NUTRIENT CYCLING IN THE ANTARCTIC MARINE ENVIRONMENT

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ABSTRACT. Samples of marine mud and sea-water obtained from several depths off the coast of Signy Island have been examined, using liquid culture-enrichment techniques, for bacteria capable of cycling nitrogen and sulphur compounds. The results obtained indicate that, with the exception of nitrifying bacteria, those capable of carrying out the specialized reactions of the sulphur and nitrogen cycles have been isolated from all the marine samples so far examined. In addition, the data show that most of these bacteria are able, albeit more slowly, to carry out these reactions at low temperatures (*c.* 5° C).

ALTHOUGH the existence of bacteria capable of growth at low temperatures has been known for many years (Forster, 1887; Ingraham and Stokes, 1959; Morita, 1966; Farrell and Rose, 1967), the effects of low temperature on the ecology, physiology and metabolism of this important group of organisms is not well understood. From the literature cited, it is clearly evident that psychrophilic (cold-loving) bacteria are widely distributed in nature. They are known to occur in temperate as well as polar regions and from the applied aspect are of considerable economic importance in the spoilage of chilled foods. However, our knowledge of the role played by these organisms in the natural environment is as yet limited, although it is probable that they play a significant role in mineralization processes, especially in cold environments. In this respect, the marine environment is of considerable interest since Zobell (1963) reported that >95 per cent by volume of oceanic waters are colder than 5° C. The mean annual temperature of the Antarctic marine environment is −0·8° C (personal communication from I. Everson) and, owing to its remoteness from the populated landmasses of the world, it is relatively free from pollution. It is therefore an ideal environment for isolating and studying bacteria capable of carrying out nutrient cycling at very low temperatures.

The literature available on bacteria in the Antarctic marine environment is sparse and almost wholly confined to preliminary observations. Walls (1967) collected sediment and water samples from various points along the Antarctic Convergence and concluded that the bacteria from the Antarctic marine sediments were similar to those found in other oceans. Preliminary data by Morita and others (1971), who also took samples along the Antarctic Convergence, indicate that significant microbial metabolic activity could be detected at temperatures as low as −3° C. No data, apart from the preliminary observations of Walls (1967) on heterotrophic bacteria, however, appear to be available on the species distribution of bacteria in the Antarctic marine environment. The objective of the present study was first to ascertain whether bacteria capable of transforming nitrogen and sulphur compounds are present in the Antarctic marine sediments and sea-water and, secondly, to determine whether or not these organisms were physiologically active at low temperatures.

METHODS

Samples of marine mud and sea-water from several depths (2, 5, 10, 20, 30 and 50 m.) were collected from off the coast of Signy Island, South Orkney Islands, in March 1972. The water samples were collected in National Institute of Oceanography reversing water bottles from near Bare Rock, Borge Bay, and the sediment samples from the vicinity of Small Rock, Factory Cove. The sediment samples were obtained by diver and care was taken to minimize contamination of the samples by organisms in the waters overlying the deposits.

The enrichment media, inoculation techniques, incubation temperatures and times used are as described previously (Herbert and Bell, 1973) with the one exception that all the enrichment media were supplemented with 3 per cent w/v sodium chloride.

Wet-film and Gram-stained preparations were made from the liquid-enrichment cultures and examined by phase-contrast and bright-field microscopy, respectively. Motility was determined by using the hanging drop technique. Identification of the isolates to genus level

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was based on nutritional requirements, Gram stain, morphology and motility according to the key devised by Skerman (1967).

Results

Examination of the liquid enrichments for heterotrophic nitrogen-fixing bacteria showed the presence of large, motile cocco-bacillary rods in those cultures inoculated with the marine muds. No growth was observed in enrichments inoculated with samples of sea-water. Each enrichment was assayed for nitrogen fixation using the acetylene-reduction technique (Stewart and others, 1967) at 5° and 20° C. All of the enrichments inoculated with marine mud samples showed positive nitrogen fixation at both temperatures. Greatest activity was observed with the 10 and 30 m. samples. No activity could be detected in the sea-water enrichments. Serial ten-fold dilutions from each of the positive mud-sample enrichments were prepared and 0·5 ml. volumes plated on to media, of the same composition, solidified by the addition of 1 per cent Ionagar No. 2 (Oxoid Ltd). The plates were incubated at 20° C for 4 days. Small translucent colonies appeared and these became yellowish on ageing. Examination of wet-film and Gram-stained preparations showed the presence of Gram-negative, large (3–5 μm.), motile cocco-bacillary rods. These data together with good growth on N-free media and the active fixation of nitrogen provide positive evidence that the bacteria involved are *Azotobacter* sp. Enrichment culture for *Clostridium pasteurianum*, a nitrogen-fixing obligate anaerobe, has failed to isolate this organism from any of the samples so far examined. Similarly, all attempts to isolate nitrifying bacteria of the genera *Nitrosomonas* (convert NH_4-N to NO_2-N) and *Nitrobacter* sp. (convert NH_4-N to NO_3-N) have so far failed.

