Population Sizes and Growth Pressure Responses of Intestinal Microfloras of Deep-Sea Fish Retrieved from the Abyssal Zone

YUTAKA YANO,¹ AKIHIKO NAKAYAMA,^{1*} AND KATSUHIKO YOSHIDA²

Marine Biochemistry Division¹ and Marine Productivity Division,² National Research Institute of Fisheries Science, Kanazawa-ku, Yokohama, Kanagawa 236, Japan

Received 25 April 1995/Accepted 26 September 1995

The intestinal floras of seven deep-sea fish retrieved at depths of from 3,200 to 5,900 m were examined for population sizes and growth responses to pressure. Large populations of culturable bacteria, ranging from 1.1×10^6 to 3.6×10^8 cells per ml of contents, were detected when samples were incubated at conditions characteristic of those of the deep sea. Culturable cell counts at in situ pressures were greater than those at atmospheric pressure in all samples. Most of the strains isolated by the spread-plating method at atmospheric pressure later proved barophilic. Barophilic bacteria were the predominant inhabitants of the abyssal fish intestines.

It has been shown that bacteria that have adapted to deep-sea environments, characterized by low temperatures and high hydrostatic pressures, inhabit various spaces of the deep sea (5, 11). In particular, microbial activities have been detected in the guts of abyssal scavenging amphipods and deposit-feeding holothurians (2, 4). By contrast, the intestinal microfloras of deep-sea fish in the abyssal zone are poorly understood, though Tabor et al. showed the presence of a barophilic gut flora in a deep-sea fish with their experiments of substrate utilization (8).

In a previous paper, we showed that obligately barophilic bacteria could be isolated from the intestinal contents of deep-sea fish captured in the abyssal zone, even if the fish were retrieved by crab-pot fishing without any special treatments to retain in situ pressure and temperature (6). In this paper, to reveal quantitatively population sizes and growth pressure responses of the intestinal microfloras of deep-sea fish, we determined total cell counts and viable cell counts at both atmospheric pressure and pressures approximately equal to in situ pressures in the intestinal contents of deep-sea fish retrieved from depths of 3,200 to 5,900 m.

Deep-sea fish. Deep-sea fish were retrieved at six stations in the northwestern Pacific Ocean in 1991 and 1992 by the R/V *Soyo Maru's* crab-pot fishing as described previously (6). Although sardines had to be used as bait, we packed them in 2-liter plastic bottles with 324 small holes (diameter, 3.8 mm) to avoid their being eaten by trapped deep-sea fish. The crab pots (steel-framed net creels) were left on the sea bottom for about 1 day to collect deep-sea fish. It took 4 to 5 h to pull up the crab pots. The characteristics of the deep-sea fish recovered are shown in Table 1, in order of depth of capture. The pressures at the depths of samples 47, 46, 36, 53, 56, 40, and 41 were approximately 32.4, 43.6, 47.6, 53.7, 56.7, 59.8, and 59.8 MPa, respectively. Instead of these pressures, however, we used the pressures shown in Table 2 as the in situ pressures for

experimental convenience. Hereafter, in situ pressures refer to these experimental pressure values unless otherwise indicated.

Sampling of intestines. The sampling of intestines from the seven deep-sea fish was carried out as described previously (6). The pouches containing the intestines were pressurized in pressure vessels at in situ pressures and kept in a refrigerator (2 to 3°C) for 2 to 3 h until viable cell counts were performed on board. For direct cell counts, 0.1-ml aliquots of the intestinal contents were fixed aboard ship with prefiltered (pore size, 0.22 μ m) formaldehyde at a final concentration of 3.7%.

Total cell counts. Total cell counts in the intestinal contents were determined by the direct count method after returning to our institute. The intestinal contents fixed with formaldehyde as described previously were appropriately diluted in artificial sea water, stained with 4',6-diamidino-2-phenylindole (DAPI) at a final concentration of 0.05%, and examined by epifluorescence microscopy (7). A minimum of 300 cells or 10 fields was counted. As shown in Table 2, total cell counts of the intestinal contents of the seven deep-sea fish ranged from 3.0×10^4 to 4.9×10^9 cells per ml.

