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Enzymatic Profiles of 11 Barophilic Bacteria under In Situ Conditions: Evidence for Pressure Modulation of Phenotype

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Barophilic bacteria are microorganisms that grow preferentially (facultative barophiles) or exclusively (obligate barophiles) under elevated hydrostatic pressure. Barophilic bacteria have been isolated from a variety of deep-sea environments. Attempts to characterize these organisms have been hampered by a lack of appropriate methodologies. A colorimetric method for the detection of 19 constitutively expressed enzymes under in situ conditions of pressure and temperature has been devised, using a simple modification of the commercially available API ZYME enzyme assay kit. By using this method, enzyme profiles of 11 barophilic isolates, including an obligate barophile, were determined. Nine of the 10 facultatively barophilic isolates examined exhibited a change of phenotype in at least one enzyme reaction when tested at 1 atm (1 atm = 101.29 kPa), compared with results obtained under in situ pressure. The assay is simple and rapid and allows for direct determination of enzyme activity under conditions of high pressure and low temperature.

The API ZYME enzyme test kit is a semiquantitative micromethod system designed for rapid detection of enzymatic activities, including phosphatase, amino peptidase, lipase, esterase, and carbohydrate hydrolase. Results of studies using tissues, cells, biological fluids, and microorganisms show that the technique is applicable to a variety of specimens. Humble et al. (6) reported the system useful for enzymatic characterization of bacteria, yielding enzyme profiles for both gram-positive and gram-negative bacteria. Since 1977, API ZYME enzyme test kits have been used successfully to characterize a wide range of microorganisms of clinical importance (1, 8, 10, 11).

In the present study, the rapid test system was extended to microorganisms isolated from environmental samples, specifically, barophilic bacteria from the deep sea. A simple modification of the manufacturer's recommended protocol permitted enzymatic characterization of these barophiles grown under conditions of low temperature and high pressure, thereby simulating their natural habitat.

Bacterial strains. Eleven strains of barophilic bacteria were examined. The source of each strain is described in Table 1. The 5S rRNA sequence has been determined for strains UM 40 and UM 145 (4) and for the obligate barophile BNL-1 (5). All strains were maintained in marine broth 2216 (Difco Laboratories, Detroit, Mich.), prepared as follows. After rehydration of the broth following the manufacturer's directions, the medium was boiled, cooled to room temperature, filtered through a 0.22-m-pore-size filter, and autoclaved. Standing cultures of the barophilic isolates were maintained in steel pressure vessels at the approximate hydrostatic pressure of the depth of collection (430 atm [1 atm = 101.29 kPa] for the facultative barophiles, and 720 atm for BNL-1, the obligate barophile). The vessels containing the cultures were incubated in a cold room adjusted to the deep-sea temperature of 4°C.

To obtain a sufficient volume of cell suspension to inoculate all of the enzyme test tubes, cultures were prepared as follows. For each isolate, duplicate polycarbonate tubes containing approximately 50 ml of sterile cold marine broth 2216, prepared as described above, were inoculated with 1.0 ml of a standing broth culture. To eliminate possible hyperbaric gas toxicity, it is necessary to eliminate air bubbles from all culture tubes. Bubbles were eliminated by tapping the tube and slightly overfilling with media. The excess media and the contained bubbles were removed when the tubes were sealed with Parafilm. The sealed culture tubes were placed into two stainless-steel pressure vessels. One vessel was then pressurized, and the other was sealed but maintained at 1 atm. To acclimate the cultures to the two pressures and to obtain the approximate turbidity recommended by API for use in the ZYME tests, cultures were grown at 4°C for 19 days and then used to fill a series of small tubes, prepared as follows.

Enzyme detection method. The API ZYME enzyme test kit (product no. 8886-070252; Analytab Products [API], Plainview, N.Y.) consists of a plastic-covered strip with a series of microcupules containing dehydrated chromogenic substrates. In this study, the strip was cut aseptically into individual microcupules, each of which was placed in a separate, sterile, 1.5-ml centrifuge tube (Sigma Chemical Co., St. Louis, Mo.). Each tube was completely filled with bacterial suspension, making sure that all air bubbles were expelled. By removing the molded snap top of each tube, it was possible to fill the tube and seal it with Parafilm, as described above. The 200 tubes for each pressure treatment (10 isolates, 20 cupules each) were placed in a single large (1.6-liter) pressure vessel and brought to the required pressure. The inoculated tubes containing the ZYME test cupules were incubated at 4°C for 5 days. Preliminary trials of the method indicated that this incubation time was the minimum for consistent color development by the substrates.

