APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1997, p. 2105–2108 Vol. 63, No. 5
0099-2240/97/$04.00+0

Evolutionary Relationships of Cultivated Psychrophilic and Barophilic Deep-Sea Bacteria

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Received 5 November 1996/Accepted 4 March 1997

Evolutionary relationships of cultivated barophilic bacteria were determined. All psychrophilic and barophilic isolates were affiliated with one of five genera of the gamma subdivision of the class *Proteobacteria* (γ-*Proteobacteria*): *Shewanella*, *Photobacterium*, *Colwellia*, *Moritella*, and a new group containing strain CNPT3. The data indicate that the barophilic phenotype has evolved independently in different γ-*Proteobacteria* genera.

Barophilic bacteria grow optimally or exclusively at hydrostatic pressures greater than 1 atm (1 atm = 0.1013 MPa = 1.103 bar). Barophilic isolates have been obtained from a variety of different deep-sea habitats by a number of different laboratories (5, 8, 9, 15, 16, 20, 34–36, 38, 39).

The phylogenetic affiliations of several barophilic isolates have been inferred by 5S rRNA (8, 22) or 16S-like rRNA sequence comparisons (9, 13, 15, 16, 20). Studies based on 5S rRNA sequences showed that two facultatively barophilic strains from a depth of 5,920 m were affiliated with the genus *Shewanella* and that one obligate barophile from a depth of 7,410 m was specifically related to a psychrophilic marine isolate, *Colwellia psychroerythrus* (9). Additionally, small-subunit rRNA sequence analysis indicated that two strains of Antarctic obligate barophiles, initially grown in mixed culture, are members of the gamma subdivision of the class *Proteobacteria* (γ-*Proteobacteria*) (20). Recently, Kato and coworkers also isolated many barophilic and barotolerant strains reported to belong to the γ-*Proteobacteria* (15, 16). In this study, we report the phylogenetic analyses and partial phenotypic characterization of a variety of psychrophilic and barophilic heterotrophic bacterial isolates.

Bacterial strains and growth media. The pressure physiologies of most barophilic bacterial strains in this study have been previously described (Table 1) (7, 35, 37–39). Isolate SS9 originated from a depth of 2,551 m in the Sulu Sea and was obtained from an amphipod homogenate enrichment (5). Barophilic strain F1A was kindly provided by Carl O. Wirsen (34). All other reference strains were obtained from the culture collection of A. A. Yayanos or the American Type Culture Collection. Nonbarophilic strains were grown at 3°C in marine broth 2216 (Difco). Barophilic strains were grown at 3°C in marine broth 2216 at the appropriate hydrostatic pressure (Table 1), as previously described (Table 1) (6, 7, 39), decompressed, harvested by centrifugation, and stored frozen at −80°C. All barophilic strains examined in this study (Table 1), with the exception of strain MT41, which was not tested, were successfully stored as frozen stock cultures at 1.1×10^5 Pa and −80°C. Strains were grown at the appropriate growth pressure at 3°C to approximately 1×10^8 cells/ml, decompressed, immediately diluted 1:1 in ice-cold 2× freeze buffer (1% yeast extract, 10% glycerol, 10% dimethyl sulfoxide, 0.1 M K_2HPO_4 [pH 7.0]), quickly frozen in a dry ice-ethanol bath, and stored at −80°C. Frozen cultures were revived by inoculation of ice-cold marine broth with scrapings from the frozen stock and incubation under the appropriate hydrostatic pressure at 3°C.

Phenotypic characterization. DNA base composition was determined from thermal melting profiles performed under standard conditions as previously described (24). Other physiological tests were performed by slight modification of the general procedures described by Baumann and Baumann (1). All high-pressure physiological tests were performed in tandem with uninoculated blank controls. Acid production from glucose was assessed in a modified OF medium (1) containing 0.5× ASW (1× ASW is 400 mM NaCl, 100 mM $MgSO_4 \cdot 7H_2O$, 20 mM KCl, 20 mM $CaCl_2 \cdot 2H_2O$), 10 mM morpholinepropanesulfonic acid (MOPS; pH 7.5) at 22°C, 1% glucose, 0.2% agar, 0.5% yeast extract, and 0.003% bromcresol purple. Cultures were inoculated as stabs and sealed with molten media containing 4% gelatin. After topping the tube with 4% gelatin, the tubes were chilled to 4°C, sealed with Parafilm, and incubated at the appropriate pressure at 3°C (Table 1). After decompression, the stabs were examined for growth and acid production. Hydrogen sulfide production from thiosulfate was assessed in triple sugar iron agar stabs (TSI; Difco) prepared with 0.5× ASW instead of water. DNase activity was tested by inoculation of stab cultures in DNase medium (Difco) containing 0.5× ASW and 0.01% methyl green. Oxidase tests were performed as previously described (1). Tests for anaerobic growth with lactate as the sole carbon source and trimethylamine oxide (TMAO) as the sole electron acceptor were performed as follows. Two parts of a defined mineral medium (SM medium [10]) was mixed with 12% gelatin plus the following combinations of electron donors and acceptors: (i) no addition, (ii) 25 mM TMAO, (iii) 15 mM lactate, and (iv) 25 mM TMAO plus 15 mM lactate. The tube was chilled to solidify the gelatin, inoculated as a stab, overlaid with 1 ml of 4% gelatin, sealed with Parafilm, and incubated at 2°C and the pressure appropriate for the specific strain. Strains which showed growth only in the presence of TMAO and lactate were scored as positive. Motility determinations were performed on cultures grown at their optimal growth pressure in marine broth. Exponentially growing cultures were decompressed to 1.1×10^5 Pa, placed on a chilled microscope slide, and immediately examined by phase microscopy for motility at 1.1×10^5 Pa.

