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The many ways of coping with pressure

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Abstract

The current paper reviews the strategies employed by microorganisms from the deep-biosphere, especially piezophiles (from the Greek piezo = to press and philo = love), to cope with the high hydrostatic pressures (HHP) prevailing in these biotopes. The aim of this review is not to constitute an exhaustive report of our current knowledge on the physiology of piezophiles. Recent reviews have covered part of these subject in details (Abe, 2007; Lauro and Bartlett, 2008; Michiels et al., 2008; Simonato et al., 2006). Here, we illustrate by a few chosen examples where we stand on the path to understanding the mechanisms that microorganisms from the depth of our planet use to cope with HHP.

Introduction

The twentieth century has been benchmarked by technological and scientific breakthroughs that have drastically modified the way we understand life on our planet. Once man-centered, our living world is nowadays unicellular, prokaryote-centered. A novel domain had to be created to accommodate for the discovery of newly isolated prokaryotic organisms, the Archaea (Woese, 1987). It was demonstrated that unicellular prokaryotic life forms are able to inhabit virtually any environment on Earth, even the most extreme in terms of temperature, pH, salinity. Prokaryotes constitute the largest diversity reservoir, the main primary producers as well as the creatures that have evolved for the longest period on Earth. The discovery of living organisms in the Mariana trench, at the bottom of 11km of ocean (Yayanos et al., 1981), or in deep sediments ca. 2 km below the seafloor have extended the biosphere to the depth of our planet (Roussel et al., 2008). We now estimate that life on Earth mostly dwells under its surface, deep in the oceans, or in the depth of the seafloor and continents, in the so-called deep-biosphere (Amend and Teske, 2005; Whitman et al., 1998).

The deep-biosphere

The deep biosphere was first defined by Jannasch as the oceanic waters below 1000m, e.g. under a pressure of 100 atmosphere or 10MPa (Jannasch and Taylor, 1984). It has been since extended to include all biotopes over 10MPa in an oceanic, sub-seafloor or continental setting (Figure 1). The different deep-biotopes share at least one characteristic which is HHP. They are otherwise very diverse in terms of physico-chemical characteristics, as is briefly summarized below. In the ocean, temperature decreases with depth until an almost constant 3°C is reached below the thermocline (30-100 m), although in some oceanic context, the temperatures be as low as -1.5°C. The deep ocean is characterized by the lack of sunlight, only about 1% of the organic carbon produced photosynthetically at its surface eventually reaches the deep-sea floor. Thus, the deep-ocean is cold and oligotroph, to the exception of hydrothermal vent systems. In the
continental or sub-seafloor systems, on the contrary, the average geothermal gradient is ca. 25°C km⁻¹. The deep-underground biosphere is variable with depth in terms of temperature and in terms composition as a function of the host rock. It lacks oxygen and light. Potential energy sources include geothermally produced reduced minerals, H₂ and CH₄ (D'Hondt et al., 2002; Engelen et al., 2008; Parkes et al., 1994). There is so far no evidence that it extends outside of the fluid fraction contained in the rock within cracks, fractures, and the intrinsic rock porosity. Amongst deep-biosphere biotopes, the hydrothermal vents may be the most intriguing. The deep-sea vents were discovered in 1977 as the result of a systematic search for active volcanism at submarine spreading centers (Corliss et al., 1979). They were shown to harbor abundant primary productivity and diversity based essentially on the chemical harvest of the energy of the geological fluids seeping through the ocean floor. Because of this, they are the only ecosystems on Earth which are not linked to photosynthesis, or photosynthesis-derived products such as oxygen (Baross and Hoffman, 1985; Erauso et al., 1993). As such they may well represent windows to our pre-photosynthesis metabolic past (Deming and Baross, 1993). Their location at the bottom of the ocean would have protected them from harmful radiation. Geological fluids would have supplied the energy and small organic molecules, while the high temperature would have facilitated chemical reactions (Daniel et al., 2006).

