Extremophiles (2002) 6:507–514
Digital Object Identifier (DOI) 10.1007/s00792-002-0288-1

ORIGINAL PAPER

Deana Desmarais Martin · Douglas H. Bartlett
Mary F. Roberts

Solute accumulation in the deep-sea bacterium *Photobacterium profundum*

Received: November 20, 2001 / Accepted: July 9, 2002 / Published online: September 7, 2002

Abstract The identity and amounts of intracellular solutes in the deep-sea bacterium *Photobacterium profundum* strain SS9 were studied using nuclear magnetic resonance techniques. *P. profundum* strain SS9, a moderate piezophile which grows optimally at 20–30 MPa primarily accumulated glutamate and betaine, with lesser amounts of alanine, β-hydroxybutyrate (β-HB) and oligomers composed of the β-HB units when grown at 0.1 MPa to early stationary phase. When grown at the optimal pressure, the cells preferentially increased intracellular concentrations of β-HB and β-HB oligomers, while the amino acid pools remained relatively constant. Since the organic solutes increased with increasing external NaCl in the medium, they are functioning as osmolytes. The β-HB molecules represent a novel class of osmolytes, termed "piezolytes," whose cellular levels responded to hydrostatic pressure as well as osmotic pressure. Factors such as cell growth stage and temperature were also examined for their effect on the solute distribution in these cells.

Key words Intracellular solutes · Osmolytes · *Photobacterium profundum* · Piezophiles · Solute accumulation

Introduction

Piezophiles (previously termed barophiles) are organisms which grow optimally at pressures above atmospheric pressure (1 atmosphere = 1 bar ≈ 0.1 megapascal (MPa) (Yayanos 1995). These microorganisms have been isolated from various deep-sea environments where the hydrostatic pressure can reach up to 110 MPa. At depths greater than 5 km (~50 MPa pressure) obligately piezophilic bacterial isolates have been recovered (Yayanos et al. 1981; Nakayama et al. 1994; Kato et al. 1995, 1998). Such microorganisms have acquired the capacity to flourish at pressures which perturb mesophile membrane structure, protein quaternary structure, and enzyme kinetics (Somero 1992; Yayanos 1995; Abe et al. 1999; Kato et al. 2000). Despite these remarkable growth characteristics, most piezophiles currently in culture collections are members of the gamma proteobacteria, and thus fall within a bacterial subdivision occupied by many well studied bacterial species which are not adapted to elevated pressure.

Although numerous studies have examined osmolytes in mesophilic bacteria, no previous studies of osmolyte type and abundance in a piezophile have been undertaken. There are several reasons to examine the osmolytes of piezophiles. Novel osmolytes have been identified in other microbial extremophiles, many of whom belong to the domain *Archaea* (Robertson et al. 1990, 1992; Sowers et al. 1990; Trüper and Galinski 1990; Lai et al. 1991; Severin et al. 1992; Somero 1992; Menaia et al. 1993; Ciulla et al. 1994; Martins and Santos 1995; Yayanos 1995; Desmarais et al. 1997; Martins et al. 1997). Also, both hydrostatic and osmotic pressure exert a profound effect on the hydration of macromolecules (Tommassen et al. 1990; Robinson and Sligar 1994, 1995a, b). Thus, exploration of pressure effects on osmolyte levels in a piezophile could reveal new facets of osmotic pressure-hydrostatic pressure interactions.

For this purpose, the deep-sea bacterium *Photobacterium profundum* strain SS9 (Nogi et al. 1998) was selected for study. It is a moderately piezophilic deep-sea isolate which grows over a wide pressure range (Bartlett et al. 1989). SS9 responds to pressure changes by modulating gene expression, protein abundance and lipid fatty acids (Chi and Bartlett 1993, 1995; Bartlett and Welch 1995; Allen et al. 1999; Allen and Bartlett 2000). Because it is amenable to a variety of genetic techniques, it has developed into a useful model system for studies of pressure adaptation (Chi and Bartlett 1993, 1995; Welch and Bartlett 1998).

Communicated by K. Horikoshi

D.D. Martin · M.F. Roberts
Merkert Chemistry Center, Boston College, Chestnut Hill, MA, USA

D.H. Bartlett (✉)
Center for Marine Biotechnology and Biomedicine, Marine Biology Research Division, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92093, USA
Tel. +1-858-5345233; Fax +1-858-5347313
e-mail: dbartlett@ucsd.edu

Nuclear magnetic resonance spectroscopy is a useful method for monitoring the identity and amounts of low-molecular-weight solutes (Fan 1995) in intact cells and in cell extracts. The technique has been used extensively to screen solutes in a wide range of organisms including extremophiles (Robertson et al. 1990, 1992; Sowers et al. 1990; Lai et al. 1991; Severin et al. 1992; Ciulla et al. 1994; Martins and Santos 1995; Desmarais et al. 1997; Martins et al. 1997). NMR analysis of extracts from *P. profundum* indicates that this piezophile, grown to early stationary phase, accumulates several amino acids as well as two unusual solutes, β-hydroxybutyrate (β-HB) and a β-HB oligomer, in response to increased hydrostatic as well as osmotic pressure. β-HB is usually found in cells in the form of polyhydroxybutyrate (PHB), a polymer used for carbon and energy storage. The mobilization of PHB monomers or oligomers in early stationary phase in response to increased growth pressure appears to be a novel environmental response in these cells.

Materials and methods

Chemicals

D_2O (99.9%) and β-HB were obtained from Sigma Chemical Company (St Louis, MO, USA). All other chemicals were ACS reagent grade and obtained from either Sigma Chemical Company or Fisher Chemical Company (Pittsburgh, PA, USA).

