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Isolation and properties of barophilic and barotolerant bacteria from deep-sea mud samples

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Several barophilic and barotolerant bacteria were isolated from deep-sea mud samples of Suruga Bay (2485 m depth), the Ryukyu Trench (5110 m depth), and the Japan Trench (land-side 6356 m, and sea-side 6269 m depth, respectively). The barophilic bacteria, strains DB5501, DB6101, DB6705 and DB6906, were able to grow better under high hydrostatic pressures than under atmospheric pressure (0.1 megapascals; MPa). The optimal growth pressures for the barophilic bacteria were approximately 50 MPa at 10°C. The barotolerant strains DSK1 and DSS12 were determined to be psychrophilic, and had optimal growth temperatures of 10°C and 8°C, respectively. The degree of barophily and barotolerance was shown to be very dependent on temperature. For example, at 4°C the barophilic strains were indistinguishable from barotolerant bacteria, whereas at 15°C the barotolerant strains behaved more like the barophilic strains. Based on sequence analysis of 16S ribosomal DNA, all of the strains included in this study belong to the gamma subgroup of the Proteobacteria. Phylogenetic relations between the isolated strains and the known gamma subgroup bacteria suggested that the isolated strains belong to a new sub-branch of this group.

Keywords: barophilic bacteria; barotolerant bacteria; deep-sea; high pressure; bacterial phylogeny.

Introduction

The deep-sea bottom is a special world under extremely high pressure and low temperature. Microorganisms living there have several special features for adapting to such an extreme environment. To investigate this environment and the biology of the deep-sea world, several manned submersibles have been constructed. The *SHINKAI 6500,* operated by the Japanese Marine Science and Technology Center (JAMSTEC), is the most capable of all, with an ability to submerge to 6500 m depth (Takagawa *et al.*, 1989).

Among the important features of the deep-sea, it is important to keep in mind the following:

First, although the deep-sea is under extremely high hydrostatic pressure, many organisms live and grow in this environment. ZoBell and Morita (1957) were among the first to attempt to isolate microorganisms which were specifically adapted to such high pressures, and they called them barophilic bacteria. The first isolates of barophilic bacteria were reported in 1979 (Yayanos *et al.*, 1979).

Second, most of the deep-sea is very cold (1–2°C), though locally it may be extremely hot (200–400°C) at hydrothermal vents. Most of the deep-sea bottom is stable and cold and it is possible that ancient life-forms may be in suspended animation in the world's largest refrigerator. The study of microorganisms isolated from the deep-sea promises to give us

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new information on the origin of life and its evolution. Hyperthermophilic bacteria have been isolated from deep-sea hydrothermal vents. Some of these organisms utilize carbon dioxide and hydrogen gas emitted from hot vents, and may therefore be functioning as primary producers in the hot vent ecological system (Jannasch and Mottl, 1985).

Third, the deep-sea bottom is the final resting place of much human generated effluent. Some of this material is recalcitrant (e.g. many plastics), whereas other materials are degraded by the bacteria of the deep-sea. Generally, the rates of decomposition for organic compounds are very slow at the deep-sea bottom compared with surface environments due to the low temperature and high pressure (Jannasch, 1979). Therefore, those bacteria, isolated from the deep-sea, which can decompose organic compounds very strongly, may be particularly well suited to decomposing them in the mild surface environment. As an example, very robust crude oil degrading bacteria were isolated from a deep-sea mud sample by the DEEP STAR Group (Moriya and Horikoshi, 1993).

Therefore, the microorganisms living in the deep-sea are very interesting and promise to have many novel capabilities. In order to investigate the mechanisms of adaptation to high pressure in the deep-sea environment, several barophilic and barotolerant bacteria were isolated from deep-sea mud samples obtained by *SHINKAI 6500*. In this paper, the isolation procedure and some properties of the barophilic and barotolerant bacteria are reported. The behaviour of these bacteria growing under a range of pressure and temperature combinations is also discussed. Finally, the phylogeny of these bacteria is described.