Considering the actual temperature limit for life, e.g. 122°C for Methanopyrus kandleri (Takai et al., 2008), and a ca. 25 to 30°C thermal gradient in the subsurface, the temperature limit for the putative continental biosphere ca. 5 km below ground on average, with the notable exceptions of subduction zones, mid-ocean spreading ridges and volcanic plumes (Figure 1). Considering the actual pressure limit for life, e.g. ca. 130-150 MPa for Pyrococcus yedianosii (Zheng et al., 2009), the pressure limit for the deep-biosphere would thus occur ca. 4 km below ground in continental settings. Pressure and temperature are limiting in specific zones. Based on a 3% average porosity of surface rock and a 5 km average thickness, we estimate its total volume to be ca. 10¹⁶ m³ (Whitman et al., 1998). Even though the maximal productivity of the high pressure continental or marine biosphere is orders of magnitude lower than that of the surface biotopes, due to their extremely large volume, these high pressure biotopes contribute significantly to the production and recycling of organic carbon on Earth (Whitman et al., 1998). The deep biosphere could represent up to 70% of all cells on Earth, as well as 50% of the primary production of biomass.

The effect of high hydrostatic pressure on biological systems

Pressure affects chemical equilibria and reaction rates, depending on the reaction ( V) and activation ( V°) volumes involved. The behavior of all systems under high pressure is governed by Le Châtelier's principle, which predicts that the application of pressure shifts an equilibrium towards the state that occupies a smaller volume, and
accelerates processes for which the transition state has a smaller volume than the ground state (Smeller, 2002). For example, if a reaction is accompanied by a $V^*$ value of $\sim 50$ ml mol$^{-1}$, it is enhanced more than 3000-fold by applying a pressure of 400MPa at ambient temperature. With the knowledge of $V$ and $V^*$ values, one can draw valuable conclusions about the nature of the reaction and its mechanism. Pressures encountered by living organisms on Earth range from 0.1MPa to less than 200MPa. Such pressures only change intermolecular distances and affect conformations, but do not change covalent bond distances or bond angles. The covalent structure of low molecular mass biomolecules (peptides, lipids, saccharides), as well as the primary structure of macromolecules (proteins, nucleic acids and polysaccharides), is not perturbed by pressures up to about 2 GPa. Pressure acts predominantly on the conformation and supramolecular structures of biomolecular systems, thus on their functionality in the cells (Balny et al., 2002) (Figure 2).

**Nucleic acids**: HHP stabilizes the DNA hydrogen bonds and the stacking interactions increasing the duplex to single strand transition temperature, e.g. the melting temperature, $T_m$. As a consequence, DNA is stabilized by increasing the pressure, and the double to single strand transition necessary for replication/transcription/translation processi may become more difficult (Macgregor, 2002).

**Lipid bilayers**: Lipid membranes are those biological structures that are among the most pressure sensitive. The complete biomembrane is a very complex lamellar phospholipid bilayer matrix, containing a variety of different lipid molecules and a host of proteins performing versatile biochemical functions. Upon compression, the lipids adapt to volume restriction by changing their conformation and packing (Figure 2). As a consequence, with increasing pressure the lipid bi-layer loses in fluidity, becomes rapidly impermeable to water and other molecules, and the protein-lipid interactions, which are essential to the optimal function of the membrane are weakened (Winter and Jeworrek, 2009).

**Proteins**: Proteins, and more so multimeric protein structures, are also amongst the most pressure sensitive macromolecules in the cell. Similarly to lipids the protein will adapt to volume restriction upon compression by changing their conformation. Almost no protein will be denatured by pressure in the range relevant to life in HHP biotopes. However, modifications will be sufficient to affect multimer association and stability, as well as catalytic sites. Thus, protein functions will be altered upon compression (Balny et al., 2002; Northrop, 2002).

**Cells**: Submitted to increasing hydrostatic pressure, organisms will experience the failure of several of their cellular functions (Figure 2): (1) loss of membrane fluidity which will lead to reduced transmembrane transport, loss of flagellar motility, (2) loss of protein and nucleic acids synthesis, (3) loss of enzymatic function and metabolism, (4)
alteration of cellular architecture, etc. which will eventually lead to the cell death, although the absolute P values for each step may differ from one organism to another. An example of such values are given here for the piezosensitive bacterium *E. coli* (table 1) and the piezotolerant microeucaryote *Saccharomyces cerevisiae* (Abe, 2007; Bartlett, 2002).

