BAROPHILIC BACTERIA IN SOME DEEP SEA SEDIMENTS[1]

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Barophile is a term coined by ZoBell and Johnson (1949) to describe bacteria which grow preferentially or exclusively at moderately high hydrostatic pressures (Johnson *et al.*, 1954). Most terrestrial bacteria appear to be affected adversely by pressures of 200 to 600 atm. Similar results were obtained by Oppenheimer and ZoBell (1952) working with marine bacteria, virtually all of which were inhibited by pressures ranging from 200 to 600 atm, although a few deep sea cultures reproduced at 1,000 atm.

Little is known regarding the occurrence, characteristics, and physiological activities of bacteria on the deep sea floor, where pressures approaching 1,100 atm prevail, although fragmentary observations indicate that shallower marine muds on the continental slopes support bacterial populations comparable to those occurring in soil (ZoBell, 1946; Limberg-Ruban, 1952). Even organic-poor pelagic sediments, including red clay and globigerina ooze collected at stations several hundred miles from the nearest land and from water depths ranging from 1,707 to 5,942 meters during the Mid-Pacific Expedition to the Marshall Islands, were shown by Morita and ZoBell (1955) to contain from 10 to 10^4 viable bacteria per gram. The Danish *Galathea* 'Round-the-World Expedition (Spärck, 1952) afforded an opportunity to demonstrate bacteria in a large number of sediment samples taken from depths of 7,000 to more than 10,000 meters. The pressures at these depths are about 700 to more than 1,000 atm, the pressure depth gradient in the sea being approximately 0.1 atm per meter. One atmosphere is equivalent to 1.0332 kg per cm^2, 1.0133 bars, or 14.696 psi.

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MATERIALS AND METHODS

The R.D.N. *Galathea*, a 1,630-ton corvette converted for studying the deep sea floor, was equipped with radar, loran, sonic depth recorders, and other modern navigational aids. Locating oceanic deeps and staying on station for prolonged periods while taking bottom samples required the best of navigation, for which a high tribute is due Captain Sven Greve and his staff of 90 Danish seamen. The ship was equipped with a tapered dredging cable having dimensions as shown in table 1. The tapering was designed to provide a cable that could support its own weight in the water with a load of 26,000 kg from the greatest depths in the sea (Wüst, 1950; Carruthers and Lawford, 1952).

Radially central subsections of bottom sediment were taken aseptically from cores or grab hauls of mud for bacteriological examination immediately after being hauled on deck. Hauling samples from a depth of 10,000 m required from 8 to 18 hr, during which the pressure changed from 1,000 atm at the bottom to about 1 atm at the surface, and the temperature changed from about 3 C at the bottom to 30 C at the surface in tropical waters. The effect of these changes in climate on the deep sea bacteria is yet to be determined. Many survived, but we have no way of knowing how many perished. Obviously our observations are on the survivors, which slowly die off at 30 C or when held in the refrigerator at normal atmospheric pressures. Most marine bacteria are thermosensitive, many being killed in 10 min at a temperature of 30 C or less (ZoBell and Conn, 1940). In order to minimize mortality, samples were processed as soon as possible. Part of the material was used at once to inoculate nutrient media for incubation at different temperatures and pressures. The remainder of the sediment sample was stored in the refrigerator (3 to 5 C) in pressure cylinders at *in situ* pressures (700 to 1,000 atm).