Materials and methods

Sample collection

The deep-sea mud samples were collected by sterilized mud samplers (Ikemoto and Kyo, 1993) from Suruga Bay (2485 m depth; 34′29.67″N 138′32.28″E), the Ryukyu Trench (5110 m depth; 24′15.23″N 126′47.30″E), and the Japan Trench (land-side 6356 m; 40′06.84″N 144′11.04″E, and sea-side 6269 m depth; 39′20.45″N 144′35.93″E) using the manned submersible *SHINKAI 6500*. Each sample was diluted with Marine broth 2216 (Difco, Co.) and pressurized at about 65 MPa in a pressure vessel (titanium, Rigo-sha, Co., Tokyo). These vessels were placed in a refrigerator (2–4°C) on the support vessel *M.S. YOKOSUKA*.

Isolation of barophilic and barotolerant bacteria

The isolation protocols for barophilic and barotolerant bacteria are shown in Fig. 1. Most bacteria adapted to the deep-sea environment are also psychrophilic, so we tried at the same time to isolate psychrophilic and pressure dependent bacteria. Subsequently, a test for barophily was conducted by comparing the growth rate of bacteria at 65 and 0.1 MPa.

Growth studies

The isolated barophilic and barotolerant bacteria were grown in pressure vessels under a range of hydrostatic pressures (0.1–70 MPa) and temperatures (4–15°C). The medium for these tests was Marine broth 2216 (autoclaved and filtered through 0.22 μm membrane filters). To supply oxygen to the cultures, fluorinert (FC–72, Sumitomo–3M, Co., Tokyo) which was saturated with the gas, was added to the cultures (25% of total volume). Bacterial densities were determined by absorbance measurements (660 nm) using a

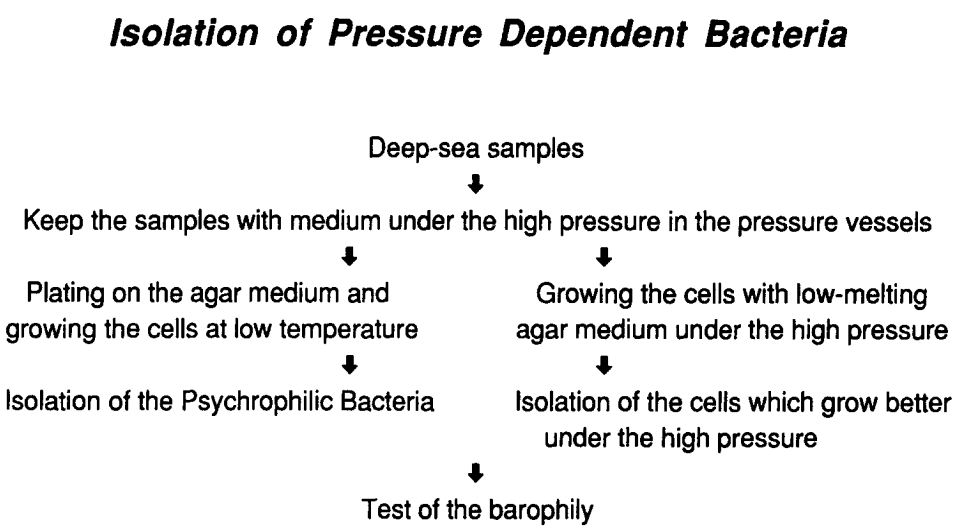

Figure 1. The protocol for the isolation of pressure dependent bacteria.

Beckman Model DU7500 spectrophotometer. Growth rates were calculated from 3–5 points along the logarithmic portion of the resulting growth curves using linear regression analysis.

Extraction of chromosomal DNA and PCR amplification of the 16S ribosomal DNA

The barophilic strains (DB5501, DB6101, DB6705, and DB6906) isolated in this study, were grown at 50 MPa and 10°C for 3 days. The barotolerant strains (DSK1 and DSS12) isolated in this study, were also grown at atmospheric pressure, and at 10°C and 8°C respectively for 2 days. Cells were harvested and the purified chromosomal DNA was prepared using extraction procedures described by Saito and Miura (1963). GC contents of the chromosomal DNAs from the isolated strains were determined by a HPLC method (Tamaoka and Komagata, 1984). 16S ribosomal DNA was amplified from purified DNA using Gene AMP kit reagents (Takara Co., Otsu). Protocols recommended by the manufacturer were observed. PCR amplification was done as described by DeLong (1992), using the synthesized oligonucleotide primers, Eubac27F (AGAGTTTGATCCTGGCTCAG) and Eubac1492R (GGTTACCTTGTTACGACTT) (Lane, 1991) and a Perkin-Elmer/Cetus model 9600 DNA thermal cycler.