Piezophily, or the need for high hydrostatic pressure

In the deep-biosphere, the hydrostatic pressure conditions often exceed that inhibitory to surface organisms such as *E. coli* or *Saccharomyces cerevisiae*. Indeed, microorganisms isolated from these biotopes are often able to grow more efficiently under elevated hydrostatic pressure than under atmospheric pressure. These have been called piezophiles (from the greek piezo = to press and philo = love). Piezophiles have optimal growth rates at pressures greater than 1 atmosphere or 0.1MPa. Inhibitory pressures for piezophiles are higher than that of surface organism, often exceeding the 100MPa observed in the deepest parts of the ocean. Piezophilic organisms have been isolated in pure culture from several high pressure environments, including the deep-ocean, hydrothermal vents, the sub-seafloor and the continental underground. They belong to a wide variety of bacterial and archeal genera (Abe and Horikoshi, 2001). In 1981, the group of Pr. Yyanos isolated the first obligate piezophile organism strain MT41 of *Colwellia* sp., a psychrophilic bacterium isolated from a decaying amphipod fished at the bottom of the Mariana Trench (Yayanos et al., 1981). In 2009, the first non bacterial, non psychrophile obligate piezophile, *Pyrococcus yayanosii* strain CH1 was isolated from the Ashadze site, the deepest hydrothermal vent field explored so far (Zeng et al., 2009). Strain MT-41 and CH1 have optimal growth pressures ca. 70MPa at 2°C and 52MPa at 98°C respectively. Neither strain can grow at pressures below or equal to 20MPa, while both can grow at pressures exceeding 100MPa. Since the isolation of MT-41 several other obligate piezophilic bacteria have been isolated (Table 2). To the exception of strain CH1, all isolates are psychrophilic, belong to the γ-proteobacteria (*Shewanella* and *Colwellia*), and are closely related to *E. coli*. The ability of piezophiles to grow under HHP inhibitory to surface organisms, and more so, the inability of the obligate piezophiles to grow at atmospheric pressure are proof that piezophiles have adapted to HHP in the course of their evolution. Three main mechanisms have been proposed to explain the ability to grow best under HHP in piezophiles: 1) Finely tuning the global gene expression to compensate for loss of biological activity (Campanaro et al., 2005); 2) Expressing HHP specific genes (Kato and Qureshi, 1999); 3) Adapting the structure of biomolecules to sustain HHP, e.g rendering the structures piezophilic or piezotolerant (Chilukuri and Bartlett, 1997).

Adapting to HHP through the fine tuning of the transcriptome
In the pressure range relevant to HHP biotopes, most cellular structures are not profoundly affected, but their biological activity might be substantially diminished. As mentioned above membranes and proteins are amongst the most pressure sensitive compartments of the cells. Lipid bi-layer tend to lose their functionality (permeability, fluidity, protein movement) due to the increased packing of its constituting lipids (Winter and Jeworrek, 2009). Enzymatic activities may be increased by low pressures. Such is the case with E. coli's aspartase activity, ethanol production from glucose in yeast or the methanotrophy in Methanocaldoccoccus jannaschii (Eisenmenger and Reyes de Corcuera, 2009), but in most systems enzymatic activities will be reduced under pressure, depending on the ground state and the activation volume of the reactants. After 4h at 60MPa, Morita and ZoBell showed that most enzymatic were inhibited by HHP in E. coli, but that the extent of this inhibition was protein and metabolic pathway dependent (Morita and Zobell, 1956; Zobell and Morita, 1957). In these conditions, the succinic dehydrogenase of E. coli had already lost 50% of its activity. This inhibition was reversible if the applied pressure did not exceed 100MPa. Transporters are also very pressure sensitive in E. coli. Up to 90% inhibition has been observed for the transport of amino acids at 50MPa (Paul and Morita, 1971). Since the overall effect of pressure on these systems is a reduction, but not a complete inhibition of the activity, one may overcome this decrease by slightly increasing the concentration of certain components, which can be obtained by finely tuning the expression of a pool of genes that would be common to the piezophile and the piezosensitive strains. Fine tuning of gene expression is expected to play an important role in low pressure environments, e.g. below 40MPa, at which most surface organisms are able to survive and grow and to respond to pressure variations.