The nutrient medium employed for demon-

TABLE 1

Dimensions of dredging cable

Section No.	Diameter	Length
	(*mm*)	*m*
1	9.3	3,600
2	11.6	1,750
3	13.2	770
4	14.7	1,330
5	17.1	1,730
6	19.6	1,080
7	20.2	980
8	21.8	1,060
Total		12,300

strating the most probable number (MPN) of bacteria by the minimum dilution method consisted of peptone (Difco), 5.0 g; soluble starch, 2.0 g; KNO_3, 1.0 g; yeast extract (Difco), 1.0 g; and $FePO_4$, 0.1 g. per L of aged sea water (salinity 25 per mille). Following autoclave sterilization the pH was 7.7. It was dispensed in 9.0-ml quantities in 15-ml screw cap bottles for preparing proper dilutions of inoculated sediment sample (diluted seriatim by powers of 10) and for incubating at 1 atm. Portions of the inoculated medium were transferred aseptically to small glass vials (10 by 50 mm) for incubation at high pressures. The vials were closed with tapered No. 000 Neoprene stoppers, which functioned as pistons when the tubes were subjected to pressure in steel cylinders (ZoBell and Oppenheimer, 1950) filled with hydraulic fluid. Air was introduced in part of the vials for estimating MPN of aerobes; free oxygen was excluded from other vials employed for estimating MPN of anaerobes. Sulfate reducing bacteria were detected and cultivated in medium M-10E: K_2HPO_4, 0.2 g; $MgSO_4 \cdot 7\ H_2O$, 0.2 g; Ca lactate, 3.5 g; ascorbic acid, 1.0 g; $FeSO_4\ (NH_4)_2SO_4 \cdot 6\ H_2O$, 0.1 g; peptone (Difco), 1.0 g; yeast extract (Difco), 1.0 g; agar (Difco), 3.0 g; and aged sea water (salinity 25 per mille), 1000 ml.

The inoculated media were incubated for a minimum of 2 weeks at the stated temperatures and pressures. Turbidity and direct microscopic examination (in questionable cases) were employed as criteria of growth or reproduction of bacteria. Portions of media in which bacteria grew were treated with iodine reagent to determine starch hydrolysis. Other portions were examined for the appearance of nitrite or for the disappearance of nitrate as criteria of nitrate reduction. An increase in the ammonium content of the medium was indicative of the activity of ammonifiers. The production of black ferrous sulfide in the anaerobic series indicated the activity of sulfate reducers.

RESULTS AND DISCUSSION

Data summarized in table 2 are representative of results which, for the first time, prove the presence of living bacteria at depths exceeding 10,000 meters. Prior to the *Galathea* Expedition, 5,942 meters was the greatest depth at which bacteria had been found. In numerous sediment samples taken at depths of about 10,000 meters were found from 10^4 to 10^5 viable bacteria per gram wet weight. In overlying water, except that closely associated with bottom deposits, the bacterial population was generally sparse, ranging from less than 1 to no more than 10^3 viable bacteria per ml. This should dispel any doubt that the bacteria detected in the bottom sediment samples could possibly be contaminants from overlying water through which the deep sea sampling devices passed. Moreover, unlike most bacteria collected from shallow depths, most of those in the deep sea sediment samples grew preferentially or exclusively at high pressures and at refrigeration temperatures. Less than 10 per cent of the aerobic bacteria from the bottom of the Philippine Trench grew at either 30 C or at 1 atm.

Direct phase contrast microscopic examinations of sediment samples showed appreciably larger bacterial populations than the MPN indicated by growth in nutrient media, probably owing to the death of some bacteria in transit to the surface and to the inadequacy of the media to provide for the cultural requirements of all deep sea bacteria. Since there is evidence for the activity of bacteria on the deep sea floor, we were not surprised to find large bacterial populations. Nor did it seem surprising that bacteria grew at the high hydrostatic pressure and low temperatures characteristic of oceanic deeps. It seems singularly significant, though, that most of the bacteria from the Philippine Trench behaved as obligate barophiles. A good many tests made on those which grew at 1,000 atm demonstrated their inability to grow in similar media incubated at 1 atm. Similarly, among the many cultures tested, none which grew at 1 atm did so when incubated at 1,000 atm.