Sub-cloning of the amplified 16S ribosomal DNAs and sequencing

The phage plasmid pUC119 was used as the vector for sub-cloning of the amplified 16S ribosomal DNAs. *Escherichia coli* strain MV1184 [*ara* Δ(*lac-pro*) *strA thi* (φ 80 *lac*ZΔM15) Δ(*sri-recA*)*306*::Tn*10*(Tet^r)F′ *traD36 proAB lacI^q*ZΔM15] was also used as a host of the recombinant plasmids, and the helper phage M13K07 was used for preparation of single stranded template DNA from *E. coli* transformants (Vieira and Messing, 1987). The procedure of recombinant DNA work was carried out as described by Sambrook *et al.* (1989). The vector pUC119 was digested by *Sma*I, and was dephosphorylated. The

amplified 16S ribosomal DNAs were blunted by T4 polymerase and treated with kinase. Then, the vector and genes were ligated and introduced into *E. coli* strain MV1184. Ampicillin resistant and *lac*Z$^-$ transformants were obtained, and the recombinant plasmids, containing an insert of about 1.5 kb of amplified DNA, were isolated. The cloned 16S ribosomal DNA was sequenced using an automated DNA sequencer model 373A (Applied Biosystems Inc.) by the dideoxy terminator procedure, as described in the ABI manual. Commercial sequencing primers and synthesized primers were used to determine the sequences of the entire 16S ribosomal DNAs, which were approximately 1500 nucleotides in length.

Analysis of DNA sequences and phylogenetic relations

The determined 16S ribosomal DNA sequences of the barophilic and barotolerant strains, isolated in this study, were checked for similarity with sequences in the DNA databases, GenBank and EMBL using the GENETYX-CD program (ver.21.0, Software Co., Tokyo). The percent similarity between each of the new isolates and other bacteria was calculated using the GENETYX-MAC program (ver.5.0, Software Co., Tokyo). A phylogenetic tree was constructed by the neighbour-joining method (Saitou and Nei, 1987) using BIORESEARCH/SINCA program (Fujitsu Co., Chiba).

Nucleotide sequence accession numbers

The amplified 16S ribosomal DNA sequences reported in this paper have been deposited in the DDBJ (Mishima, Japan), EMBL (Heidelberg, Germany), and GenBank (Mountain View, California, USA) nucleotide sequence databases. The accession numbers of the amplified DNA sequences from the barophilic strains DB5501, DB6101, DB6705, and DB6906 are D21229, D21221, D21222, and D21223, respectively. The accession numbers of the DNA sequences of the barotolerant strains DSK1 and DSS12 are D21224 and D21225, respectively.

Results and discussion

Effect of temperature and pressure on the growth of barophilic and barotolerant strains

Several novel strains of barophilic and barotolerant bacteria were isolated from deep-sea mud samples collected by the manned submersible *SHINKAI 6500*. The barophilic strains DB5501, DB6101, DB6705 and DB6906 were isolated from the samples of Suruga Bay, the Ryukyu Trench, the Japan Trench land-side, and the Japan Trench sea-side, respectively. The optimal pressure for growth of these barophilic strains was about 50 MPa at 10°C. It is particularly noteworthy that DB6705 and DB6906, isolated from samples collected at more than 6000 m depth, were not able to grow under atmospheric pressure (0.1 MPa) at the same temperature of 10°C (Fig. 2, A–D). The barotolerant strains, DSK1 and DSS12, were also isolated from the samples of the Japan Trench land-side and the Ryukyu Trench, respectively. The optimal growth temperatures of these barotolerant strains were at 10°C for DSK1 and 8°C for DSS12 and these strains were not able to grow at temperatures above 20°C. The growth rates of these barotolerant strains, which were also determined to be psychrophilic, were very stable under high pressure. Even at 70 MPa the specific growth rate was 80% of the maximum observed rate (Fig. 2, E, F).