Cell growth

High pressure cultivation of *P. profundum* SS9 was conducted as previously described (Chi and Bartlett 1993). Cultures were grown to stationary phase in three-quarter strength 2216 Marine Medium (Difco Laboratories, Detroit, MI, USA) at 0.1 MPa, 15°C. Stationary-phase cultures typically were diluted 1/200 into 2216 medium buffered with HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 100 mM, pH 7.5, containing 22 mM glucose (2216HG medium). Growth in this medium is the default condition; any other growth conditions are identified in the text and Figure captions. The diluted culture was used to fill polyethylene bags (Kapak Corporation, Minneapolis, MN, USA). The bags were filled completely, then heat-sealed with a hand-held heat sealing clamp (Nalgene, Rochester, NY, USA). Cells were incubated in stainless steel pressure vessels which could be pressurized using a hydraulic pump and which were equipped with quick-connect fittings for rapid decompression and recompression as described by Yayanos and Van Boxtel (1982). "Early stationary" phase represented cultures harvested during the ascending portion of the growth curve where the cell numbers were continuing to increase, but at a decreased rate. Thus "early stationary" phase cultures in 2216HG medium were harvested at A_{600} values between 0.8 and 1.3. For the experiments where cells were grown at 0.1 MPa to different stages, the A_{600} for the early log cells was 0.13; A_{600} = 1.2 for "early stationary" phase and A_{600} = 1.1 (descending part of the growth curve 39 h after the "early stationary" phase cells were harvested) for "late stationary" phase. For growth at increased sodium chloride concentration, the concentration of sodium chloride was increased from 250 to 500 mM.

Extraction of intracellular solutes

The lyophilized cell pellets were extracted with 70% ethanol according to previous protocols (Lai et al. 1991; Robertson et al. 1992). Each pellet was disrupted using a sealed glass disposable pipette, vortexed briefly, bath-sonicated for 5–10 min, and then centrifuged for 10 min at 10,000 rpm in an Eppendorf Microcentrifuge (Brinkman Laboratory Products, Westbury, NY, USA). The extraction procedure was repeated three or four times to ensure complete disruption of the pellet. Supernatants were pooled and the bulk of the ethanol removed under vacuum. The sample was then frozen and lyophilized overnight.

Preparation of samples for NMR

The lyophilized extracts were dissolved in 500 µl 99.9% D_2O. The pH-meter reading of the sample was adjusted to fall between 6.8 and 7.2, using deuterated acid (DCl) or base (NaOD). The sample was centrifuged to remove any particulate material and the supernatant was used for NMR analysis.

NMR spectroscopy

^1H NMR spectra with presaturation of the residual water resonance [and parameters similar to those used previously (Robertson et al. 1990, 1992; Lai et al. 1991; Robertson and Roberts 1991; Ciulla et al. 1994; Desmarais et al. 1997)] were obtained with a Varian Unity 500 spectrometer (Varian Scientific Instruments, Cary, NY, USA) using a 5 mm inverse probe. Natural abundance ^1H WALTZ decoupled ^{13}C (125.7 MHz) and ^{31}P NMR experiments were carried out with the same spectrometer (^{13}C also on a Varian Unity 300 spectrometer) using a broadband probe and parameters described previously (Martin et al. 2000). For all experiments the instrument temperature was 30°C. Typically, 10,000–20,000 transients were acquired for ^{13}C spectra and 256 transients for ^{31}P. For processing, line-broadening functions of 1, 5, and 10 Hz were used for ^1H, ^{13}C, and ^{31}P spectra, respectively. Two-dimensional experiments were performed using standard Varian software with residual water suppression by presaturation.

Quantitation of solutes

The amount of organic solutes present in the extracts was determined by adding either 6 µmol of dioxane, or 4–12 µmol of imidazole, to the sample as an internal standard. The resonances for the solutes were then integrated and

compared to those obtained for the standards. These values were then normalized (μmol solute/mg protein) using total protein concentration in the cell pellet (determined with a Bradford assay of the pellet resuspended in 10– 20 ml water and sonicated for 20–30 min (Bradford 1976). For most different pressure, salt, and temperature conditions, at least two cell samples were extracted and analyzed. The error bars on the graphs reflect the difference in solute amounts from this analysis. Only one sample was examined for the cells grown at 0.1 MPa at temperatures of 4° and 17°C (333 mM NaCl) and 9°C (666 mM NaCl).

Hydrolysis of samples

The lyophilized ethanol extract was dissolved in a small amount of 6N HCl and heated at 113°C for 24 h to hydrolyze any ester or amide linkages of solutes. The sample was brought to pH 7 using NaOH and lyophilized. The dried, hydrolyzed sample was then dissolved in D_2O for NMR analysis.

Results

Hydrostatic pressure effects on solute accumulation in *P. profundum*

P. profundum cells were grown under glucose fermentation conditions at both low (0.1 MPa) and high (28 MPa) pressure and harvested in late log/early stationary phase. The ethanol extracts of these cultures were examined by natural abundance ^{13}C NMR for amounts and types of intracellular solutes accumulated. In cells grown at both pressures, readily identifiable solutes included betaine ($^{13}CH_2$ resonances at 66.9 and 54.1 ppm) and glutamate ($^{13}CH_2$ resonances at 55.4, 34.1, and 55.4 ppm) with low levels of other amino acids (alanine $^{13}CH_2$ resonances at 51.1 and 16.8 ppm and glycine $^{13}CH_2$ at 42 ppm). As shown in Fig. 1, other very intense resonances (indicated by asterisks in Fig. 1) were observed in the extract from cells grown at 28 MPa: resonances at 19.8, 22.7, 44.5, 46.9, 66.3, and 71.4 ppm. These were sometimes observed at low levels in cultures grown at atmospheric pressure as well. The APT (attached proton test) indicated that the ^{13}C resonances ~20 ppm belonged to CH_3 groups, the resonances ~45 ppm to CH_2 groups, and those ~65–70 ppm were CH moieties. Identification of two spin systems (via TOCSY experiment) and connectivity of the three types of protons involved (COSY experiment) suggested β-HB-type structures [CH_3-CH(OR)-CH_2COOR] for both of the spin systems. Addition of authentic β-HB to a portion of the extract and analysis by ^1H NMR confirmed that this was in fact one of the solutes present. However, the second β-HB-like system had a significantly downfield-shifted methine carbon, suggestive of an ester linkage. Also, the natural abundance ^{13}C spectrum showed that all of the carbons corresponding to the second spin system were closely spaced asymmetric multiplets. This behavior is suggestive of a relatively small polymer of β-HB