Phylogenetic characterization. Nucleic acids were purified by standard methods (30). In some cases (SC2A, F1A, *Shewanella hanedai* ATCC 33224, MT41, PT48, and PT99), rRNA templates

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TABLE 1. Deep-sea bacterial strains used in this study

Isolate	Source	Physiology	Geographic origin	Depth (m)	Approx growth pressure optimum (10^5 Pa)
MT41	Yayanos (38)	Obligate barophile	Mariana Trench	10,476	1,034
C. psychroerythrus	ATCC 27364 (9)	Psychrophile	Norwegian fjord	Surface	1.1
CNPT3	Yayanos (37)	Facultative barophile	Central North Pacific	5,800	517
SC2A	Yayanos (39)	Psychrophile	California coast	1,957	1.1
F1A	Jannasch and Wirsen (34)	Facultative barophile	North Atlantic	4,900	414
PT99	Yayanos (7)	Obligate barophile	Philippine Trench	8,600	621
S. benthica	ATCC 43991 (8)	Facultative barophile	Puerto Rico Trench	5,920	414
PT48	Yayanos (7)	Obligate barophile	Philippine Trench	6,163	621
Photobacterium sp. strain SS9	DeLong (5)	Facultative barophile	Sulu Sea	2,551	276
M. marinus MP1	ATCC 15381 (4)	Psychrophile	Oregon coast	1,200	1.1
PE36	Yayanos (35)	Facultative barophile	California coast	3,584	414

were directly sequenced with reverse transcriptase (Seikagaku) as previously described (17, 18). All other small subunit rRNA sequences (PE36, *Moritella marinus* MP1, CNPT3, SS9, *S. hanedai* ATCC 35256, *S. benthica* ATCC 43991, and *Shewanella putrefaciens* ATCC 8072) were obtained by direct sequencing of PCR-amplified ribosomal DNA genes.

Phylogenetic analyses were conducted with reference sequences and software (GDE 2.2 [32]) obtained via anonymous file transfer program from the Ribosomal RNA Database Project (23) and the software package Phylip, version 3.5 (12). Distance matrices were calculated with the Phylip program DNAdist by using the Kimura two-parameter model, assuming a transition/transversion ratio of 2.0. Distance matrix trees were then inferred from the estimated evolutionary distances by neighbor-joining analyses. Bootstrap neighbor-joining analysis was conducted on 100 replicates with random taxon addition. Maximum likelihood phylogenetic analyses (11) were performed with fastDNAml, version 1.0 (26), employing empirical base frequency, global branch swapping, and bootstrapping options.

Nucleotide sequence accession number. The sequences of the ribosomal DNA genes used in this study have been deposited in GenBank under accession no. U91586 to U91600.

Results and discussion. Strain designations, sites and depths of origin, and pressure optima for barophilic strains characterized in this study are shown in Table 1. Phenotypic characteristics of barophilic isolates and reference strains are shown in Table 2. Physiological tests conducted at 3°C and in situ growth pressures (Tables 1 and 2) were consistent with the placement of F1A, PT99, PT48, and SC2A within the genus *Shewanella*. Characteristics common to *Shewanella* species (19) and found in *S. benthica* strains PT99, PT48, and F1A included H_2S production on TSI agar, lactate oxidation coupled to TMAO reduction, being oxidase and DNase positive, and showing no fermentation of glucose. At 1.1×10^5 Pa and 8°C, *S. benthica* ATCC 43991 tested positive for anaerobic growth on lactate with Fe^{3+} but not sulfite as a terminal electron acceptor. Strain SC2A, phylogenetically affiliated with *S. putrefaciens*, grew anaerobically on lactate with Fe^{3+}, TMAO, or sulfite as the terminal electron acceptors. Unlike most other barophilic strains examined, strain CNPT3 fermented glucose vigorously as indicated by both acid production and gas production on glucose. Strain CNPT3 was the only strain examined which tested positive for arginine dihydrolase.

