibrio* 18.48%. The majority of the isolates (61.95%) were found to be gram-positive nonsporing rods, probably belonging to coryneforms (*Brevibacterium*).

Physiological Properties

Most of the isolates were able to grow in a wide range of salinities—i.e., from 0% to 10.0% NaCl concentrations (Table 2). However, it is clear from the results that the majority preferred an elevated concentration of NaCl. In the case of the nodules, as many as 94% of the isolates tested exhibited moderate growth at 10% NaCl, whereas at 0% NaCl only 58.9% showed weak growth. Likewise, all the sediment isolates tested exhibited growth at 10% NaCl, indicating the preference of isolates to higher concentration of NaCl (Table 2). Of the two sugars tested, sucrose was preferred by most of the isolates, which produced acid. A higher number of sucrose-positive strains were recorded from sediments than from nodules. The greatest number of nitrate reducers were encountered at the surface of the nodule, and the next greatest were found in the core region (Table 2). The sediments also harbored nitrate reducers. The lowest percentage (25.0%) occurred in the sediment sample collected from the non-nodule area.

The isolates were found to form a number of hydrolytic enzymes (Table 2). Sediment from the non-nodule area harbored the greatest percentage of lipolytic, gelatinolytic, amylolytic, and phosphate-solubilizing bacteria. Around 50% of the total isolates were lipolytic. Amylolytic strains associated with the nodules and the nodule area were strikingly low in number when compared with those from the non-nodule area. The greatest numbers of gelatinolytic and phosphate-solubilizing bacteria (68.7% and 81.2%, respectively) were also observed in sediment from the non-nodule area. The smallest percentage of bacteria exhibiting these properties was recorded in the core of the nodules.

Even though more than 3200 isolates were recovered, only 50

nodule isolates and 36 sediment isolates were tested for their reaction to manganese. Based on their reaction, they could be divided into three categories: Mn(II) oxidizers, MnO₂ reducers, and inactive forms. MnO₂ reducers dominated over Mn(II) oxidizers both in nodule and sediment samples (Table 3). Maximum percentage (32.2%) of oxidizers was noticed on the surface of the nodule, and the minimum (6.7%) was found in the non-nodule area. Compared with sediments, nodules always harbored a greater number of oxidizers. Similarly, the greatest amount of reducers was found to be associated with the nodule. In general, sediments harbored more inactive forms. The greatest percentage (66.7%) of inactive forms was encountered in the non-nodule area sediment. The generic distribution of oxidizers, reducers, and inactive forms isolated from nodules and sediments is given in Table 4.

Discussion

The foregoing results suggest that the microbial populations associated with Indian Ocean nodules are, in general, comparable to those of the Pacific and Atlantic Oceans (Ehrlich et al., 1972). The present study clearly shows that heterotrophic bacteria capable of oxidizing Mn(II) and reducing MnO₂ are commonly present in nodules and sediments. No attempt was made in the present study to isolate autotrophic or obligate manganese-requiring organisms. It may also be pointed out here that all the

Table 3
Percentage Occurrences of Mn(II) Oxidizers, MnO₂ reducers,
and Inactive Forms in Nodule and Sediment

Sample	Area	No. of Isolates Tested	Mn(II) Oxidizers	MnO ₂ Reducers	Inactive Forms
Nodule	Surface	31	32.2	48.3	19.3
	Core	19	26.3	52.6	21.0
Sediment	Nodule area	21	9.5	33.3	57.0
	Non-nodule area	15	6.7	26.6	66.7

Table 4
Generic Distribution of Oxidizers, Reducers, and Inactive Forms
from Nodule and Sediment

Genera	Total No. of Isolates	No. of Isolates Tested	Reaction group (percent)			
			Oxidizers	Reducers	Combination Oxidizers-Reducers	Inactive
<i>Vibrio</i>	17	17	23.5 (4)*	23.5 (4)	17.7 (3)	35.3 (6)
Enterobacteriaceae	2	2	50.0 (1)	50.0 (1)	—	—
<i>Micrococcus</i>	1	1	—	—	—	100.0 (1)
<i>Arthrobacter</i>	2	2	—	50.0 (1)	—	50.0 (1)
<i>Staphylococcus</i>	3	3	—	33.3 (1)	—	66.7 (2)
<i>Bacillus</i>	10	10	20.0 (2)	30.0 (3)	20.0 (2)	30.0 (3)
Coryneforms	57	51	19.6 (10)	33.3 (17)	5.9 (3)	41.2 (21)
Total	92	86	19.8	31.4	9.3	39.5

*Number of positive isolates.

