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Pressure effects on *Clostridium* strains isolated from a cold deep-sea environment

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Abstract Three *Clostridium* strains were isolated from deep-sea sediments collected at a depth of 6.3–7.3 km in the Japan Trench. Physiological characterization and 16S rDNA analysis revealed that the three isolates were all closely related to *Clostridium bifermentans*. The spores of all three isolates were resistant to inactivation at high pressure and low temperature. However, despite the fact that the vegetative cells were halotolerant and eurythermal they did not appear to be adapted for growth or viability under the conditions prevailing in the deep-sea sediments from which they were obtained. The results suggest that the isolates had survived as spores in the deep-sea sediments and that the marine benthos could be a source of clostridia originating in other environments.

Keywords *Clostridium* · *Clostridium bifermentans* · Deep sea · Japan Trench · Pressure

The bacterial genus *Clostridium* is a phylogenetically diverse group of Gram-positive spore-forming anaerobes. Certain *Clostridium* species have been isolated from the marine environment to assess the ecological impact of sewage disposal (Edwards et al. 1998; Hill et al. 1993; Takizawa et al. 1993) and to assess the distribution of human pathogens (Miller 1975). But, truly "marine" clostridia are rare. In only a few instances have any clostridia species exhibited optimal growth under seawater conditions (Fendrich et al. 1990). *Clostridium* spores may remain dormant for hundreds or even thousand of years prior to germination into vegetative cells (Braun et al. 1981; Cano and Boruki 1995). Thus, the capacity to produce spores resistant to a broad range of physiochemical stresses is likely to be a major factor in the broad distribution of these bacteria. In this report we describe the recovery and characterization of clostridia from 6.3 to 7.3 km depth within the Japan Trench, the effects of pressure on their growth and survival, and the survival of their spores.

To characterize obligate anaerobic deep-sea bacteria from marine sediments, samples from the Japan Trench sediments were inoculated and subsequently subcultured in a buffered anoxic marine medium at 9 °C. Sediments were collected by the remote-operated vehicle "KAIKO" (Kato et al. 1997) during dives 10K-83 (latitude 40°06′50″ N; longitude 144°11′05″ E; at a depth of 6,340 m) and 10K-87 (latitude 40°06′40″ N; longitude 144°11′30″ E; at a depth of 7,325 m) by means of sterilized mud samplers (Kato et al. 1997). Upon recovery the sediments were maintained at a low temperature, and subcored and subsequently shipped to the USA in sterile 50-ml centrifuge tubes (Corning, Corning, N.Y., USA). Initial enrichments were performed using basal carbonate buffered medium 3 (Widdel and Bak 1992) amended with glucose (20 mM) in Hungate tubes under nitrogen gas. Strain isolation, routine culturing, and physiological studies were accomplished using a derivative of Postgate's basal medium (Widdel and Bak 1992) containing sea salts (0.5%; Sigma, St. Louis, Mo., USA), glucose (20 mM), and HEPES buffer (100 mM, pH 7.5). Two strains, FL2 and FL3, were isolated from Japan Trench sediment collected at a depth of 7,325 m. Another strain, FL4, was isolated from sediment collected near a clam colony of *Calyptogena phaseoliformis* in the Japan Trench at a depth of 6,340 m.

FL2–4 were all strictly anaerobic, Gram-positive, spore-forming rods which were β-hemolytic on blood

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agar plates. Their morphologies are presented in Fig. 1. Thin-layer electron microscopy of spore suspensions was performed by imbedding in Dow epoxy resin (Lockwood 1964), staining with uranyl acetate and lead citrate, and examination in a Hitachi HS9 electron microscope. This analysis revealed that the spores of FL4 contained a particularly thick exosporium and cortex (Fig. 1E).

The biochemical profile of the isolates was obtained using the API ID32A test kit and database (Biomerieux, Marcy-L'etoile, France). All of the isolates exhibited striking similarity to one another and to *Clostridium bifermentans*, being positive for indole production and proline arylamidase, and negative for 27 other enzymes. Phylogenetic placement derived from16S rDNA sequence analysis corroborated the API test results and further indicated that all three strains belong to cluster XI of the genus *Clostridium* (Fig. 2) as defined by Collins et al. (1994). Genomic DNA was extracted as previously described (Maniatis et al. 1982). 16S rDNA was amplified using bacteria domain-specific primers 27F and 1492R (DeLong 1992) and cloned into pCR2.1 using the TOPO-TA cloning kit (Invitrogen, San Diego, Calif., USA). Plasmids were purified with a plasmid miniprep kit (Qiagen, Valencia, Calif., USA) and sequenced using an ABI 373 automated sequencer (PE Applied Biosystems, Foster City, Calif., USA). All steps of phylogenetic tree reconstruction were performed within the ARB software package in conjunction with the LINUX operating system (Strunk et al. 1998). The 16S rDNA sequences determined have been deposited in GenBank under accession numbers AF224673–AF224675, for FL2–4, respectively. 16S rDNA sequence identity between each of the isolates and *C. bifermentans* was more than 97%.