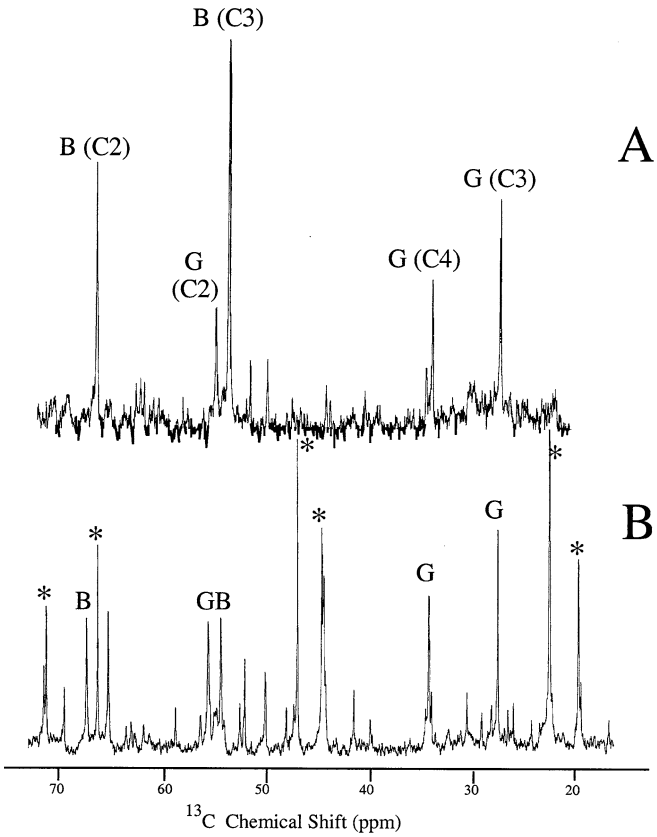

Fig. 1. ^1H decoupled natural abundance ^{13}C (125.7 MHz) spectrum of extracts from *Photobacterium profundum* grown to early stationary phase at **A** 0.1 MPa and **B** 28 MPa. Resonances are identified by solute (*B* betaine, *G* glutamate) and carbon number. The resonances marked by an *asterisk* indicate unusual solutes that did not correspond to known osmolytes

units where the end moieties would have different chemical shifts from internal units. The hypothesis that the second spin system belonged to a β-HB polymer was tested by hydrolyzing a portion of the extract. The ^1H NMR spectrum of the hydrolyzed sample indicated that only β-HB was present.

The distribution among the diverse solutes and the total amount of solute changed with growth pressure. For cells grown at 28 MPa, there was a large increase in both β-HB as well as the β-HB oligomer, while the three amino acids remained relatively constant (Fig. 2). Under these conditions, the total solute concentration increased approximately two-fold with the increase in hydrostatic pressure.

The *P. profundum* extracts were also analyzed by ^{31}P NMR spectroscopy for the presence of phosphorus-containing organic solutes. Cell extracts from "early stationary" phase cultures at both low and high pressure contained inorganic phosphate (P_i), as well as small amounts of unknown phosphodiester compounds (~0.5 μmol/mg protein). However, it was unlikely that the phosphodiester was related to the β-HB oligomer since increased hydrostatic pressure did not alter the amounts of the phosphorus-containing compounds (Fig. 3). ^{31}P spectra of extracts from log phase cells exhibited mostly P_i (1.4 μmol/mg protein)

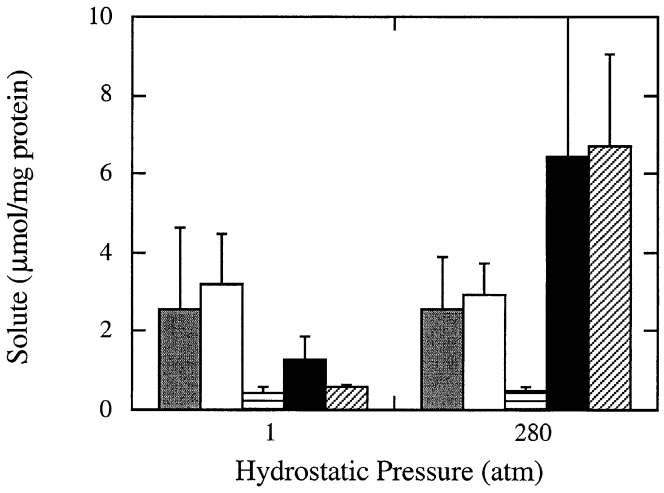

Fig. 2. Osmolyte distribution as a function of hydrostatic pressure for *P. profundum* cells grown to early stationary phase at 9°C: ▩, betaine; □, glutamate; ▤, alanine; ■, β-HB; ▨, β-HB oligomer