TABLE 2

MPN of different physiological types of bacteria detected per gram of wet sediment from the Philippine Trench in selective media incubated at different hydrostatic pressures at 3 to 5 C

	Galathea Station							
	No. 418		No. 419		No. 420		No. 424	
Latitude Longitude Water depth	10°13 N 126°43′ E 10,190 m		10°19 N 126°39′ E 10,210 m		10°24′ N 126°40′E 10,160 m		10°28′ N 126°39′ E 10,120 m	
	Incub. pressure (atm)							
	1	1,000	1	1,000	1	1,000	1	1,000
Total aerobes	10^3	10^6	10^3	10^5	10^4	10^5	10^4	10^6
Total anaerobes	10^3	10^5	10^4	10^5	10^3	10^5	10^4	10^5
Stareh hydrolyzers	10^2	10^3	10	10^2	10	10^2	10^2	10^3
Nitrate reducers	10^2	10^5	10^2	10^4	10^3	10^5	10^2	10^5
Ammonifiers	10^3	10^5	10^3	10^4	10^3	10^5	10^3	10^5
Sulfate reducers	0	10^2	0	10	0	10^2	0	0

The significance of these latter cultures from the deep sea floor which grow at 1 atm but not at 1,000 atm nor at 30 C is unknown. Whether they would grow at other pressures or temperatures in media providing different combinations of nutrients or cultural conditions has not been examined exhaustively. These apparently alien bacteria may represent species which have settled from the surface to the sea floor where they remain dormant or, more probable, they are passive mutants. Incidentally, only a small percentage of them form endospores. There are numerous analogous anomalies on the apparent temperature requirements of bacteria. Bartholomew and Rittenberg (1949), for example, report finding up to 800 thermophilic bacteria per gram in long cores of deep sea mud (3,000 to 4,000 feet) having a temperature of about 3 C. These bacteria grew in nutrient medium at 60 C, but not at 20 C nor at 37 C. In frozen Arctic soils McBee and McBee (1956) similarly found many thermophilic bacteria which grew in nutrient medium at 55 C.

Nearly as many bacteria from the Kermadec-Tonga Trench (table 3) grew in nutrient media at 1 atm as at high *in situ* pressures. Actually, more anaerobes were detected in nutrient medium inoculated with sediment from a depth of 6,790 m (Station No. 650) when incubated at 1 atm than at 700 atm. Likewise, more aerobes were detected in medium incubated at 1 atm than at 1,000 atm in a sediment sample from a depth of 10,080 m (Station No. 686). Bacteria which are unable to grow at the high pressures where they are found may be alien to the deep sea floor or they may be passive mutants. Finding numerous aerobes buried at depths where ostensibly there is no free oxygen suggests their dormancy. From 10^4 to 10^6 aerobes per gram were found in mud cores 25 to 30 cm long, and from 10^3 to 10^4 aerobes per gram were found in 85- to 90-cm strata at the bottom of the Kermadec-Tonga Trench (ZoBell and Morita, 1957).

In most cases only a small percentage of the bacteria isolated from aerobic enrichment cultures grew when transferred to a similar medium from which oxygen was excluded. Conversely, a majority of the "anaerobes" grew readily when transferred to oxygenated medium, thereby indicating that they are either facultative aerobes or pseudo-anaerobes which can activate nitrate as an hydrogen acceptor. Many of the deep sea bacteria reduced nitrate at high hydrostatic pressures. This is noteworthy because not only is nitrate reduction by most surface dwelling bacteria, which we have investigated, inhibited when compressed to 400 to 600 atm, but their nitratase dehydrogenase system is irreversibly inactivated in a few hours at 1,000 atm (ZoBell and Budge, unpublished manuscript, 1957). This suggests that the enzyme systems of certain deep sea bacteria may differ from such systems of surface-dwelling prototypes.