One of the best example of how microorganisms counteract the effects of high pressure may be found in the membrane. Low temperature and high hydrostatic pressure have related and synergistic effects on biological membranes (Winter and Jeworrek, 2009), reducing their fluidity by increasing the packing of fatty acyl chains. The felt impact of combined HHP and low T at the bottom of the Marianna trench (100MPa, 2°C) is similar to that of a temperature of -18°C at atmospheric pressure. Deep-sea microbes are thought to preserve membrane functionality at high pressure and low temperature by increasing the proportion of unsaturated fatty acids in their lipids. In fact, DeLong and Yanoos observed a positive correlation between the proportion of mono and polyunsaturated fatty acids in the membrane of deep-sea microorganisms and the depth of isolation (Delong and Yanoanos, 1985, 1986). Mono- and poly-unsaturated fatty acid increase membrane fluidity by increasing membrane disorder, thus reducing the pressure-dependent packing of the lipid by-layer. Substitution of saturated with unsaturated fatty acids is also observed as a response to increased pressure in yeast or E. coli. After a 30 min 200MPa treatment Saccharomyces cerevisiae up-regulates the expression of the ole1 gene (stearoyl- CoA desaturase), which activity could increase the proportion of unsaturated fatty acids (Fernandes et al., 2004). Yeast exposure to sub lethal pressure (30MPa) also results in an up-regulation of genes
involved in the response to membrane structure stresses such as inol, opi3, pst1, rta1, sed1 and prm5 (Iwahashi et al., 2005). In the piezophile Photobacterium profundum SS9, Allen and colleagues observed an increased proportion of both mono- and poly-unsaturated fatty acids, correlated with an up-regulation of the corresponding synthesis genes, when grown at elevated pressure, although only mono-unsaturated fatty acids are required for HHP growth (Allen and Bartlett, 2002; Allen et al., 1999; Vezzi et al., 2005). A similar trend is also observed in obligate piezophiles. Shewanella sp. strain DB21MT-2 ($P_{opt} = 70$MPa) and Moritella sp. Strain DB21MT-5 ($P_{opt} = 80$MPa) contain high proportions of the mono-unsaturated fatty acid C18:1 and tetradeccenoic acid (14:1), respectively, compared with the type strain of Shewanella benthica and the type strain of Moritella marina (Kato et al., 1998; Nogi et al., 1998). Thus, far only modifications of the relative proportions of membrane lipids between high and low pressures have been reported. No novel fatty acid synthesis genes have been described in piezophiles. The concentration variations always resulted from the differential expression of the cell's fatty acid synthesis genes. While evidence is accumulating about the role of mono-unsaturated fatty acids in membrane piezoadaptation, the role of poly-unsaturated fatty acids remain to be confirmed. It should be noted that due to the protocols employed for their isolation, it remains to demonstrate that these lipids are integral part of the membrane. In absence of mutants with a HHP-sensitive phenotype their biological and ecological importance can only be inferred by their prevalence in deep-sea bacteria (Delong and Yayanos, 1986). The absence of a direct link with HHP adaptation would rather support a physiological role for these lipids, for example as a reserve molecule.

In addition to lipid synthesis genes, transcriptome analyses in E. coli and yeast have identified several genes that are up-regulated by a sub-lethal HHP treatment. Most significantly, HHP induces chaperone-encoding genes, which are proposed to help in maintaining protein folding, and thus protein function after the shock in pressure. Metabolic genes also comprise a large set of the genes up-regulated by a HHP shock. In S. cerevisiae genes involved in glycolysis, gluconeogenesis and glycogen metabolism are up-regulated during HHP exposure which could correspond to a response to the need to quickly manage energy and osmotic stability (Fernandes et al., 2004). Opposite to what is observed in piezotolerant organisms, in the moderately piezophilic strain SS9 of P. profundum, the pool of genes that is up-regulated at 28MPa compared to 0.1MPa is modest. Indeed, most differentially expressed genes are up-regulated at 0.1MPa compared to the optimal growth conditions of 28MPa (Campanaro et al., 2005). However, similarly to what is observed in yeast the set of up-regulated genes include most transport operons, a large number of metabolic enzymes, signal sensing systems, or membrane associated proteins. Thus, up-regulation can also be explained in the piezophile and the piezotolerant/piezosensitive strains as a way to compensate for the loss of activity of the proteins and/or the membrane. These latter results also highlight that if finely tuning one's genome may help respond to
Adapting to HHP through the expression of piezophile specific genes