Long-chain polyunsaturated fatty acids (PUFAs) are found in a large percentage of barophilic isolates distributed among distinct genera (Table 2) (7, 34), although these fatty acids are not found exclusively in deep-sea barophilic strains (25, 26, 28, 40, 41). Psychrophilic or barophilic *Photobacterium*, *Shewanella*, *Colwellia*, and *Moritella* strains have all been reported to contain high amounts of the PUFA eicosapentaenoic acid ($C_{20:5}$) or docosahexaenoic acid ($C_{22:6}$) in their membrane lipids. Eicosapentaenoic acid is found in the genus *Shewanella* (strains SC2A, PT99, PT48, and F1A) (7, 34), as well as in *Photobacterium* sp. strain SS9 (32a). Docosahexaenoic acid is found in members of two distinct barophile-containing groups, *Moritella* (*M. marinus* and sp. strain PE36 [7]) and *Colwellia* (sp. strain MT41) (7). The incidence of high PUFA content in a variety of phylogenetically distinct psychrophilic and barophilic genera suggests an important role for PUFAs in maintenance of membrane fluidity at low temperature and high pressure (7).

Phylogenetic analysis of psychrophilic and barophilic deep-sea bacteria consistently placed them within one of five groupings (Fig. 1). All deep-sea heterotrophic, barophilic bacteria examined were affiliated with one of the following five γ-*Proteobacteria* genera: *Shewanella*, *Photobacterium*, *Colwellia*, *Moritella* (former *Vibrio* sp.) (4), and one as-yet-undescribed group containing strain CNPT3 and one Antarctic barophile from the Weddell Sea (Fig. 1). Facultative barophiles were distributed among all five of these barophile-containing phylogenetic groups (Fig. 1). Obligate barophiles fell within one of three clades: *Shewanella*, *Colwellia*, or the CNPT3 group (Fig. 1).

rRNA sequence similarity values determined from 1,043 unambiguously aligned positions (Fig. 1) relating the barophilic strains examined in this study to previously sequenced strains were as follows: SS9 and *Photobacterium phosphoreum*, 96.2%; PE36 and *M. marinus*, 97.7%; CNPT3 and WHB46-2, 97.5%; MT41 and *C. psychroerythrus*, 92.6%; SC2A and *S. putrefaciens* 8071, 95.3%; PT48 and *S. benthica* 43992, 97.1%; PT99 and *S. benthica* 43991, 95.8%; and F1A and *S. benthica* ATCC 43991, 96.9%. Other barophilic and barotolerant strains recently isolated by the Japanese DEEPSTAR group (29, 30) were also closely affiliated with the groups described above (Fig. 1) and include strains affiliated with *Moritella* (DSK1), *S. benthica* (DSS12, DB9606, and DB6101), and *Photobacterium* (DSJ4) (Fig. 1).

The genus *Shewanella* is composed of an ecologically diverse group of obligately respiratory, gram-negative *Proteobacteria* isolates of both marine and terrestrial origin (9, 13, 14, 21, 22, 27, 29, 31, 33, 37). A large number of barophilic isolates, obtained by five different laboratories working in disparate locales (7, 8, 15, 16, 20, 34), are *S. benthica* strains or extremely close relatives (Fig. 1). The data indicate that *S. benthica* is the most commonly isolated barophilic species, recovered from a variety of abyssal to hadal environments in Pacific, Atlantic, and Antarctic seas. It is difficult to judge from available data how numerically abundant or ecologically significant this species actually is, since its representation in deep-sea bacterial