As shown in Fig. 2, the specific growth rate profiles of the barophilic strains indicate that these bacteria show the strongest barophilic response near their upper temperature for

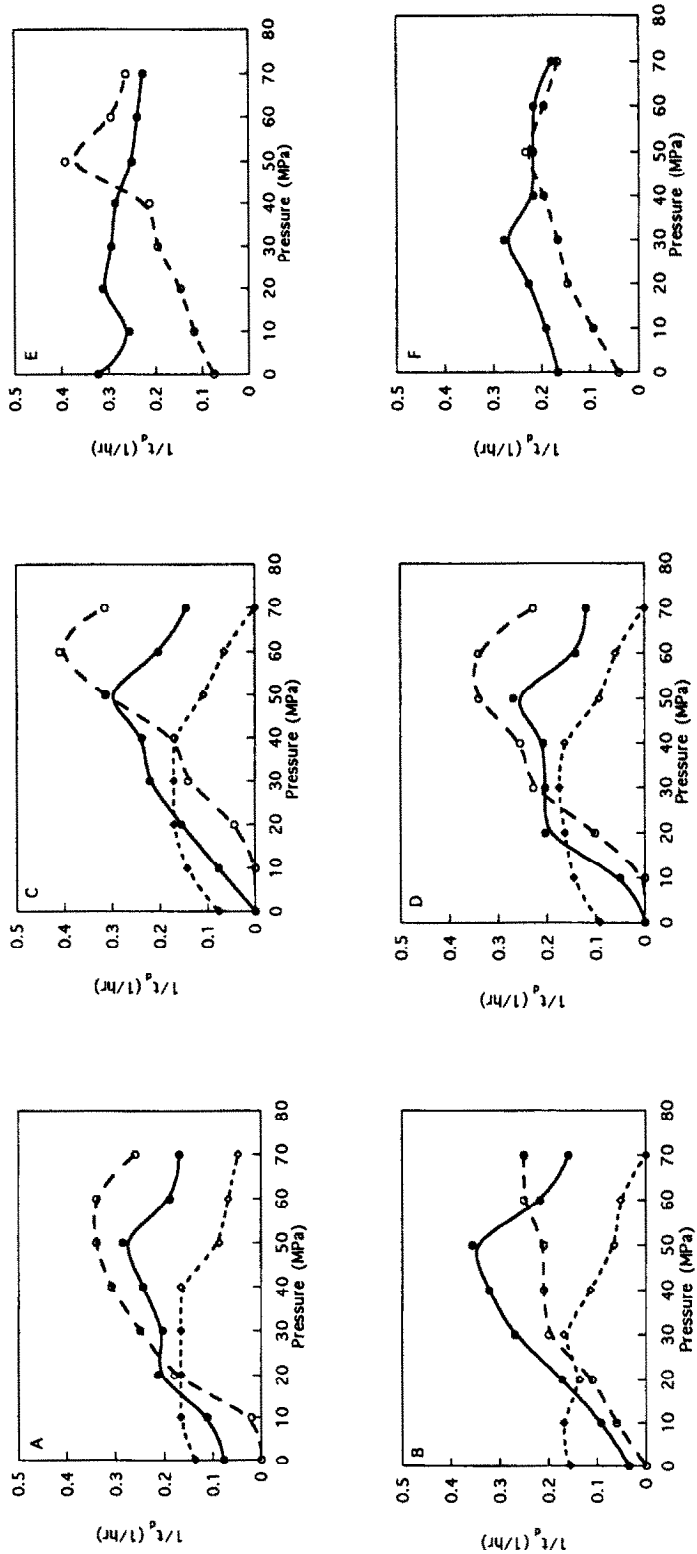

Figure 2. The growth profiles of the barophilic bacteria, strains DB5501(A), DB6101(B), DB6705(C), and DB6906(D), and the barotolerant bacteria, strains DSK1(E) and DSS12(F) at several pressures and temperatures (○--○, 15°C; ●—●, 10°C for A–E, 8°C for F; ◇----◇, 4°C). t_d indicates doubling time (h). Growth rate is shown as $1/t_d$(1/h).

growth (15°C). For example, the optimal growth pressures of the strains DB6101 and DB6705 at 15°C were around 70 MPa and 60 MPa respectively, whereas at 4°C the specific growth rate profiles of the barophilic strains were similar to the profiles of the barotolerant bacteria. On the other hand, the specific growth rate profiles of the barotolerant strains were very similar to those of the barophilic strains, because at 15°C the optimal pressure for strains DSK1 and DSS12 was approximately 50 MPa, much like the barophilic bacteria. In total, the effects of several pressures and temperatures on cell growth for these six strains may be similar, in that all strains become more barophilic at higher temperatures.