isolates tested in the present study were of psychrotrophic nature (capable of growing between 5° and 30°C). In general, Mn(IV)-reducing bacteria seemed to be more dominant than Mn(II)-oxidizing bacteria in both sediments and nodules, as was reported earlier for Pacific and Atlantic nodules by Ehrlich et al. (1972). The ratios of oxidizers to reducers from the four types of samples (nodule surface, nodule core, and sediments from the nodule area and non-nodule area) are 0.66, 0.50, 0.30, and 0.26, respectively, suggesting that the nodule surface is the site of greatest oxidation. The greatest number of inactive forms was seen in the non-nodule area sediment. Some isolates exhibited both oxidizing and reducing properties (Table 4). As suggested by Ehrlich (1972), the observation that oxidizers are exceeded by the reducers does not indicate that the nodules were undergoing active Mn(IV) reduction. Besides, both these forms can grow heterotrophically without metabolizing Mn compounds. It is also true that both processes (i.e., oxidation and reduction) occur simultaneously. The direction in which the reaction should proceed is greatly dependent on certain physicochemical conditions and the extent of availability of organic matter (Ehrlich, 1972). Since deep sea waters are generally oligotrophic, it is possible that some of the oxidizers are mixotrophic. There is evidence to show that nodules contain sufficient amounts of organic matter to support a diversified microbial community (Greenslate, 1974). The present study also supports this from the fact that a variety of heterotrophic bacteria could be isolated and that bacteria capable of elaborating a number of hydrolytic enzymes were present (Table 2). In the nodule, both lipolytic and proteolytic bacteria were more common than amylolytic forms. Schütt and Ottow (1978) also reported a similar pattern in the case of Pacific nodules.

Nearly 90% of the nodule isolates and 100% of the sediment isolates grew well, even at 10% NaCl concentration. This property seems to be common even with nodule strains isolated from other areas. For example, Schütt and Ottow (1978) reported that 100% of the isolates grew well at 5% NaCl, and nearly 70% of them exhibited good growth even at 15% NaCl. Tolerance to higher NaCl concentrations may be a common property among

marine bacteria (Nealson, 1978), but it is interesting to note that NaCl is needed for MnO₂ reduction by *Bacillus* sp. (Ghiorse and Ehrlich, 1974).

The two nodules examined differed in the size of populations of nitrogen-fixing bacteria that they supported. Although one nodule showed a total lack of these bacteria, the other nodule contained a population of 10⁶ to 10⁷ g⁻¹. This observation supports our contention that no nitrogen source from the original sample was carried over in dilution and allowed some non-nitrogen fixing bacteria to grow on the media. Sediments from the nodule and the non-nodule areas harbored a very low population of nitrogen fixers. Interestingly, only when the sediment samples were incubated at room temperature could these bacteria be detected. Psychrophilic nitrogen fixers could, on the other hand, be recovered from both the surface and the core of the nodules. The nodule surface yielded the greatest number of nitrogen fixers, compared with other types of samples.

Even though both gram-positive and gram-negative bacteria were present in the nodule samples, the gram-positive group dominated (79%). A comparison of bacterial types isolated from the Pacific and Atlantic Oceans clearly indicated the dominance of gram-positive groups in Atlantic nodules (Ehrlich et al., 1972) and gram-negative bacteria in Pacific nodules. The dominance of gram-positive bacteria in Atlantic nodules and sediment was attributed to the proximity of the collecting site (Black Plateau) to the U.S. coast. A similar reasoning can also be applied to the present study, since the samples were collected from a site that is known to be influenced by terrigenous supply (Cronan and Moorby, 1981). It is also possible that the predominance of gram-positive coryneform bacteria, some of which are well-known psychrophiles, and spore-forming bacteria, which are well known to resist temperature extremes, may have been influenced by freezing and thawing. Schütt and Ottow (1978) reported that all 67 cultures isolated from Central Pacific Ocean nodules were gram-negative. More interestingly, they also found that 94% of the isolates were *Pseudomonas* spp. and the rest were *Aeromonas* spp. On the basis of the bacterial types, the Indian Ocean nodules appear to be more like the Atlantic Ocean nodules than

the Pacific ones. More often, species of *Bacillus*, *Staphylococcus*, *Micrococcus*, *Arthrobacter*, coryneforms, and *Vibrio* were encountered in manganese nodules (Table 4) (present study and Ehrlich, 1963, 1972).

Besides containing these bacteria, nodules also have been shown to harbor foraminifera and other shelter-building protozoa (Dugolinsky et al., 1977). Thiel (1978) also feels that some of the agglutinating foraminiferal species actively contribute to the metalliferous growth of manganese nodules. However, in the present study such associations were not recorded. Perhaps the limited number of nodules examined might have been the major reason. Unless a larger number of specimens is examined, such associations can not be ruled out. As pointed out earlier (Ehrlich, 1972), these observations should be interpreted with caution because of the following reasons: (1) only limited number of culture media were used for primary isolations; (2) no special care was taken to avoid contamination by sea water while the nodules were lifted from the bottom of the sea; (3) the estimation of bacterial populations was done at atmospheric pressure, which might have eliminated a number of obligate barophiles; and (4) the analyses were carried out with stored samples, which could have eliminated some sensitive bacteria and allowed others that were not harmed by freezing and thawing to survive. Our results also could have been influenced by storage, including the magnitude of plate counts, the proportion of salt-tolerant bacteria, and even the proportion of manganese oxidizers and reducers. Because several workers employed methods of sampling similar to those in the present study, we were able to make some reasonable comparisons.

It is clear from the present study that Indian Ocean nodules harbor a variety of heterotrophic bacteria that are, as reported from the Pacific and Atlantic Ocean nodules, capable of mobilizing and immobilizing the element manganese. The microbial characteristics differ from one nodule to another, even for specimens collected from the same area. Perhaps this may be one of the other reasons that can be attributed to the observed variations in the elemental composition of nodules (Siddiquie et al., 1978; Sharma and Somayajulu, 1983) besides varying depositional

conditions (Mallik, 1980). Further studies under in-situ conditions would throw more light on the extent and activity of microbial flora associated with nodules.

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