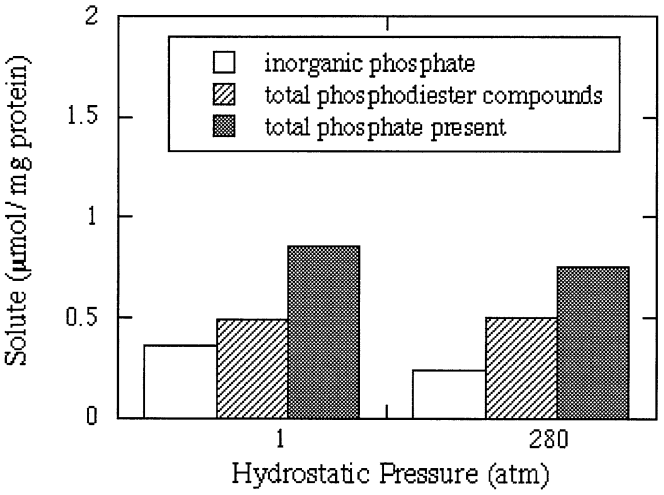

Fig. 3. Distribution of phosphorus-containing metabolites in *P. profundum* grown at 0.1 or 28 MPa to early stationary phase at 9°C under glucose fermentation conditions

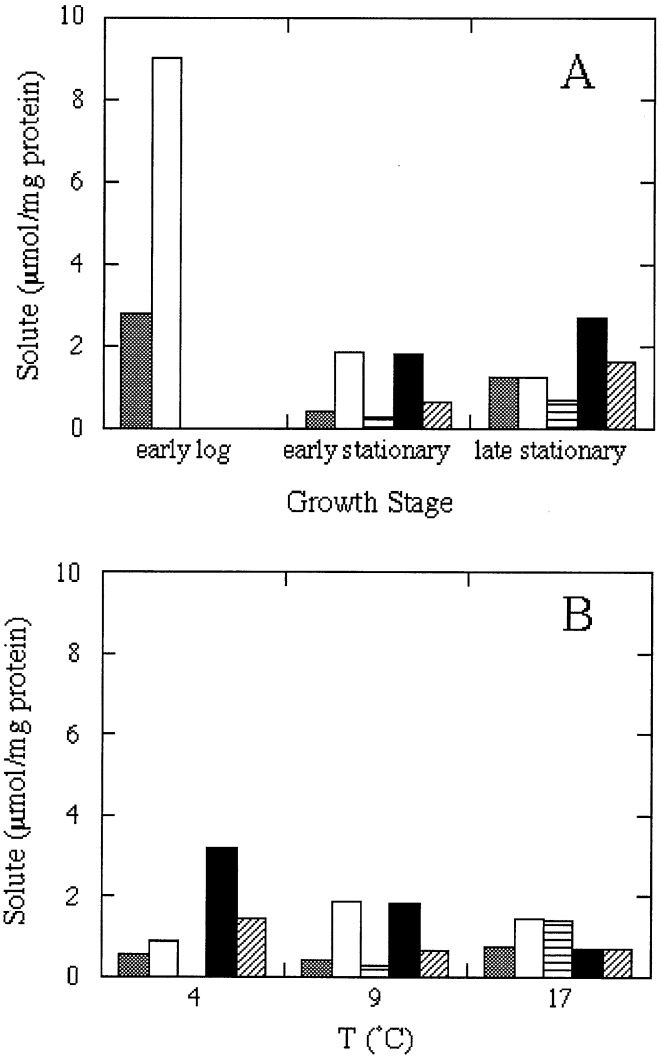

Fig. 4. Distribution of intracellular solutes in *P. profundum* cells grown at 0.1 MPa and **A** harvested at different stages of growth (9°C growth temperature), or **B** grown to early stationary phase at different temperatures: ▩, betaine; □, glutamate; ▤, alanine; ■, β-HB; ▨, β-HB oligomer

and little phosphodiester (0.8 μmol/mg protein). The amounts of all soluble phosphorus species present were three- to four times lower than the organic solutes in 1 atm cultures of "early stationary" cells and an even smaller proportion of solutes in the log phase cells, indicating that they did not play an important role in the response to pressure.

To our knowledge, this is the first time that β-HB or an oligomer composed of β-HB units has been used by a microorganism in response to an environmental physical cue. Given their enhanced accumulation under high hydrostatic pressure, these molecules are termed "piezolytes."

Effect of growth conditions on osmolytes

Initial extracts were from cells grown at 0.1 and 28 MPa at 9°C under conditions that support glucose fermentation. Altering the growth conditions (with the hydrostatic pressure maintained at 0.1 MPa) could affect the accumulation of some or all of the observed solutes. When the *P. profundum* cells were grown at 0.1 MPa, but harvested at different stages of growth, there were variations in the solute distribution that mimicked the effect of increased hydrostatic pressure (Fig. 4A). In early log phase, the cells preferred to accumulate betaine and glutamate. However, upon reaching stationary phase, β-HB and the oligomer were accumulated and contributed to the bulk of the osmotic balance at that pressure.

Growth temperature also affected solute accumulation. Cells grown to early stationary stage but at different temperatures exhibited a distinct change in solute distribution (Fig. 4B). There was an increase in β-HB production at lower temperatures, while accumulation of amino acids did not change dramatically.

Lastly, cells were grown under fermentative conditions, but glucose was eliminated from the media. In this case the cells did not grow very well, due to reduced carbon and energy availability. Extracts of the cells showed that all the same solutes were present, but the large increase in β-HB and the oligomer with increased hydrostatic pressure was lost (data not shown). This may suggest that when the cells are not growing well, they are not able to produce these solutes as efficiently.