Colorimetric reactions were developed as follows. The pressure vessels were decompressed and the tubes were removed, opened, and tested within 2 h. To make room in the tubes for the ZYME reagents, small (0.5-ml) volumes of the bacterial suspension were discarded. Four drops each of ZYME reagents A and B were added to each tube and the contents were thoroughly mixed. The color reactions were allowed to develop for 5 to 10 min in the dark. The intensity

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TABLE 1. List of strains used in the study

Strain designation	Sample source	Reference
UM 40	Animal gut	4
UM 145	Animal gut	4
BBG3	Animal gut	3
BBG4	Animal gut	3
BBS2	Sediment core	3
BBDT1	Sediment core	3
BBDT2	Sediment trap	3
BBP5	Sediment trap	3
BBP6	Sediment trap	3
BNL-1	Sediment trap	5

of the reaction was graded 0 to 5, according to the API ZYME color reaction chart supplied by the manufacturer. The two vessels subjected to different pressure treatments were incubated side by side without agitation. The obligate barophile, BNL-1, was tested in triplicate at 720 atm, the approximate pressure of the ocean depth at which it was collected.

To assess any possible effects due to our modifications of the established ZYME kit protocol, a control experiment was conducted with *Escherichia coli* K-12. A single culture was used to inoculate both an intact ZYME strip and one which had been modified as described above. Both were incubated at 37°C for 4 h. Scoring of the colorimetric reactions showed only one difference: esterase (C_4) reaction in the intact strip was scored 3, while the tube test yielded a score of 1. Uninoculated media, heat-killed suspensions of UM 40 and BBDT2 (60°C for 1 h), and autoclaved (15 min, 121°C) suspensions of UM 40 and BBP5 served as negative controls.

The API ZYME color chart allowed for enzyme reactions to be graded as negative (grade 0), weak (grade 1), and positive (grades 2 through 5). Positive reactions for the 10 facultatively barophilic strains under the described conditions are shown in Table 2. These results, combined with results of two preliminary trials with the 10 facultative barophiles and different pressure equipment, showed a degree of variability very close to that of the obligate barophile results. The enzymatic profile of the obligate barophile is given in Table 3. The 11 strains tested in this study yielded negative reactions for esterase (C_4), lipase (C_{14}), valine aminopeptidase, cystine aminopeptidase, chymotrypsin, α-galactosidase, β-glucuronidase, α-glucosidase, α-mannosidase, and α-fucosidase.

Although the difference between a negative and a weak positive reaction was considered significant when comparing results obtained under the two pressures, due to the inherently subjective nature of grading, differences of <2 in positive results were not recorded as significant. By these criteria, pressure affected the phenotypes of 9 of the 10 facultative barophiles. Pressure had no effect on strongly positive enzyme reactions, such as those observed for acid and alkaline phosphatase and trypsin (with one exception). However, enzymes such as esterase lipase, leucine aminopeptidase, β-galactosidase, and *N*-acetyl-β-glucosaminidase, which were only weakly expressed, were affected by pressure. In five of the isolates, pressure changed the phenotype for leucine aminopeptidase, and all showed higher leucine aminopeptidase activity under pressure, com-

TABLE 3. Enzymatic activities of an obligate barophile, *Colwellia hadaliensis*[a]

Phosphatase		Esterase lipase	Leucine amino-peptid-ase	Trypsin	Phospho-hydro-lase	β-Galac-tosidase	*N*-Acetyl-β-glu-cosamini-dase
Acid	Alkaline						
5	5	0	1	4	5	0	0
2	3	0	0	3	1	0	0
2	4	0	0	3	0	0	0

[a] Enzymatic profile of an obligate barophile growing at 4°C and under its in situ pressure (720 atm). Results of three trials are shown to demonstrate the range of variability of the results. Numbers indicate intensity of reaction.