Viable cell counts by MPN method at atmospheric and in situ pressures. Viable cell counts were determined by the most-probable-number (MPN) method at atmospheric and in situ pressures. The intestinal contents were well mixed and diluted serially by 10-fold dilutions with chilled marine broth (Difco). Then, 0.1-ml aliquots of serial dilutions of intestinal contents were inoculated into pouches containing 1 ml of chilled marine broth. Ten pouches were inoculated per one dilution step and heat-sealed, taking care to avoid the entrapment of air bubbles in the pouch. Five pouches at each dilution step were incubated at atmospheric pressure, and the other five were incubated at in situ pressures in a pressure vessel at 5°C. This procedure was carried out in duplicate. During these procedures, the intestinal samples in tubes were kept immersed in an ice bath. We observed growth in the pouches after incubation for 1 and 2 months. When the growth of bacteria was not obvious by visible turbidity, the cultures were examined by microscopy and reinoculated into fresh, chilled marine broth. Growth in the broths was assessed after 1 month. After assessing growth in the pouches, MPN estimates were

* Corresponding author. Mailing address: Marine Biochemistry Division, National Research Institute of Fisheries Science, 2-12-4, Fukuura, Kanazawa-ku, Yokohama, Kanagawa 236, Japan. Phone: 045-788-7660. Fax: 045-788-5001.

TABLE 1. Characteristics of deep-sea fish retrieved from depths of 3,200 to 5,900 m in the northwestern Pacific Ocean

Sample no.	Scientific name	Collection					Total length (cm)	Total wt (g)
		Date of retrieval	Site (°N, °E)	Depth (m)	Temp (°C) ^a	Time ^b		
47	<i>Coryphaenoides armatus</i>	28 June 1992	41.2, 144.0	3,200	13.8	2 h 10 min	58.1	770
46	<i>Coryphaenoides yaquinae</i>	26 June 1992	41.2, 144.3	4,300	13.3	3 h 5 min	56.7	800
36	<i>Ilyophis</i> sp.	23 July 1991	30.0, 134.3	4,700	29.6	3 h 5 min	91.8	810
53	<i>Coryphaenoides yaquinae</i>	8 July 1992	39.6, 114.6	5,300	17.8	3 h 30 min	49.7	510
56	<i>Coryphaenoides yaquinae</i>	10 July 1992	40.1, 149.4	5,600	19.1	3 h 45 min	46.2	400
40	<i>Coryphaenoides yaquinae</i>	2 August 1991	27.6, 144.3	5,900	30.5	4 h 0 min	56.6	845
41	<i>Coryphaenoides yaquinae</i>	2 August 1991	27.6, 144.3	5,900	30.5	4 h 0 min	65.2	1,525

^a Temperature of the surface water when crab pots (steel-framed net creels) were retrieved from the deep-sea bottom.^b Time required for the retrieval of crab pots from the seafloor.

determined from a published five-tube table for decimal dilutions (1).

As shown in Table 2, viable cell counts were obtained from samples 47, 46, 36, 53, and 56 at atmospheric pressure and in situ pressures. Viable cell counts by the MPN method at atmospheric pressure and in situ pressures ranged from 3.8×10^2 to 1.5×10^7 and from 1.1×10^6 to 3.6×10^8 per ml of intestinal contents, respectively. Viable cell counts at in situ pressures were approximately 2 to 1,000,000 times greater than those at atmospheric pressure. However, no growth was detected for intestinal samples 40 and 41 by this method, though they yielded total cell counts of 4.3×10^4 and 3.0×10^4 cells per ml, respectively.

Viable cell counts by spread-plating method at atmospheric pressure. One hundred-microliter aliquots of serial dilutions of intestinal contents were spread with sterile styrene plastic rods on chilled marine agar (Difco) plates in duplicate and incubated at 5°C and atmospheric pressure. The counts were recorded as CFU after incubation for 2 months. During these procedures, the intestinal samples in tubes were kept immersed in an ice bath. As shown in Table 2, CFU per ml of the intestinal contents of samples 47, 46, 36, 53, and 56 ranged from 2.5×10^2 to 2.6×10^5 . However, no colonies were found on the plates of samples 40 and 41, though total cell counts of more than 10^4 /ml were found in these intestinal contents as described above.

Isolating bacteria at atmospheric pressure and their responses to pressure. Colonies were picked from the agar plates mentioned above and purified by streaking them in a series of three transfers on marine agar plates. Thus, 12, 13, 14, 10, and

19 strains were isolated from samples 47, 46, 36, 53, and 56, respectively. Ten strains from each of the isolates of samples 47, 36, and 53, eight strains from the isolates of sample 46, and nine strains from isolates of sample 56 were randomly selected and preincubated in small pouches containing 5 ml of marine broth at the in situ pressure corresponding to the habitat of the deep-sea fish and at 5°C for 1 week. However, as 10 strains from sample 53 grew very slowly at the preincubation pressure (55.2 MPa), these strains were preincubated at 27.6 MPa for 2 weeks instead. Then, a 250- μ l aliquot of each culture was inoculated into a pouch containing 25 ml of marine broth. The pouch was heat sealed and divided into five pouches by the seal-and-cut method as described previously (6). Of these five pouches, two were incubated at the in situ pressure corresponding to the habitat of the deep-sea fish and at 5°C in a pressure vessel and two others were incubated at atmospheric pressure and 5°C. The culture in the last pouch was immediately fixed with formaldehyde as described above and used to determine the initial cell concentration without incubation. After 2 days, the cultures were fixed with formaldehyde and used to determine total cell counts by the direct count method.