Effect of external NaCl on solute accumulation

The changes in solute distribution shown in Fig. 1 were the result of changes in hydrostatic pressure. Do the same solutes that respond to altered hydrostatic pressure respond to alterations in external osmotic pressure? To address this, *P. profundum* cells were grown on glucose at both low and high pressure, but in the presence of twice the normal NaCl level. For cells grown at 0.1 MPa there was an increase in betaine, glutamate, alanine, β-HB, and oligomer levels, with the total solute amount increasing approximately two-fold with increased NaCl (Fig. 5A). Similarly, when cells were grown at 28 MPa the solutes were present at much higher levels in the sample with the higher NaCl (Fig. 5B). At 28 MPa, the response to doubled NaCl was a five-fold increase in the total solutes. These results suggest that the solutes that respond to hydrostatic pressure ("piezolytes") also function as osmolytes. However, osmolytes such as betaine and glutamate do not necessarily function as "piezolytes".

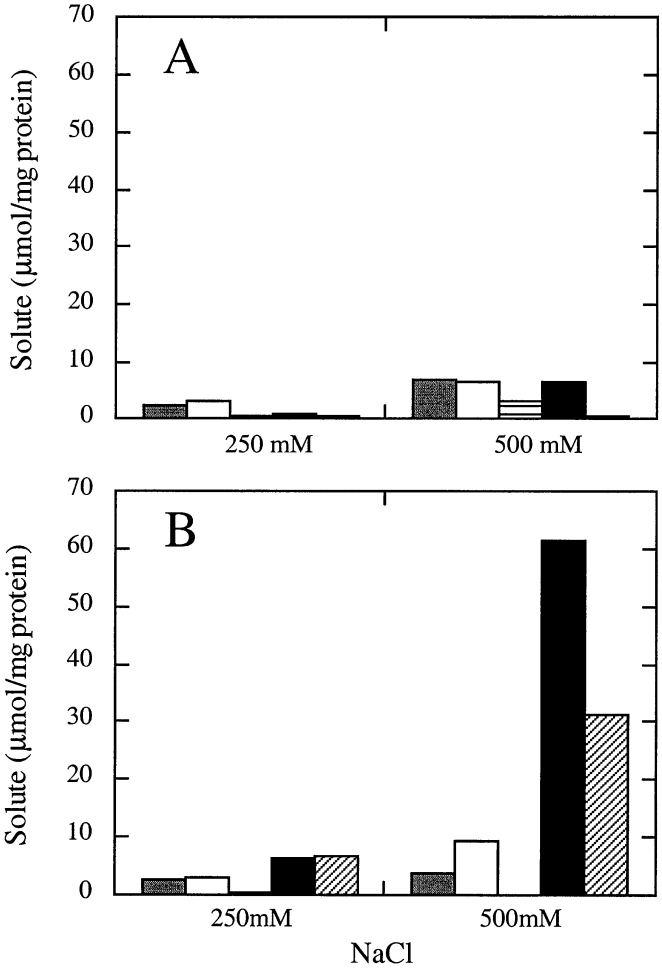

Fig. 5. Distribution of intracellular solutes in *P. profundum* cells grown at **A** 0.1 MPa or **B** 28 MPa to early stationary phase as a function as external NaCl: ▩, betaine; □, glutamate; ▤, alanine; ■, β-HB; ▨, β-HB oligomer

Discussion

The question of how an organism adapts to life in a high-pressure environment is an area of current research interest. Pressure has an effect on many cell functions involving macromolecules, including growth, cell division, and protein expression, among others (Abe et al. 1999; Kato et al. 2000; Allen and Bartlett 2001; Bartlett 2002). Adaptation to increased external NaCl (where the osmotic pressure is initially high outside) involves changes in turgor pressure and uptake or synthesis and accumulation of small solutes by the cells. In contrast, increased hydrostatic pressure is not predicted to result in changes in the pressure differential across the cell membrane. However, increased hydrostatic pressure can alter macromolecule conformation, packing, and intermolecular interactions in such a way that the cell needs to offset these effects. It may do so by increasing the intracellular concentration of small molecule solutes. *Photobacterium profundum* does indeed increase its total concentration of intracellular solutes when grown at high hydrostatic pressure (28 MPa). The average value of total organic solutes (including analyses of all the different environmental effects except higher external NaCl) for cells grown at 0.1 MPa is 8.2 ± 2.4 μmol/mg protein; the average total solute level for cells grown at 28 MPa is 20 ± 5.0 μmol/mg protein. Solutes preferentially accumulated at high hydrostatic pressure also behaved as osmolytes when the cells were challenged with higher external NaCl (in which case total osmolytes more than doubled at 0.1 MPa and increased four-fold at 28 MPa).

Photobacterium profundum preferentially accumulated β-HB and an oligomer composed of β-HB units as a response to changes in hydrostatic as well as osmotic pressure. β-HB is the basic unit of poly(3-hydroxybutyrate) (PHB). These PHB polymers are often accumulated by bacteria as carbon and energy storage units (Anderson and Dawes 1990; Wang and Lee 1997). PHB has been found in a number of microorganisms, including *Alcaligenes eutrophus*, *Bacillus megaterium*, *Halobacterium mediterranei*, various *Staphylococci*, and several *Pseudomonas* species (Doi et al. 1989; Anderson and Dawes 1990; Szewczyk 1992; Das et al. 1997; Wang and Lee 1997). PHB is typically localized in granules, and in most cases is accumulated when cell growth conditions are stressed (i.e., nitrogen or phosphorus limiting conditions; Doi et al. 1989; Anderson and Dawes 1990; Wang and Lee 1997), although there are some organisms, such as *Alcaligenes latus*, that accumulate poly-

hydroxyalkanoates under normal growth conditions (Wang and Lee 1997). In some organisms, PHB accumulation is accompanied by polyphosphate accumulation, another form of energy storage. However, based on the ^{31}P NMR results, there does not appear to be significant polyphosphate accumulation in the case of *P. profundum*. There are minor amounts of phosphodiesters present in extracts, but these are not enhanced with increased hydrostatic pressure.