Enrichment cultures for denitrifying bacteria using Giltay's medium showed rapid growth of denitrifiers at both 5° and 20° C. Denitrifying bacteria were found in all the mud and water samples examined. Examination of wet-film and Gram-stained preparations showed the predominance of Gram-negative, asporogenous motile rods and Gram-positive cocci. Preliminary biochemical tests on 25 pure cultures of the Gram-negative rods show that they are all oxidase and catalase positive and use glucose oxidatively. These data suggest that the Gram-negative rods are most probably *Pseudomonas/Achromobacter* sp., whilst the non-motile cocci are *Micrococcus* sp. Bacterial growth and gas production was observed in all the liquid enrichments for *Thiobacillus denitrificans* inoculated with the marine mud samples. No growth or gas evolution was observed in enrichments inoculated with samples of sea-water. Serial ten-fold dilutions were prepared from the positive enrichments and 0·5 ml. volumes plated out on media of the same composition solidified by the addition of 1 per cent Ionagar. Small translucent colonies appeared after 4 days at 20° C and examination of slide preparations showed the presence of small, motile Gram-negative rods. Inoculation of pure cultures from the plates into the liquid medium resulted in growth and gas production. Tests with Griess-Llosvay reagent showed the disappearance of NO_3-N from the medium. These data provide positive evidence for the presence of *Thiobacillus denitrificans* in the mud samples.

The results in Table I show that considerable numbers of proteolytic bacteria, as measured by gelatin hydrolysis, are present in the marine muds and sea-water samples examined. No attempt, however, has been made to classify these bacteria although Gram-stained preparations show that they are predominantly Gram-negative rods.

Results of the liquid enrichments for sulphate-reducing bacteria show that these organisms were present in all the mud samples but none of the water samples. Slide preparations from the positive enrichments showed the predominance of Gram-negative, motile pleomorphic rods. These features together with the extremely active formation of H_2S indicate the presence of sulphate-reducing bacteria of the genus *Desulfovibrio*. No Gram-positive, spore-forming sulphate-reducing bacteria of the genus *Desulfotomaculum* have been observed in any of the positive enrichments examined.

Enrichment cultures for purple and green photosynthetic sulphur bacteria have so far only yielded purple sulphur bacteria from the mud samples. These organisms are slow to develop, requiring incubation periods in excess of 12 weeks at 20° C before growth was apparent. Slide preparations from the positive enrichments showed the presence in all of the mud samples of Gram-negative, motile, single small (1 μm. diameter) cocci which contained refractile inclusions. Pigmentation varies from a pale pink to deep red suggesting that several species

TABLE I. DISTRIBUTION OF PROTEOLYTIC BACTERIA IN ANTARCTIC MARINE MUDS AND SEA-WATER

Sample and depth	*Proteolytic bacteria* Incubation temperature* 5° C	20° C
Water (2 m.)	$21 \cdot 3 \times 10^3$	33×10^3
Water (20 m.)	27×10^3	38×10^3
Mud (5 m.)	$13 \cdot 9 \times 10^4$	$91 \cdot 1 \times 10^4$
Mud (10 m.)	31×10^3	61×10^3
Mud (20 m.)	17×10^3	71×10^3
Mud (30 m.)	29×10^3	43×10^3
Mud (50 m.)	25×10^3	60×10^3

* Bacterial count/ml. of sea-water or 1 g. of wet mud.

may be present. As yet we have not succeeded in isolating any of these organisms in pure culture.

Bacterial growth was observed in all the aciduric and non-aciduric *Thiobacillus* enrichments inoculated with the marine muds and sea-water. Bacterial growth was initially slow and appeared as a pellicle on the surface of the medium, whilst in ageing cultures the whole medium became turbid. Ten-fold dilutions were prepared from each of the positive enrichments and 0·5 ml. volumes plated out on to media of the same composition solidified by the addition of 1 per cent Ionagar. Small translucent colonies appeared after 3–4 days incubation at 20° C and slides prepared from these colonies showed the presence of Gram-negative, regularly shaped, small motile rods. 50 colonies were picked off the plates and inoculated into the liquid enrichment medium and the disappearance of thiosulphate monitored by titration with 0·1 N iodine using starch as the indicator (Vogel, 1961). Thiosulphate was observed to disappear from the medium as bacterial growth occurred in the inoculated enrichments but not in the uninoculated controls. These data provide positive evidence for the presence of *Thiobacillus* sp. in the mud and water samples examined.