Besides a few clostridia, sulfate reducers were the only strict anaerobes from deep sea sediments which developed at high hydrostatic pressures. From 10 to 100 bacteria per gram of sediment

TABLE 3

MPN of bacteria detected per gram of wet sediment from the Kermadec-Tonga Trench in selective media incubated at different hydrostatic pressures at 3 to 5 C

	Galathea Station							
	No. 649		No. 650		No. 658		No. 686	
Latitude	35°15′ S		32°20′ S		35° 51′ S		28°30′ S	
Longitude	178°40′ W		176°54′ W		178°31′ W		176°53′ W	
Water depth	8,500 m		6,790 m		7,900 m		9,800 m	
	Incub. pressure (atm)							
	1	850	1	700	1	800	1	1,000
Total aerobes	10^5	10^6	10^6	10^6	10^4	10^6	10^6	10^5
Total anaerobes	10^4	10^5	10^6	10^5	10^5	10^5		
Starch hydrolyzers	0	0	10	10				
Sulfate reducers	0	0	0	0				

TABLE 4

MPN of bacteria detected per gram of wet sediment from deeps in Indian Ocean in selective media incubated at different hydrostatic pressures

	Pressure	*Galathea* Station	
		No. 463 (Soenda Deep): 10°16′ S x 109°51′ E; water depth 7,020 m	No. 492 (Weber Deep): 5°31′ S x 131°01′ E; water depth 7,250 m
	atm		
Total aerobes	1	690,000	810,000
	700	1,050,000	2,300,000
	1,000	4,800	17,000
Starch hydrolyzers	1	100	10
	700	1,000	1,000
	1,000	0	0
Nitrate reducers at	1	10,000	1,000
	700	10,000	10,000
	1,000	10	10
Sulfate reducers at	1	0	0
	700	100	10
	1,000	0	0

from the Philippine Trench (table 2) reduced sulfate at 1,000 atm. A like number of bacteria from the Soenda Deep (7,020 m) and the Weber Deep (7,250 m) in the Indian Ocean reduced sulfate in medium incubated at 700 atm (table 4). There was no activity of sulfate reducers in these deep sea sediments incubated at 1 atm, although sulfate reducers which grow at 1 atm have been recovered from depths down to nearly 5,000 m (ZoBell and Rittenberg, 1948; Morita and ZoBell, 1955).

Growth of the obligate barophilic sulfate reducers from the Philippine Trench which grew at 1,000 atm but not at 1 atm was retarded at 1,200 atm. These cultures were killed during 2 weeks' compression at 1,500 atm at 3 C. They survived for several weeks in nutrient medium and in raw mud stored at 1 atm in the refrigerator. Although these cultures grew preferentially or exclusively when compressed to a pressure approximating that of their native habitat, like most barophilic bacteria that we have studied, their best growth is generally much slower than the normal growth of surface-dwelling bacteria at atmospheric pressure. Similarly, psychrophiles from the deep sea, high latitudes, or from frozen foods whose optima for growth are under 20 C reproduce much more slowly at these lower temperatures than mesophiles multiply at their higher optima, 20 to 45 C.

Incubation for from 2 weeks to 2 years in the refrigerator (3 to 5 C), or compression to from 700 to 1,000 atm may be required for psychrophilic or barophilic bacteria, respectively, to reproduce sufficiently to be detected by increased turbidity in nutrient media. Routinely an attempt is made in this laboratory to hold barophilic and psychrophilic cultures for at least 2 months before discarding as negative, and some cultures have developed only after being incubated for 2 years. Part of this prolonged period of incubation required for evidence of activity is attributable to a long lag phase or to slow acclimatization, but it is often attributable to a long generation time at low temperatures and high pressures.