Generally, PHB production requires an excess carbon source, such as glucose (Wang and Lee 1997). *P. profundum* cells produce small amounts of β-HB and oligomer in the absence of glucose. However, it is only when the cells are grown with glucose that there are large changes in β-HB accumulation in response to hydrostatic pressure. The biosynthetic pathway for β-HB starts with acetyl CoA; conversion of β-HB to PHB occurs via PHB synthase (Poirier et al. 1995). The polymer can be degraded back into β-HB units by PHB depolymerase, an activity that can be secreted into the extracellular space (see, for example, Kasuya et al. 1997), or present within the cell for intracellular PHB degradation (Huisman et al. 1991). In the case of *P. profundum* grown at 28 MPa, whether β-HB is converted to the oligomer or both osmolytes are produced by PHB degradation is not known at present. However, the levels of β-HB and the oligomer found in the cell extracts at 0.1 MPa were found to be dependent on stage of growth. β-HB and oligomer levels were highest when the organism was grown into stationary phase. It is possible that when the cell reaches early stationary phase some of the nutrients become limiting, causing an increase in β-HB that translate to increased oligomer and PHB production. Increased PHB levels could make it more energetically favorable to use β-HB and oligomers to increase turgor pressure under high hydrostatic pressure and even high osmotic pressure. It will be interesting to see whether a PHB synthase and/or depolymerase is the species that is responding to increased hydrostatic pressure.

The "solute cocktail" which *P. profundum* chooses to accumulate is fascinating, with the choice of β-HB possibly being unique to this organism. Bacteria of the genus *Vibrio*, but not related bacteria of the genus *Photobacterium*, have previously been found to synthesize the osmolyte ectoine (Schmitz and Galinksi 1996). Thus, the absence of ectoine synthesis by *P. profundum* SS9 (at least at levels detectable by NMR spectroscopy) is consistent with its phylogenetic position. β-HB is an interesting anionic molecule for use as an osmolyte and a "piezolyte". This is not the first time that an organism has used a β-substituted molecule in a pressure response; several β-amino acids accumulate in methanogenic archaea in response to increased osmotic pressure (Lai et al. 1991; Robertson and Roberts 1991; Robertson et al. 1992). β-HB has a similar structure to β-glutamate and β-glutamine, known osmolytes in several methanogens. β-Amino acids make good compatible solutes in these organisms because they are not used by the cell, and therefore do not interfere with any of the normal cell functions (Robertson et al. 1992). Perhaps this characteristic also applies to β-HB.

To our knowledge, the effects of hydrostatic pressure on solute accumulation in a piezophile, or indeed any organism, have not been examined before, although Yancey and coworkers have shown that the organic osmolyte trimethylamine oxide (TMAO) occurs at elevated levels in diverse deep-sea animals compared with related shallow-water species (Gillett et al. 1997; Kelly and Yancey 1999), and that TMAO stabilizes protein structure and function at high pressure (Yancey and Siebenaller 1999; Yancey et al. 2001). The fact that *P. profundum* shows an increase in solute accumulation that is dependent on hydrostatic pressure is very intriguing. When one considers the effects of hydrostatic versus osmotic pressure, the question that immediately comes to mind is what purpose are these solutes serving if they are not used to offset a pressure differential, as in the case of osmotic pressure. Perhaps this can be explained when one considers the effect that pressure has on protein structures. The case of osmotic pressure is relatively straightforward: an increase in pressure causes the organism to synthesize or transport compatible solutes, which can displace the water molecules bound to protein (Robinson and Sligar 1995b). This process is sometimes called preferential hydration. When protein is exposed to increased hydrostatic pressure, the tendency is for the protein to denature (Robinson and Sligar 1995b). The mechanism is thought to involve water molecules penetrating and hydrating the interior of the protein, causing the protein to denature (Hummer et al. 1998). Elevated hydrostatic pressure and elevated osmotic pressure have been documented to have opposing effects on enzyme activity and stability. For example, increased hydrostatic pressure counteracts the effects of high osmotic pressure on the cleavage specificity of several restriction enzymes (Robinson and Sligar 1995a) and Arc repressor protein will not denature under pressure in the absence of water (Oliveira et al. 1994).

Obviously an organism that prefers to exist in a high-pressure environment must have adapted in such a way that its proteins are more stable under these extreme conditions. Perhaps one method of adaptation involves interactions of proteins with organic solutes. The role of these small molecules would be to aid in maintaining protein structure and function (and to resist pressure-driven water penetration of the protein core), a trend which has been seen before (Yancey et al. 1982). The increased amounts of TMAO in deep-sea animals compared with related shallow-water organisms may well represent such an adaptation to high pressure that is critical for maintenance of protein structure and function (Gillett et al. 1997). Given that the osmolyte TMAO has been shown to aid in the folding process of an thermodynamically unstable version of the protein ribonuclease T1, this possibility appears quite likely (Baskakov and Bolen 1998). Small solutes, particularly neutral solutes such as sugars and polyhydric alcohols, have been shown to protect proteins against denaturation by heat or chemicals (Robinson and Sligar 1995b). In most cases, the protecting molecule is a neutral compound, but there have been some cases where charged molecules are used. Negatively charged di-*myo*-inositol-1,1′-diphosphate has been suggested to serve as a thermoprotectant in a variety of hyperthermophilic archaea (Ciulla et al. 1994; Martins and Santos 1995). Therefore, it is possible that β-HB may be used as an extrinsic factor to protect against high-pressure-induced protein structure changes. In this context it is note-

worthy that PHB degradation is important for stress resistance in *Pseudomonas oleovorans* (Huisman et al. 1991). It will be interesting to look at the effects of β-HB on protein unfolding and protein-protein interactions for several proteins from *P. profundum* in vitro, in order to test this hypothesis.