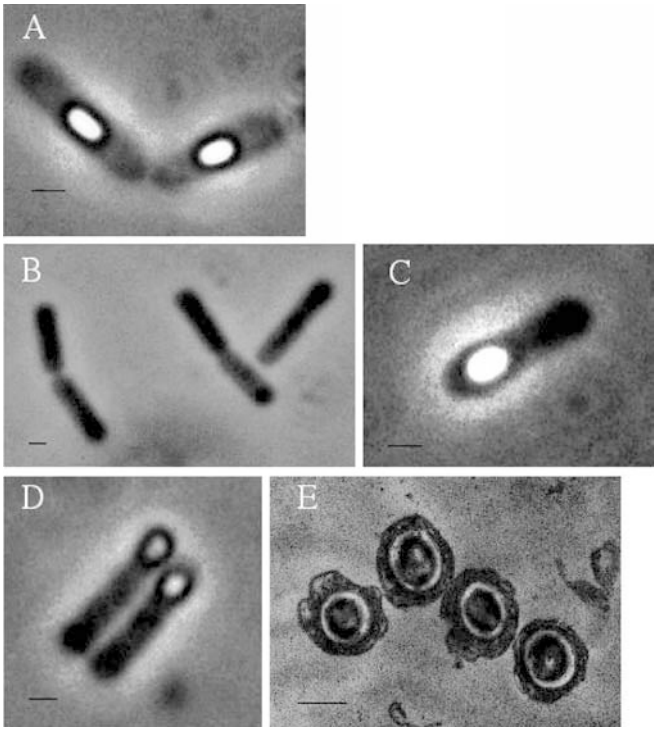

Fig. 1A–E Phase contrast and electron microscope micrographs of the FL strains. The *size bars* are 1 μm in length. **A** Phase contrast micrograph of FL2 sporulating cells. **B** Phase contrast micrograph of FL3 vegetative cells. **C** Phase contrast micrograph of FL3 sporulating cells. **D** Phase contrast micrograph of FL4 sporulating cells. **E** Thin-layer electron micrograph of FL4 spores

The isolates were able to grow in a range of NaCl concentrations from 0% to 5% with 0–2% being optimal, indicating that the strains were halotolerant. Growth studies performed as a function of temperature indicated that all three strains were eurythermal, exhibiting growth from 4 to 45 °C in liquid medium. Arrhenius plots showed the presence of broad peaks for the optimal temperature of all the strains (data not shown).

The effects of high pressure on cells and spores were also investigated. Inoculated cultures (1:50 dilution of overnight cultures) were transferred from Hungate tubes to heat-sealable polyethylene bulbs under an N_2 atmosphere. The bulbs were then heat-sealed inside pouches of low oxygen permeability (SilverPak metalized polyester barrier; Kapak, Minn., USA) that were filled with a solution of 0.15% Na_2S. High pressure incubation was performed using a custom-designed stainless steel pressure vessel/pumping system (2l inside volume, temperature controlled, with attached hydraulic pump, serial number 97151811-1; Autoclave Engineers, Erie, Pa., USA) for 17 h at 23 °C, after which time the optical density of the cultures was measured. Inocula for high pressure inactivation experiments were prepared using log phase ($OD_{600\ nm} \leq 0.4$) vegetative cells or stationary phase sporulating cells ($OD_{600\ nm} \geq 0.6$; examined microscopically to verify that nearly 100% of the cells had sporulated) obtained following 1 or 4 days growth, respectively, at 23 °C. The sporulating cells were pasteurized by heating at 72 °C for 15 min. The cultures were then incubated at either 69 MPa (23 °C) for 30 h or 150 MPa (23 °C) for 76 h, and colony counts were obtained at 0 and 76 h. All culture experiments were performed in triplicate. Pressurization of the FL cultures at 10.3 MPa and 20.7 MPa resulted in drastic reduction of cell yield at the latter pressure (Fig. 3). In contrast to the effect of pressure on FL2–4 strain growth, the spores of these isolates were observed to be extremely pressure resistant to inactivation. Vegetative cells and spore suspensions were pressurized to 69 MPa for 30 h as well as to 150 MPa for 76 h. Both high pressure treatments resulted in more than 3 log units decrease in colony-forming ability of the vegetative cells. Conversely, the spore suspensions, while giving more variable results, were always more than 2 log units more resistant to high pressure killing (Table 1). Furthermore, while the vegetative cells gave a 200–1,300% decrease in optical density following pressurization, the spore suspensions only decreased by 8–40%.