Ratios of total cell counts of these strains at the in situ pressures to those at atmospheric pressure are shown in Fig. 1A. The ratios of all of the strains from samples 47, 46, and 56 and nine strains from sample 36 were greater than 1.0. Therefore, these strains were considered to be facultative barophiles. As the ratios for 10 strains from sample 53 and 1 strain (strain T3609) from sample 36 were less than 1.0, these strains appeared to be barotolerant or to have pressures for optimal growth lower than 55.2 MPa in the case of sample 53 and 44.8

TABLE 2. Total and viable cell counts of intestinal contents of the deep-sea fish

Sample no.	Viable cell count (cells/ml)			Total cell count (cells/ml)	Viable cell count by MPN at in situ pressure (%) ^a
	CFU ^b at:	MPN ^c at:			
	Atmospheric pressure	Atmospheric pressure	In situ pressure ^d		
47	2.6 × 10 ⁵	5.2 × 10 ⁵	1.1 × 10 ⁶	2.2 × 10 ⁹	0.050
46	3.5 × 10 ²	1.0 × 10 ⁴	7.4 × 10 ⁶	4.9 × 10 ⁹	0.15
36	1.3 × 10 ⁵	1.5 × 10 ⁷	1.1 × 10 ⁸	9.3 × 10 ⁸	12
53	2.5 × 10 ²	3.8 × 10 ²	3.6 × 10 ⁸	8.0 × 10 ⁸	45
56	6.7 × 10 ⁴	1.2 × 10 ⁵	4.9 × 10 ⁷	1.1 × 10 ⁸	45
40	NG ^e	NG	NG	4.3 × 10 ⁴	ND
41	NG	NG	NG	3.0 × 10 ⁴	ND

^a Viable cell counts were determined by the MPN method at in situ pressures as a percentage of total cell counts. ND, not determined.^b CFU were determined by the spread-plating method, for which plates were incubated at 5°C (see the text).^c Viable cell counts were determined by the MPN method, for which pouches were incubated at 5°C (see the text).^d Pouches for samples 47, 46, 36, 53, 56, 40, and 41 were incubated at 34.5, 44.8, 44.8, 55.2, 55.2, 58.6, and 58.6 MPa, respectively.^e NG, no growth of bacteria.

FIG. 1. Growth pressure responses of the strains isolated by the spread-plating method at atmospheric pressure. (A) Each circle shows the ratio of the total cell count at the in situ pressure to that at atmospheric pressure when a strain was incubated at both pressures for 2 days. Circles for strains isolated from a given deep-sea fish are placed on one horizontal line. A ratio greater than 1.0 indicates barophily. A ratio less than 1.0 indicates barotolerance or that, for optimal growth, the strain requires a pressure lower than the incubation pressure. (B) Growth pressure responses of all of the strains from sample 53 and strain T3609 from sample 36, which were incubated at values approximately half those of the in situ pressures.

MPa in the case of sample 36. Therefore, all the strains from sample 53 and strain T3609 from sample 36, which were not initially confirmed as barophilic, were incubated at pressures approximately half those of the in situ conditions, namely, 27.6 and 20.7 MPa, respectively, for 2 days. As shown in Fig. 1B, at least two strains from sample 53 and strain T3609 from sample 36 proved to be barophilic at these lower pressures, because the ratios of total cell counts at the lower pressures to those at atmospheric pressure were greater than 1.0.

Pressure-related characteristics of the intestinal microfloras of deep-sea fish. The results of direct counting showed that large populations of bacteria were present in the intestines of the abyssal deep-sea fish (Table 2), consistent with the counts of those in the guts of deep-sea amphipods and holothurians (2, 3). In these intestinal samples, large populations of bacteria of more than 10^8 cells per ml of intestinal contents must have been able to grow at the in situ pressures, as shown by the determination of viable cell counts by the MPN method. Since the viable cell counts determined at the in situ pressures were much greater than those determined at atmospheric pressure in all of the samples, we infer that most of the culturable bacteria at in situ pressures were obligate barophiles unable to grow at atmospheric pressure. These results indicate that large populations of obligate barophiles may be able to survive even

after sample collection without any special treatment to maintain the pressures and temperatures of the deep sea.