TABLE 2. Enzymatic activities of 10 facultatively barophilic bacteria

Strain	Incubation pressure (atm)[a]	Intensity of reaction							
		Phosphatase		Esterase lipase	Leucine aminopeptidase	Trypsin	Phospho-hydrolase	β-Galac-tosidase	*N*-acetyl-β-glucosaminidase
		Acid	Alkaline						
UM40	430	3	4	0	0	3	3	0	1
	1	3	4	0	0	2	3	0	0
UM 145	430	3	3	0	0	2	2	0	0
	1	3	4	0	0	2	3	1	0
BBG3	430	2	3	0	1	1	1	0	0
	1	3	3	0	0	2	3	1	0
BBG4	430	3	4	0	1	2	3	0	0
	1	3	4	0	1	2	3	0	0
BBS2	430	2	5	0	1	3	2	0	0
	1	2	4	1	1	2	2	0	1
BBS3	430	1	2	0	1	1	2	0	0
	1	2	3	0	0	3	2	0	1
BBTD1	430	4	5	1	1	2	3	0	0
	1	3	4	0	0	2	3	0	0
BBTD2	430	4	5	0	2	2	4	0	0
	1	3	5	1	0	3	2	0	1
BBP5	430	2	5	0	1	3	2	0	0
	1	3	5	1	0	2	3	0	1
BBP6	430	2	3	0	0	2	2	0	0
	1	3	4	2	0	2	3	0	1

[a] 1 atm = 101.29 kPa.

pared with the equivalent reaction at 1 atm. In contrast, pressure-induced change in esterase lipase, *N*-acetyl-β-glucosaminidase (each with one exception), and β-galactosidase was increased activity at 1 atm. Most strains showed a change in the intensity of reaction for two or more of the tested enzymes. One strain, BBDT2, exhibited changes in four of the enzymatic tests, with two more strongly active under pressure and two more strongly active at 1 atm.

Barophilic bacteria are those which grow more rapidly (facultative barophiles) or exclusively (obligate barophiles) at pressures of >1 atm. While a number of barophilic bacteria have been isolated (3, 4, 6, 7, 12), they remain relatively incompletely characterized because of technical problems, such as the limited space in pressure vessels, the long doubling time of psychrophilic barophiles (4 to 12 h), and lack of simple assay methods for enzyme activity under pressure.

By modifying API ZYME enzyme test kits, it is possible to obtain enzyme profiles for a set of barophiles growing at the temperature and pressure of the deep ocean from which they were originally isolated. The sensitivity and specificity of the API ZYME system allow for detection of low levels of enzyme activity (9), thereby shortening the incubation period required to achieve adequate enzyme production.

The API ZYME test system is designed to be semiquantitative, with the strength of each enzyme reaction being correlated to a range of units of activity. In this study, modification of the established ZYME protocol may have affected the ZYME test results in terms of units of enzyme activity. However, since the described protocol was used throughout this study, grading of the colorimetric reactions should provide a valid basis for the comparison of relative enzyme activities for the various strains under the two incubation pressures.

Some barophiles showed enzyme activity at 1 atm which was not evident under pressure. It is possible that barophiles grown at 1 atm may be more susceptible to lysis by ZYME reagent A. In tests conducted at 1 atm, such an effect might elevate low-level expression of the enzyme above the detection threshold. On the other hand, the observation of enzyme activity at 1 atm but not under pressure may indicate that adaptation to growth under elevated pressure may not involve all enzyme systems of the microorganism and the expression or activity of some enzyme systems in barophiles may indeed be inhibited by high pressure. Those enzymes for which the substrate may not be available or abundant in the oligotrophic environment of the deep sea may be particularly susceptible to pressure inhibition.

The physiology of barophilic adaptation has recently been explored. For example, Delong and Yayanos reported that pressure stimulates the activity of the glucose transport system of a barophilic bacterium (2). The study reported here suggests that pressure affects the expression or activity of a variety of other enzyme systems as well.

The method used in this study to obtain enzyme profiles for deep-sea bacteria should prove useful for the characterization of barophilic bacteria. Although some aspects of the method are necessarily cumbersome, it does provide a simple, relatively rapid method for testing barophilic bacteria under their natural growth conditions.

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