Acknowledgments This work has been supported by grant DE-FG02-91ER20025 (to M.F.R.) from the Department of Energy Biosciences Division and by grants NSF/MCB 96-30546 and NSF/MCB 99-74528 (to D.H.B.) from the National Science Foundation.

References

Abe F, Kato C, Horikoshi K (1999) Pressure-regulated metabolism in microorganisms. Trends Microbiol 7:447–453

Allen EE, Bartlett DH (2000) FabF is required for piezoregulation of *cis*-vaccenic acid levels and piezophilic growth of the deep-sea bacterium *Photobacterium profundum* strain SS9. J Bacteriol 182:1264–1271

Allen EE, Bartlett DH (2001) Piezophiles: microbial adaptation to the deep-sea environment. In: Gerday C (ed) Extremophiles. EOLSS Publishers, Oxford, UK

Allen EE, Facciotti D, Bartlett DH (1999) Monounsaturated but not polyunsaturated fatty acids are required for growth at high pressure and low temperature in the deep-sea bacterium *Photobacterium profundum* strain SS9. Appl Environ Microbiol 65:1710–1720

Anderson AJ, Dawes EA (1990) Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol Rev 54:450–472

Bartlett D, Wright M, Yayanos A, Silverman M (1989) Isolation of a gene regulated by hydrostatic pressure. Nature 342:572–574

Bartlett DH (2002) Pressure effects on in vivo microbial processes. Biochem Biophys Acta 1595:367–381

Bartlett DH, Welch TJ (1995) *ompH* Gene expression is regulated by multiple environmental cues in addition to high pressure in the deep-sea bacterium *Photobacterium* species strain SS9. J Bacteriol 177:1008–1016

Baskakov I, Bolen DW (1998) Forcing thermodynamically unfolded proteins to fold. J Biol Chem 273:4831–4834

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 7:248–254

Chi E, Bartlett DH (1993) Use of a reporter gene to follow high pressure signal transduction in the deep-sea bacterium *Photobacterium* SS9. J Bacteriol 175:7533–7540

Chi E, Bartlett DH (1995) An *rpoE*-like locus controls outer membrane protein synthesis and growth at cold temperatures and high pressures in the deep-sea bacterium *Photobacterium* SS9. Mol Microbiol 17:713–726

Ciulla RA, Burggraf S, Stetter KO, Roberts MF (1994) Occurrence and role of di-*myo*-inositol-1-1′-phosphate in *Methanococcus igneus*. Appl Environ Microbiol 60:3660–3664

Das S, Lengweiler UD, Seebach D, Reusch RN (1997) Proof for a non-proteinaceous calcium-selective channel in *Escherichia coli* by total synthesis from (R)-3-hydroxybutanoic acid and inorganic phosphate. Proc Natl Acad Sci USA 94:9075–9079

Desmarais D, Jablonski P, Fedarko NS, Roberts MF (1997) 2-Sulfotrehalose, a novel osmolyte in haloalkaliphilic archaea. J Bacteriol 179:3146–3153

Doi Y, Kawaguchi Y, Nakamura Y, Kunioka M (1989) Nuclear magnetic resonance studies of poly(3-hydroxybutyrate) and polyphosphate metabolism in *Alcaligenes eutrophus*. Appl Environ Microbiol 55:2932–2938

Fan TNM (1995) Metabolic profiling by one- and two-dimensional NMR analysis of complex mixtures. Prog NMR Spectr 28:161–219

Gillett MB, Suko JR, Santoso FO, Yancey PH (1997) Elevated levels of trimethylamine oxide in muscles of deep-sea gadiform teleosts: a high-pressure adaptation? J Exp Zool 279:386–391

Huisman GW, Wonink E, Meima R, Kazemeier B, Terpstra P (1991) Metabolism of poly(3-hydroxy)alkanoates by *Pseudomonas oleovorans*: identification and sequences of genes and function of the encoded proteins in the synthesis and degradation of PHA. J Biol Chem 266:2191–2198

Hummer G, Garde S, Garcia AE, Paulaitis ME, Pratt LR (1998) The pressure dependence of hydrophobic interactions is consistent with the observed pressure denaturation of proteins. Proc Natl Acad Sci USA 95:1552–1555

Kasuya K, Inoue Y, Tanaka T, Akehata T, Iwata T, Fukui T, Doi Y (1997) Biochemical and molecular characterization of the polyhydroxybutyrate depolymerase of *Comamonas acidovorans* YM1609, isolated from freshwater. Appl Environ Microbiol 63:4844–4852

Kato C, Sato T, Horikoshi K (1995) Isolation and properties of barophilic and barotolerant bacteria from deep-sea mud samples. Biodivers Conserv 4:1–9

Kato C, Li L, Nogi Y, Nakamura Y, Tamaoka J, Horikoshi K (1998) Extremely barophilic bacteria isolated from the Mariana Trench, Challenger Deep, at a depth of 11,000 meters. Appl Environ Microbiol 64:1510–1513

Kato C, Nakasone K, Qureshi MH, Horikoshi K (2000) How do deep-sea microorganisms respond to changes in environmental pressure? In: Storey KB, Storey J (eds) Environmental stressors and gene responses. Elsevier Science, Amsterdam, The Netherlands, pp 277–291

Kelly RH, Yancey PH (1999) High contents of trimethylamine oxide correlating with depth in deep-sea teleost fishes, skates, and decapod crustaceans. Biol Bull 196:18–25