Data presented in Table II show the incidence of bacteria capable of degrading organic

TABLE II. INCIDENCE OF H_2S-PRODUCING BACTERIA IN ANTARCTIC MARINE MUDS AND SEA-WATER

Sample and depth	*H_2S-producing bacteria* Incubation temperature* 5° C	20° C
Water (2 m.)	6×10^3	23×10^3
Water (20 m.)	35×10^3	1×10^4
Mud (5 m.)	49×10^3	16×10^4
Mud (10 m.)	56×10^3	21×10^4
Mud (20 m.)	3×10^4	39×10^3
Mud (30 m.)	41×10^3	9×10^4
Mud (50 m.)	18×10^3	79×10^3

* Bacterial count/ml. of sea-water or 1 g. of wet mud.

material with the release of hydrogen sulphide (H_2S) gas. Higher counts were obtained from the sediments than from the water samples. No attempt has been made to classify these organisms, although Gram-stained preparations show that the organisms involved are Gram-negative rods.

Discussion

The data show that, with the exception of nitrifying bacteria, bacteria capable of carrying out the specialized reactions of the nitrogen and sulphur cycles are present in Antarctic marine muds and sea-water. Whilst nitrifying bacteria are known to occur in marine waters and sediments (Issatschenko, 1914; Watson, 1963), their absence has also been noted. Thus, Gran (1903) was unable to isolate nitrifying bacteria from Norwegian fjord sediments. Spencer (1956) was of the opinion that the inability to isolate nitrifying bacteria from open ocean waters may not be due to the absence of these organisms but more a failure to supply iron in sufficient quantity in the growth medium. It is unlikely that iron is the limiting factor in the media we have used for the isolation of *Nitrobacter* and *Nitrosomonas* sp. since the Fe^{++} concentration, in the form of ferrous sulphate, is in excess of that suggested by Spencer (1956). The inability to isolate from liquid enrichments any strains of spore-forming N-fixing (*Clostridium pasteurianum*) and SO_4-reducing (*Desulfotomaculum* sp.) anaerobic bacteria suggests that these organisms may not be present in the Antarctic environment. There appears to be a general absence of spore-forming bacteria in the samples of marine mud, sea-water and fresh-water sediments and fresh water from Signy Island so far examined (unpublished data of R. A. Herbert) and this leads to the conclusion that the absence of these organisms may be a characteristic of cold environments. Other workers, notably Jensen (1951), who indicated that spore-forming bacteria were rare in Greenland soils, and Mishustin and Mirzoeva (1964), who obtained similar results from their studies of the Soviet tundra, support this view.

Using the acetylene-reduction technique (Stewart and others, 1967), the data show that the *Azotobacter* sp. are physiologically active at 5° C, which is of some interest since *in vitro* studies of nitrogenase indicate that this enzyme is cold-labile (personal communication from W. D. P. Stewart). Further studies are now in progress to determine whether the *Azotobacter* sp. isolated will fix nitrogen at lower temperatures (*c.* 0° C). Data from the denitrification enrichments show that this process is appreciable even at 5° C and indicate that many of the bacteria present in the marine mud and sea-water samples have the capacity to utilize nitrate as the terminal electron acceptor in the absence of oxygen. Proteolytic bacteria capable of degrading proteinaceous material also appear to be widely distributed in the mud and water samples. Greater activity, as measured by the size of the zones of gelatin hydrolysis and higher counts, was observed at 20° C than at 5° C. However, insufficient samples have been analysed to determine whether or not the higher bacterial counts obtained at 20° C are statistically significant.

The results show that hydrogen sulphide (H_2S) in the marine sediments can be generated by two groups of organisms. Thus H_2S was produced from inorganic sulphate by *Desulfovibrio* sp. and from proteinaceous material by heterotrophic bacteria. The viable counts obtained for the H_2S-producing bacteria are not absolute figures, since growth will inevitably have occurred from the time when the samples were collected to when they were examined. Nevertheless, the data show the relative distribution of these bacteria in the mud and water samples. As is to be expected, the main activity was found in the sediments where anaerobic conditions favouring H_2S production prevail. Purple sulphur-photosynthetic bacteria, which use H_2S as an electron donor in photosynthesis, have been isolated from all of the marine sediment samples, indicating that sufficient radiant energy penetrates even to a depth of 50 m. for these organisms to develop. These bacteria, under laboratory conditions, are extremely slow growing and would appear to have rather exacting light-energy requirements. For this reason, we have so far failed in our attempts to isolate these organisms in pure culture.

The presence of the facultative anaerobe *Thiobacillus denitrificans* in all the sediment samples is probably of some importance since it provides a link between the nitrogen and sulphur cycles. This organism is able to oxidize reduced sulphur compounds under anaerobic conditions using NO_3-N as the terminal electron acceptor. Under aerobic conditions, such as those found at the surface of the sediments and in the water, aciduric and non-aciduric

Thiobacillus sp. are probably actively involved in the oxidation of reduced sulphur compounds.

With the exception of the purple sulphur-photosynthetic bacteria all the bacteria will grow, albeit more slowly, and carry out the reactions of the nitrogen and sulphur cycles at 5° C. Further data are now required to determine the effect of lowering the temperature to 0° C on the growth rate and metabolism of these organisms. In addition, *in situ* field experiments are required to determine the rates at which these nutrient transformations occur in the natural Antarctic marine environment.

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