The designation “piezophile specific genes” may be understood in two different ways. The genomic view would designate a set of genes that are specific of piezophile genomes, that would no be found in the genomes of non-piezophiles, and for which we could identify a link with HHP growth. In the genetic view, one would consider genes that are specifically expressed in the genomes of piezophiles under HHP, and would not be expressed under suboptimal pressure conditions for which a link with HHP growth could be documented. The first view implies that piezophiles have had to adapt special features to sustain HHP, that would not exist in other organisms.

The search for a “piezophilic gene set” was made possible when the first genomes of true piezophiles, such as *P. profundum, P. abyssi* or *S. benthica* became available. This question is closely linked to the search for a genomic marker of piezophily which could be used to characterized the autochtonous vs. allochtonous origin of the microorganisms sampled in the deep-biosphere. Whole genome comparative studies have documented several major differences between the surface and deep-ecotypes of the genera *Photobacterium, Pyrococcus* or *Shewanella*. Deep-ecotypes were shown to lack several functions essential for growth at the surface (Campanaro et al., 2005; Gunbin et al., 2009). The most noticeable missing functions were DNA-damage repair systems. Many metabolic loci involved in the degradation of low complexity organics were missing in the deep-ecotypes, while degradation genes for organic polymers were present. With a few exceptions, in the *Pyrococcus* cluster all the modifications/rearrangements could be associated to the difference of physico-chemical conditions between the different ecological niche of origin of the strain, while none was clearly linked to HHP (Gunbin et al., 2009). Hence, it has become clear the the adaptation to life under HHP does not require novel functions that would not exist in other organisms. It is interesting to note that the same trend of genome rearrangement, e.g. lack of UV resistance, DNA repair, photosynthesis genes in the deep-sea samples, and presence in the surface samples, has been observed when comparing environmental libraries obtained from the surface or the depth of the ocean (DeLong et al., 2006).

One of the most deeply studied example of HHP specific gene expression may be found in the study of the respiratory chains of the piezophilic *Shewanella violacea* strain DSS12 and *Shewanella benthica* DB6705 (Kato et al., 1995). DSS12, is a psychrophilic facultative piezophilic bacterium that was isolated from the mud of the Ryukyu Trench (5110 m depth). DSS12 displays optimal growth at a temperature of 8 °C and a pressure of 30MPa, and it can grow from 0.1
to 70MPa. DB6705 is an obligate piezophile displaying an optimal growth at 70MPa and 8°C. It is well known that many bacteria change their respiratory systems in order to adapt to a particular environment. In piezophilic *Shewanella*, the external growth pressure significantly alters the respiratory chain components, leading to the presence of two kinds of respiratory chains regulated in response to pressure (Kato and Qureshi, 1999).

Two HHP-regulated promoters have been isolated and characterized from the two strains. The second operon encodes the CydD protein, which was shown to be required in *E. coli* for the assembly of the cytochrome bd complex one of the components of the aerobic respiratory chain (Kato et al., 1996b). The cytochrome bd protein complex of strain DSS12 is observed only under HHP growth conditions (Tamegai et al., 1998). Two additional c-types cytochromes, namely c-551 and c-552, are expressed constitutively or only at 0.1MPa respectively (Qureshi et al., 1998; Yamada et al., 2000). Piezophilic *Shewanella* also code for a novel membrane bound ccb-type quinol oxidase which is expressed only under HHP conditions, while cytochrome c oxidase activity only is present at 0.1MPa (Qureshi et al., 1998). The reconstruction of the complete respiratory chains of *Shewanella* under low or high hydrostatic pressure drawn from these observations are schematized in figure 3 (Kato and Qureshi, 1999). At low pressure, three respiratory chain enzyme complexes are present, NADH-dehydrogenase, bc1-complex and terminal cytochrome c oxidase, which is the archetypical respiratory chain in mitochondria and mesophilic bacteria. At high pressure, the three respiratory chain enzyme complexes include the NADH-dehydrogenase, the membrane-bound cytochrome c-551 and the terminal oxidase enzyme is the quinol oxidase. Whether the pressure regulation of these respiratory systems in piezophilic *Shewanella* plays a significant role in cell growth under HHP conditions raises little doubts. However, whether this is a response to pressure-induced membrane modifications remains to be elucidated.