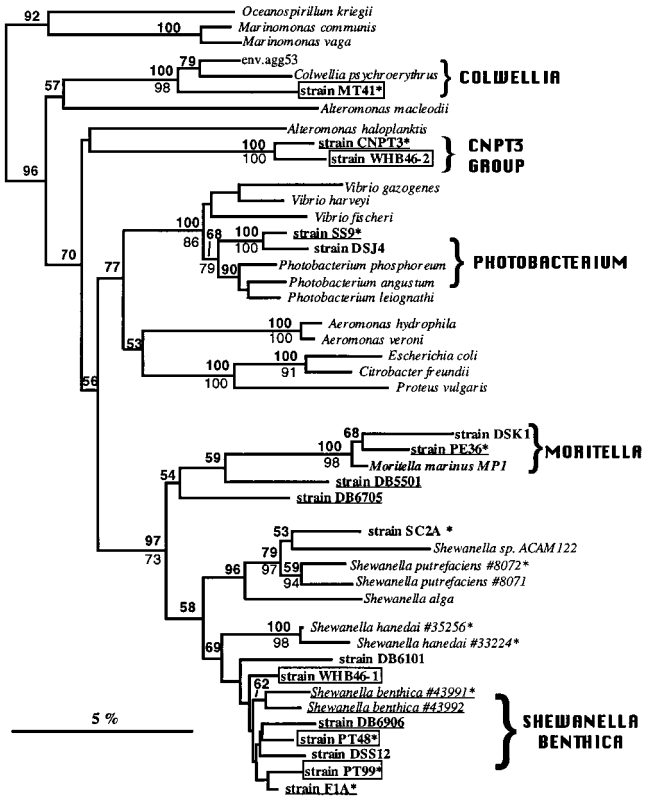

FIG. 1. Phylogenetic relationships of heterotrophic psychrophilic and barophilic deep-sea bacterial isolates. Tree topology was inferred from neighbor-joining analysis of evolutionary distances determined with the Kimura two-parameter model. Results from neighbor-joining bootstrap analysis (100 replicates) are indicated by numbers (as percentages) in boldface at the top of each node. Only nodes supported in greater than 50% of the bootstrap replicates are indicated. Nodes supported in maximum likelihood bootstrap analysis (100 replicates) in greater than 50% of the replicates are shown below the node. The scale bar corresponds to 5 fixed mutations per 100 sequence positions. rRNA sequences determined in this study are indicated with asterisks. All strains indicated in boldface are of deep-sea origin. Underlined strains have been shown to be facultatively barophilic at in situ deep-sea temperatures of 2 to 4°C. Boxed strains are obligately barophilic at all growth temperatures tested. env.agg53, environmental rRNA clone from marine aggregate clone 53.

TABLE 2. Phenotypic characteristics of psychrophilic and barophilic marine isolates and closely related reference strains

Test or characteristic[a]	Result [reference] for[b]:										
	Photobacterium phosphoreum	SS9	*S. putrefaciens* ATCC 8071	SC2A	*S. benthica* ATCC 43991	PT99	PT48	F1A	*M. marinus* ATCC 15381	PE36	CNPT3
G+C (mol%)	42 [3]	43 ± 1.0	43.8 [19, 27]	42.7 ± 0.9	47 [8]	46.4 ± 0.6	ND	ND	42.2 [2, 4]	41.9 ± 0.9	39.6 ± 0.8
Cell shape	sr	sr (1 μm)	r	r	r (1.5–3 μm)	r (3–4 μm)	r (2–3 μm)	r	r	r (1–3 μm)	sp (2–4 μm)
Oxidase	+	+	+	+	+	+	+	+	+	+	+
Growth at 1 atm	+	+	+	+	+	–	–	+ (w)	+	+ (w)	+ (w)
Motility	+	+	+	+	+	–	+	+	+	+	+
Acid from glucose	+	+ (w)	–	+	–	–	–	–	–	–	–
H_2S on TSI media	–	–	+	+/–	+	+	+	+	–	–	–
Anaerobic growth on lactate-TMAO	ND	ND	+	+	+	+	+	+	–	–	–
DNase	ND	+	+	+	+/–	+	+/–	+	–	–	–
PUFA[c]	–	+ (20:5)	ND	+ (20:5)	ND	+ (20:5)	+ (20:5)	+ (20:5)	+ (22:6)	+ (22:6)	+
Arginine dihydrolase	–	–	–	–	–	–	–	–	–	–	+

[a] All tests were performed at 3°C and at the pressure indicated in Table 1 unless otherwise indicated.

[b] ND, not determined; sr, short, plump rods; r, rods; sp, spirillum; w, weakly positive.

[c] PUFAs in membrane lipids. All data on PUFAs are from reference 10, except for those for strain SS9 (32a). 20:5, eicosapentaenoic acid; 22:6, docosahexaenoic acid.

culture collections may simply reflect its selective enrichment in culture and not its numerical abundance.

In total, our analyses indicate that the evolution of barophilic phenotype is not limited to one or a few genera, but may be fairly widely distributed among the γ-*Proteobacteria* and possibly other eubacterial or archaeal phyla. Considering that many naturally occurring microorganisms have proven difficult or impossible to recover by standard cultivation techniques, it is likely that many new lineages of deep-sea barophilic bacteria remain to be discovered and described.

We thank Carl Wirsen and Holger Jannasch for the loan of pressure vessels and hydraulic pumps at Woods Hole, Dennis Bazylinski and Jeffrey Hodges for performing the DNA thermal melting profiles, and Tom DiChristina for advice on physiological tests for anaerobic respiratory capability.

This work was supported by an Office of Naval Research Young Investigator grant and NSF grants OPP94-18442 and OCE95-29804 to E.F.D.

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