The effect of pressure and temperature on the growth rate of several deep-sea barophilic bacteria, isolated from different depths of the ocean, has been reported (Yayanos, 1986). These results showed that the maximum rate of reproduction was at 8–10°C for all isolates at different pressures, and most notably optimal growth was at a pressure near that of their capture depth. For the barophilic and barotolerant strains described in this paper, the growth rate was usually higher at 15°C than at lower temperatures, under high pressure. Strains DB6101 and DSS12 did not follow this pattern. But even for these strains, at 60–70 MPa for DB6101, and at 50 MPa for DSS12, specific growth rates were faster or equal to those at 15°C than at lower temperatures (Fig. 2, B, F).

It is important to consider the differences between previously reported barophilic bacteria and our strains. Different types of barophilic bacteria may show a range of responses to pressure and temperature, but they may have basically a very similar pattern. We have shown that psychrophilic barotolerant bacteria also have a similar profile. In fact, *Escherichia coli* also responds in a similar way to variations in temperature and pressure (Marquis, 1976). At the present time, we do not have sufficient data to draw a general conclusion, but it is possible that bacterial growth rates may generally be stimulated by high pressure near their maximum temperature for growth.

Phylogenic relationships of isolated bacteria determined on the basis of 16S ribosomal DNA sequences

From a comparison of 16S ribosomal DNA sequences held in GenBank and EMBL databases, it was shown that barophilic and barotolerant strains reported in this paper belong to the Proteobacteria gamma subgroup (Martinez-Murcia *et al.*, 1992; Table 1, Fig. 3). The GC (mol% G + C) contents of chromosomal DNA from the strains DB5501, DB6101, DB6705, DB6906, DSK1, and DSS12 were 45.5, 43.5, 44.8, 45.9, 39.6, and 46.1% respectively. The GC content of *Vibrio* spp. which belong to the gamma proteobacteria is between that 40–50% (Kreig and Holt, 1984). Therefore, the GC contents data are supportive evidence to suggest that the isolated strains belong to the gamma Proteobacteria, because representatives of the genus *Vibrio* are typical species of this group. The similarity between the 16S ribosomal DNA sequences of the isolated bacteria and 6 strains of the gamma sub-group are shown in Table 1. More than 85% similarity was detected between the isolated strains and the gamma subgroup strains. Deming *et al.* (1988) have reported a taxonomic study of the obligately barophilic bacterium *Colwellia hadaliensis* based on its 5S ribosomal RNA sequence. This barophilic bacterium also belongs to the Proteobacteria gamma subgroup. DeLong and Franks (1992) have also reported barophilic and psychrophilic deep-sea bacteria which belong in this group as indicated by 16S ribosomal DNA sequence data.

Interestingly, the 16S ribosomal DNA sequences of the barophilic strain DB6906 and the barotolerant strain DSS12 showed highest homology of all, indicating that these strains

Table 1. Levels of similarity (%) between the barophilic and barotolerant bacteria strains and representatives of the Proteobacteria gamma sub-group

Strains	5501	6101	6705	6906	DSK1	DSS12	*Ahyd*	*Ecol*	*Pshi*	*Prvu*	*Smar*	*Vang*
DB5501	100	95.4	92.4	93.4	94.2	93.5	88.2	86.4	87.0	87.0	86.2	86.3
DB6101		100	94.6	96.3	92.3	95.2	89.8	86.9	87.1	87.1	86.3	87.5
DB6705			100	94.2	92.9	95.0	90.0	88.1	88.4	87.7	86.7	88.4
DB6906				100	89.9	97.7	89.8	87.7	88.2	87.8	87.3	87.5
DSK1					100	90.3	87.4	86.4	87.0	85.9	85.5	86.2
DSS12						100	90.5	88.2	88.8	88.6	87.6	88.0
A. hydrophia							100	85.7	85.3	84.9	86.0	87.4
E. coli								100	89.6	91.2	93.2	86.2
P. shigelloides									100	89.6	89.0	86.4
Pr. vulgalis										100	88.8	85.4
S. marcescens											100	88.0
V. anguillarum												100

Ahyd; Aeromonas hydrophia (accession number; M59148), *Ecol; Escherichia coli* (J01695, #1518–3059), *Pshi; Plesiomonas shigelloides* (M59159), *Prvu; Proteus vulgalis* (X07652), *Smar; Serratia marcescens* (M59160), *Vang; Vibrio anguillarum* (X16895).