Culturable percentages, which were defined as the proportions of viable cell counts determined by the MPN method at the in situ pressures to total cell counts, ranged from 12 to 45% in the cases of samples 36, 53, and 56 (Table 2). These percentages were not extraordinary, considering that Yayanos and Dietz (10) described a 90% kill-off in 5 h of a barophilic organism kept at atmospheric pressure. On the other hand, the culturable percentages were, relatively speaking, very low in other samples. As seen in Table 2, there was no apparent relationship between the culturable percentages and surface water temperatures or the degree of decompression, in contrast to earlier studies (9). The effect of decompression seemed to be inverse. The fluctuation of culturable percentages among the samples might mean that the survival and the growth of barophiles are more strongly affected by other factors, for example, the nutritional requirements or growth stage of the intestinal microfloras, than by decompression or temperature increases. Special regard should be paid to such factors for the investigation of deep-sea bacteria hereafter.

In the cases of samples 36, 53, and 56, for which the culturable percentages were relatively high (Table 2), most of the culturable bacteria at in situ pressures could be considered obligate barophiles, as mentioned above. Moreover, in the cases of samples 36 and 56, all of the representative strains isolated by the spread-plating method at atmospheric pressure later proved barophilic in growth experiments, as shown in Fig. 1. In the case of sample 53, eight of the ten strains appeared barotolerant (Fig. 1B). However, the viable cell count of only 2.5×10^2 cells per ml by the spread-plating method at atmospheric pressure was considerably lower than the viable cell count of 3.6×10^8 cells per ml by the MPN method at an in situ pressure (Table 2). This result suggests that the population of barotolerant bacteria in the intestinal sample was very small, compared with the population of barophiles. From all of the results, we conclude that barophiles are a more important component of the intestinal microfloras of abyssal deep-sea fish than barotolerant bacteria. Furthermore, Tabor et al. (8) showed the presence of a barophilic gut flora in a deep-sea fish (*Bathyporeia* sp.) retrieved from a depth of 5,240 m by their experiments of substrate utilization. We confirm their findings by the different means described above.

In conclusion, facultative and obligate barophiles play an important role in the intestinal microfloras of abyssal deep-sea fish while barotolerant bacteria play a minor role. The intestines of deep-sea fish as relatively closed spaces may supply sufficient nutrients to sustain significant bacterial growth in the deep sea. Therefore, intestinal conditions should be considered in explaining unique microfloras well adapted to deep-sea environments characterized by high hydrostatic pressure.

We thank the officers and crew of the R/V *Soyo Maru* for skilled cooperation.

This study was partially supported by a grant from the Science and Technology Agency and a grant (BRP) from the Ministry of Agriculture, Forestry, and Fisheries.

REFERENCES

- Alexander, M. 1982. Most probable number method for microbial populations. *Agronomy (Madison)* 9:815-820.
- Deming, J. W., and R. R. Colwell. 1982. Barophilic bacteria associated with digestive tracts of abyssal holothurians. *Appl. Environ. Microbiol.* 44:1222-1230.
- Deming, J. W., P. S. Tabor, and R. R. Colwell. 1981. Barophilic growth of bacteria from intestinal tracts of deep-sea invertebrates. *Microb. Ecol.* 7:85-94.
- Hessler, R. R., C. L. Ingram, A. A. Yayanos, and B. R. Barnett. 1978.

- Scavenging amphipods from the floor of the Philippine Trench. *Deep-Sea Res.* **25**:1029–1047.
5. **Jannasch, H. W., C. O. Wirsen, and C. D. Taylor.** 1982. Deep-sea bacteria: isolation in the absence of decompression. *Science* **216**:1315–1317.
 6. **Nakayama, A., Y. Yano, and K. Yoshida.** 1994. New method for isolating barophiles from intestinal contents of deep-sea fishes retrieved from the abyssal zone. *Appl. Environ. Microbiol.* **60**:4210–4212.
 7. **Porter, K. G., and Y. S. Feig.** 1980. The use of DAPI for identifying and counting aquatic microflora. *Limnol. Oceanogr.* **25**:943–948.
 8. **Tabor, P. S., J. W. Deming, K. Ohwada, and R. R. Colwell.** 1982. Activity and growth of microbial populations in pressurized deep-sea sediment and animal gut samples. *Appl. Environ. Microbiol.* **44**:413–422.
 9. **Yayanos, A. A., and A. S. Dietz.** 1982. Thermal inactivation of a deep-sea barophilic bacterium, isolate CNPT-3. *Appl. Environ. Microbiol.* **43**:1481–1489.
 10. **Yayanos, A. A., and A. S. Dietz.** 1983. Death of a hadal deep-sea bacterium after decompression. *Science* **220**:497–498.
 11. **ZoBell, C. E., and R. Y. Morita.** 1957. Barophilic bacteria in some deep sea sediments. *J. Bacteriol.* **73**:563–568.