Away from areas of seafloor spreading, deep-sea sediments are characterized by a relatively constant low temperature (approximately 2 °C) and a high

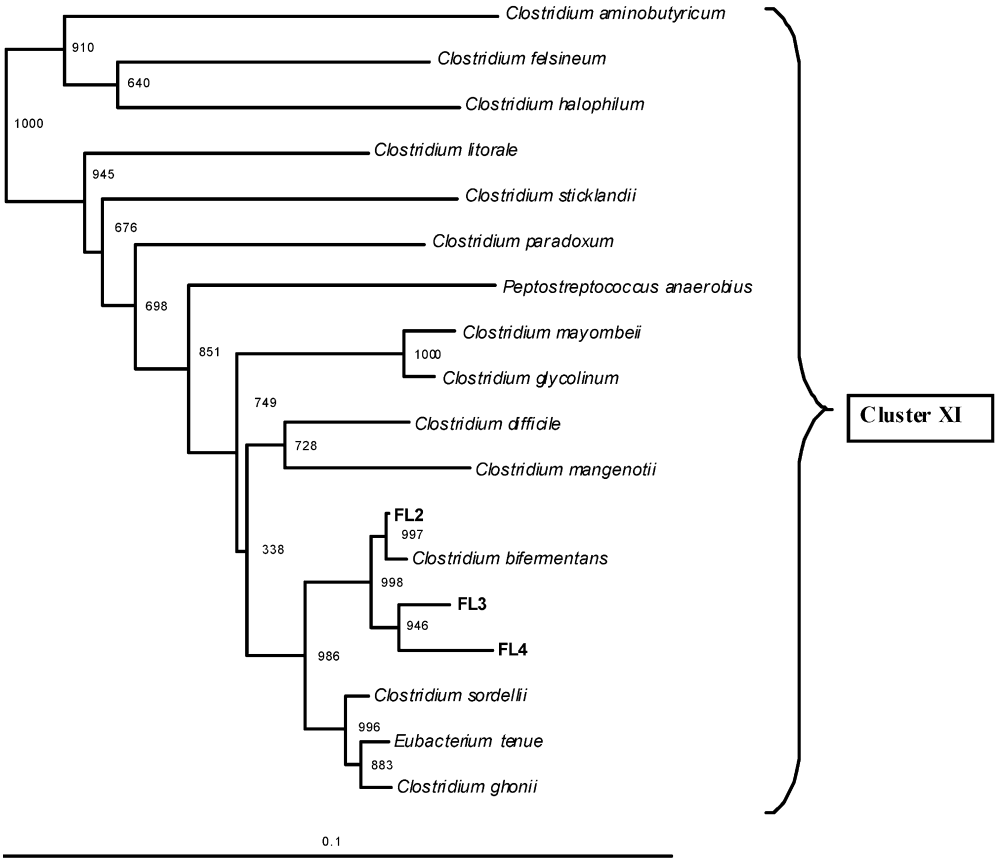

Fig. 2 Phylogenetic tree showing the position of the FL strains in cluster XI of the genus *Clostridium*. The stability of the tree was tested by 1,000 bootstrap replicates. The *scale* represents the average number of nucleotide substitutions per site

hydrostatic pressure which increases by approximately 0.1 MPa with each 10 m of depth. Many bacteria autochthonous to the deep-sea environment have been isolated which display adaptations to these conditions (Yayanos 1986). However, despite the fact that anaerobes play a central role in deep-sea benthic ecosystems (Lomholt 1980; Reimers et al. 1986), the anaerobes obtained in this study appear to be of allochthonous origin. The growth characteristics of the FL strains and the pressure resistance of their spores suggest that they are not adapted for growth under deep-sea conditions, but rather that their spores can survive for a prolonged period in this environment. The eurythermal growth of the isolates is in contrast to the psychrophilic nature of a previously isolated *Clostridium* species isolated from another permanently cold environment, namely that of an Antarctic pond sediment (Mountfort et al. 1997), and to the stenothermal character of truly piezophilic deep-sea bacteria (Yayanos 1986). Their high pressure sensitivity is consistent with that previously documented for one *Clostridium* species (Baross et al. 1975).