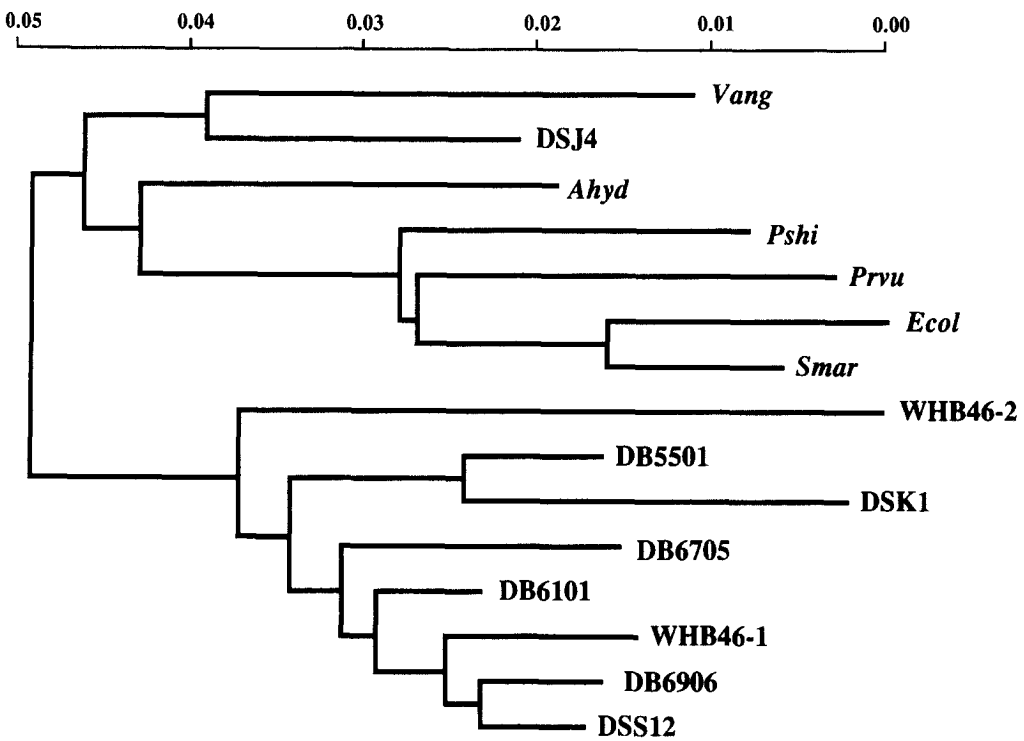

Figure 3. Phylogenetic tree showing the relationships of isolated barophilic and barotolerant strains within the Proteobacteria gamma subgroup, as determined by a 16S ribosomal DNA sequence comparison, using the neighbour-joining method. BWHB46-1 and BWHB46-2 are 16S ribosomal DNA sequences of barophilic bacteria reported by Liesack *et al.* (1991), and these accession numbers are X54744 and X54745, respectively. The strain DSJ4 is *Vibrio* sp., isolated from the Ryukyu Trench sample, and is not discussed in this paper. The accession number of 16S ribosomal DNA sequence of the strain DSJ4 is D21226. The scale represents the average number of nucleotide substitutions per site.

are very closely related species. The relationship between the isolated strains and some strains of the Proteobacteria gamma subgroup is shown in Fig. 3 in the form of a phylogenetic tree. The isolated barophilic and barotolerant strains in this study and other strains were separated into two sub-branches in the gamma subgroup. The barophilic organisms, strain WHB 46–1 and WHB 46–2 reported by Liesack *et al.* (1991) were also included in the sub-branch containing the isolated strains in this study. These results suggest that, even between closely related species, the properties of barophily and barotolerance may vary considerably. As we discussed above, the difference between the properties of barophily and barotolerance may be dependent on the growth conditions.

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