Lai M-C, Sowers KR, Robertson DE, Roberts MF, Gunsalus RP (1991) Distribution of compatible solutes in the halophilic methanogenic archaebacteria. J Bacteriol 173:5352–5358

Martin DD, Ciulla RA, Robinson PM, Roberts MF (2000) Switching osmolyte strategies: response of *Methanococcus thermolithotrophicus* to changes in external NaCl. Biochim Biophys Acta 1524:1–10

Martins LO, Santos H (1995) Accumulation of mannosylglycerate and di-*myo*-inositol phosphate by *Pyrococcus furiosus* in response to salinity and temperature. Appl Environ Microbiol 61:3299–3303

Martins LO, Huber R, Huber H, Stetter KO, Da Costa M, Santos H (1997) Organic solutes in hyperthermophilic Archaea. Appl Environ Microbiol 63:996–902

Menaia JAGF, Duarte JC, Boone DR (1993) Osmotic adaptation of moderately halophilic methanogenic archaebacteria, and detection of cytosolic *N*,*N*-dimethylglycine. Experientia 49:1047–1054

Nakayama A, Yano Y, Yoshida K (1994) New method for isolating barophiles from intestinal contents of deep-sea fishes retrieved from the abyssal zone. Appl Environ Microbiol 60:4210–4212

Nogi Y, Masui N, Kato C (1998) *Photobacterium profundum* sp. nov., a new moderately barophilic bacterial species isolated from a deep-sea sediment. Extremophiles 2:1–7

Oliveira AC, Gaspar LP, Da Poian AT, Silva JL (1994) Arc repressor will not denature under pressure in the absence of water. J Mol Biol 240:181–187

Poirier Y, Nawrath C, Somerville C (1995) Production of polyhydroxyalkanoates, a family of biodegradable plastics and elastomers, in bacteria and plants. Biotechnology 13:142–150

Robertson DE, Roberts MF (1991) Osmoregulation in methanogens. Biofactors 3:1–9

Robertson D, Roberts MF, Belay N, Stetter KO, Boone D (1990) Occurrence of β-glutamate, a novel osmolyte, in methanogenic bacteria. Appl Environ Microbiol 56:1504–1508

Robertson DE, Noll D, Roberts MF (1992) Free amino acid dynamics in marine methanogens: β-amino acids as compatible solutes. J Biol Chem 267:14893–14901

Robinson CR, Sligar SG (1994) Hydrostatic pressure reverses osmotic pressure effects on the specificity of *Eco*RI–DNA interactions. Biochemistry 33:3787–3793

Robinson CR, Sligar SG (1995a) Heterogeneity in molecular recognition by restriction endonucleases: osmotic and hydrostatic pressure effects on *Bam*HI, *Pvu*II, and *Eco*RV specificity. Proc Natl Acad Sci USA 92:3444–3448

Robinson CR, Sligar SG (1995b) Hydrostatic and osmotic pressure as tools to study macromolecular recognition. Methods Enzymol 259: 395–427

Schmitz RPH, Galinksi EA (1996) Compatible solutes in luminescent bacteria of the genera *Vibrio*, *Photobacterium* and *Photorabdus* (*Xenorabdus*) – occurrence of ectoine, betaine and glutamate. FEMS Microbiol Lett 142:195–201

Severin J, Wohlfarth A, Galinski EA (1992) The predominant role of recently discovered tetrahydropyrimidines for the osmoadaptation of halophilic eubacteria. J Gen Microbiol 138:1629–1638

Somero GN (1992) Adaptations to high hydrostatic pressure. Annu Rev Physiol 54:557–577

Sowers KR, Robertson DE, Noll D, Gunsalus RP, Roberts MF (1990) Nε-Acetyl-β-lysine: an osmolyte synthesized by methanogenic archaebacteria. Proc Natl Acad Sci USA 87:9083–9087

Szewczyk EM (1992) Poly-β-hydroxybutyrate in staphylococci. FEMS Microbiol Rev 9:165–168

Tommassen J, Vermeij P, Struyvé M, Benz R, Poolman JT (1990) Isolation of *Neisseria menignitidis* mutants deficient in class 1 (PorA) and class 3 (PorB) outer membrane proteins. Infect Immun 58:1355–1359

Trüper HG, Galinski EA (1990) Biosynthesis and fate of compatible solutes in extremely halophilic phototrophic eubacteria. FEMS Microbiol Rev 75:247–254

Wang F, Lee SY (1997) Production of poly(3-hydroxybutyrate) by fed-batch culture of filamentation-suppressed recombinant *Escherichia coli*. Appl Environ Microbiol 63:4765–4769

Welch TJ, Bartlett DH (1998) Identification of a regulatory protein required for pressure-responsive gene expression in the deep-sea bacterium *Photobacterium* species strain SS9. Mol Microbiol 27:977–985

Yancey PH, Siebenaller JF (1999) Trimethylamine oxide stabilizes teleost and mammalian lactate dehydrogenases against inactivation by hydrostatic pressure and trypsinolysis. J Exp Biol 202:3597–3603

Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN (1982) Living with water stress: evolution of osmolytes. Science 217:1214–1217

Yancey PH, Fyfe-Johnson AL, Kelly RH, Walker VP, Auñon MT (2001) Trimethylamine oxide counteracts effects of hydrostatic pressure on proteins of deep-sea teleosts. J Exp Zool 289:172–176

Yayanos AA (1995) Microbiology to 10,500 meters in the deep sea. Annu Rev Microbiol 49:777–805

Yayanos AA, Van Boxtel R (1982) Coupling device for quick high pressure connections to 1,000 MPa. Rev Sci Instrum 53:704–705

Yayanos AA, Dietz AS, Van Boxtel R (1981) Obligately barophilic bacterium from the Mariana trench. Proc Natl Acad Sci USA 78:5212–5215