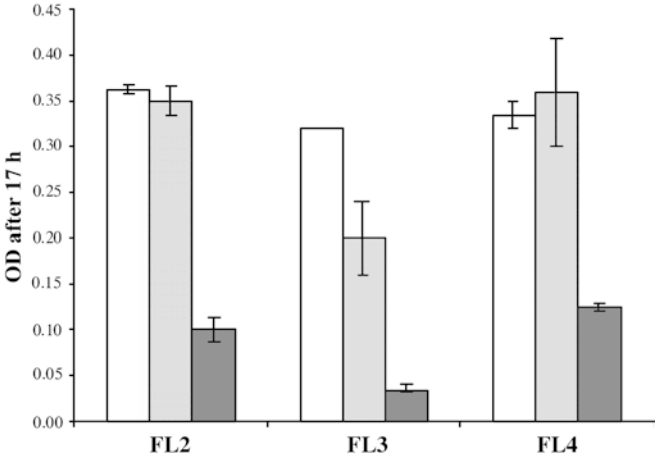

Fig. 3 Growth yields measured at an optical density (*OD*) of 600 nm for the FL strains after cultivation at various hydrostatic pressures for 17 h. Each experimental point is the mean of at least two independent determinations and the *error bars* indicate the standard deviation. *Unfilled bars* indicate 0.1 MPa, *lightly shaded bars* indicate 10.3 MPa, *darkly shaded bars* indicate 20.7 MPa

The closest relative of strains FL2–4, *C. bifermentans*, has been isolated from both soils and shallow marine sediments (Matches and Liston 1974), as well as from reptiles and fish (Dezfulian et al. 1994; Hoffmann et al. 1995). Clostridia and other Gram-positive bacteria have also previously been recovered from or identified in deep-sea sediments. Prior to this study the deepest depth previously reported for the isolation of *Clostridium* species was 2.4–2.7 km, for the recovery of *C. perfringens* from the Deep Water Municipal Sludge Disposal Site off the coast of New Jersey (Hill et al. 1993; Takizawa et al. 1993). Li and Kato (1999) found evidence for both *Clostridium* and *Bacillus* species in sediments obtained from a variety of depths, up to approximately 6.3 km in the Japan Trench, based upon 16S rDNA sequence

Table 1 Effect of high pressure treatments on the viability of FL2–4 vegetative cells and spores

Culture	Percent survival after 69 MPa for 30 h[a]	Percent survival after 150 MPa for 76 h
FL2 vegetative cells	0.025 ± 0.02	0.24 ± 0.11
FL3 vegetative cells	0.032 ± 0.02	0.006 ± 0.003
FL4 vegetative cells	0.019 ± 0.01	0.04 ± 0.006
FL2 spores	15 ± 3.5	29 ± 19
FL3 spores	70 ± 113	46 ± 20
FL4 spores	140 ± 57	52 ± 43

[a]Standard deviation included

analyses of sediment DNA. Takami and coworkers have isolated thermophiles and alkaliphiles from various deep-sea sediments near the south of Japan, including many spore-forming *Bacillus* species (Takami et al. 1999). Spore-forming thermophilic sulfate-reducing bacteria have been recovered from the North Sea (Rosnes et al. 1991), and numerous mesophilic actinomycete species have been recovered from deep-sea sediments (Colquhoun et al. 1998). Allochthonous bacteria arriving with phytodetritus from surface waters into the deep sea has been used to explain why the metabolic activity of mixed populations of microbes from the deep sea is almost always higher at atmospheric pressure than the in situ pressure (Jannasch and Taylor 1984). So, in this regard it is not surprising that it is possible to find many allochthonous Gram-positive bacteria, as well as other microbes in the deep sea. Even in the deepest ocean trench, the Mariana Trench, it has been possible to isolate bacteria which appear to have been brought down from other habitats (Takami et al. 1997).

The survival of *Clostridium* spores in deep ocean sediments is consistent with the well-documented resistance of many spores to inactivation by high pressure (Mills et al. 1998). For example, while high pressure (100–600 MPa for short periods) is now being used to sterilize some foods, it is only effective against bacterial spores in conjunction with some other agent such as hydrogen peroxide (Marquis 1997, 1998). The pressure resistance of the FL spores is likely to be related to both the dehydration of the spore protoplast and its dormancy. High pressure exerts multiple effects on cells, inhibiting metabolic processes that result in positive volume changes, disrupting multimeric proteins, and altering membrane structure (Bartlett 2002; Somero 1990). Protein and membrane stabilization by dehydration of the protoplast and the lack of metabolism within spores (Beaman et al. 1984) could counteract the destabilizing effects of pressure. Finally, the recovery of the FL strains from the deep sea might not only reflect their resistance to high pressure, but their responsiveness to compression/decompression. The exposure of some bacterial spores to pressures of 60–150 MPa followed by return to 0.1 MPa can induce their germination (Gould and Sale 1972).

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