Sulfate reducers from the Weber Deep have been maintained compressed to 700 atm at 3 to 5 C for more than 5 years and are still under observation at the Scripps Institution. Upon decompressing and opening the pressure vessels for examination of some of the original sediment sample, the emanation of hydrogen sulfide indicated the activity of sulfate reducers during storage. Shortly after collection, several small quantities of the mud were used to inoculate appropriate medium in piston stoppered vials for enrichment in the refrigerator at 700 atm. After incubation for 60 days there was no evidence of sulfate reduction, but after 10 months sulfide was found in most vials inoculated with 0.1 or 0.01 g of mud from the Weber Deep. The uninoculated controls were negative. Likewise there was no evidence of sulfate reduction after incubation for 26 months at 1 atm in similar medium inoculated with some of the original mud sample or with enrichment cultures which developed at 700 atm.

The obligate barophilic sulfate reducing bacteria from the Weber Deep have been transplanted five successive times and kept growing when compressed to 700 atm at 3 to 5 C. Morphologically they appear as small ovoid cells about 0.3 μ in width and 0.5 to 0.8 μ in length, thus differing in both size and shape from sulfate reducers commonly classified as species of the genus *Desulfovibrio*. Though highly pleomorphic (Butlin *et al.*, 1949; Pochon and Chalvignac, 1952), known *Desulfovibrio* species are ordinarily comma shaped organisms 0.5 to 1.0 μ wide and 3 to 5 μ long. The barophilic sulfate reducer from the Weber Deep is gram negative and has neither flagella nor endospores. It is believed to be a new species, but further studies must precede its naming. Pressure tolerance is probably not a sufficiently distinctive characteristic to delineate species, particularly since deep sea bacteria may be remnants of surface dwelling forms which have become acclimatized to high pressures while sinking to the sea floor. Pressure is known to affect the morphology of bacteria (ZoBell and Oppenheimer, 1950) and it may have mutagenetic effects.

Barophilic bacteria in abundance were also found on the integument and in the gut of many deep sea animals. The observations on such bacteria, though, have little quantitative significance because of the crushed or contaminated condition of the animals. All of the deep sea animals were dead upon reaching the ship's deck, presumably killed by the rigorous change in climate during transit from the deep sea floor to the surface, coupled with mechanical attrition with marine material in nets, dredges, and trawls. Many of the animals are normally mud eaters or detritus feeders, and may obtain an appreciable part of their nutrition from the ingestion and digestion of bacteria. The "standing crop" of bacteria in the topmost 1-cm layer of deep sea mud was estimated (ZoBell, 1954) to contain between 0.001 and 0.005 g of organic carbon per m^2. If these bacteria reproduce at rates comparable to rates observed in the laboratory under normal conditions of temperature, pressure, and nutrients, the annual "crop" or yield may amount to 0.5 g of organic (bacterial) carbon per m^2 in the topmost 1 cm of mud.

Spärck (1952) estimated that the total animal life on the deep sea floor amounts to 1 g per m^2. Among the many animals shown by the *Galathea* Expedition to live at depths of 10,000 m or more were Actinia, Amphipoda, Holothuriodea, Lamellibranchiata, Sipunculoidea, and Tanaidacea. Additionally from depths of 7,000 to 9,000 m were recovered Asteroidea, Branchyura, Cirripedia, Crinoidea, Echinoidea, Echiouroidea, Gastropoda, Hydroidea, Pisces, Polychaeta and Spongia.

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SUMMARY

Bacterial populations ranging from 10^3 to 10^6 per g of wet mud were demonstrated in several samples taken from depths of 7,000 to 10,000 m or more. Some of the deep sea bacteria appear to be obligate barophiles which grow when com-

pressed to 700 to 1,000 atm. Deep sea bacteria which grow at 1 atm but not at the high pressures of the environment where found may be dormant aliens from shallower depths, or they may be passive mutants, or all of their cultural requirements may not have been satisfied by the laboratory conditions.

Even under optimum conditions for reproduction barophilic bacteria, like psychrophilic forms, grow very slowly. Cultures of obligate barophilic sulfate reducing bacteria have been transplanted several times and maintained in the laboratory for more than 5 years.

Many barophilic bacteria were found associated with deep sea fauna. On the deep sea floor bacteria may contribute substantially to the nutrition of animals

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