The second example of well-documented pressure regulated set of genes has been described in the *P. profundum* strain SS9, in which the first pressure-regulated gene was described (Bartlett et al., 1989). This gene was shown to encode a porin named OmpH for outer membrane protein high pressure. A second porin, named ompL was expressed at low pressure. OmpH is maximally expressed at 28MPa, the optimum SS9 growth pressure, whereas OmpL is preferentially expressed at 0.1MPa (Welch and Bartlett, 1996). OmpH is thought to function as a nutrient transporter in nutrient-limited environments such as the deep sea, which makes its expression at high pressure relevant in the current context. Subsequent studies identified transmembrane proteins bearing similarity to the ToxR and ToxS proteins as the transcriptional regulator involved in pressure sensing and OmpH/OmpL regulation. Members of this family of proteins are involved in responses to temperature, pH or osmolarity. ToxR is a multimeric transmembrane protein which activity is modulated by ToxS and binds directly to genes under its control via a cytoplasmic DNA binding domain. To date, the ToxR/S two-component system is the only pressure sensor that has been described. A *toxR* mutant has no growth defect
at high pressure, while the over-expression of the toxR/S sensing system leads to pressure-sensitive growth. It should be
noted that the toxR mutants have not been tested in nutrient limited environment such as would be found in the deep-
sea. Thus, if OmpH and other ToxR regulated genes are required for life in these nutrient limited conditions, the
putative pressure sensitive phenotype of the toxR mutant might not express properly under laboratory conditions. It is
also interesting to note that transferring the toxR/S system from strain SS9 to another bacterium does not confer high
pressure adaptation. Thus, if the system centralizes the response to pressure, the putative adaptation to HHP is encoded
by ToxR/S regulated genes not by the sensor itself.

Adapting to HHP through the molecular adaptation of gene products to HHP

The expression of different porins with similar features in Photobacterium or different respiratory chains in Shewanella
as a function of HHP strongly suggests that these proteins/protein complexes have evolved to express an optimal
activity under the pressure condition under which they are expressed. Furthermore, if one can envision finely tuning the
expression of one's genome to compensate for loss of efficiency of the proteome, it may not be appropriate when the
function of a protein, enzyme, lipid, or any other structure is essentially abolished by HHP. For instance, since E. coli
ribosomes are totally dissociated at a pressure below 60MPa, there cannot be any protein translation at or above that
pressure. Increasing the number of ribosomal proteins and rRNAs will not suffice to maintain protein translation under
non permissive pressure conditions. Thus, in piezophiles maintaining protein translation necessarily involves more than
a fine adjustment of the expression levels of the proteome.

The best proof that a structural adaptation exists stems from the expression data of the Photobacterium strain cluster
(Campanaro et al., 2005; Simonato et al., 2006; Vezzi et al., 2005). If the proteome of a given microorganism was
adapted to HHP, one would expect the pressure optimum of its activity to coincide with its pressure optimum for
growth. Increasing or decreasing the pressure from that optimum should lead to a reduced proteome activity. Reduced
activity could be compensated for through the fine regulation of the proteome expression, in the same way fine tuning is
proposed to help maintain protein activity in piezosensitivies. In the moderate piezophile Photobacterium strain SS9,
which is able to grow efficiently at 28MPa, but also at atmospheric pressure, this may be the case. Indeed, decreasing
the pressure from 28MPa to 0.1MPa leads to the up-regulation of the largest number of genes in that strain, when
compared to an increase in pressure from 28MPa to 45MPa (Campanaro et al., 2005). The majority of these up-
regulated genes are transporter or metabolic enzymes, stressing the sensibility and importance of metabolism and transport to high pressure resistance. In addition, four stress-response genes involved in protein folding and in response to stress conditions: *htpG, dnaK, dnaJ*, and *groEL* are up-regulated at atmospheric pressure, indicating that the proteins of this piezophilic bacterium require the help of these chaperones to fold correctly at 0.1MPa, and thus may be optimized for high pressure. The proteome expression of SS9 at 0.1MPa is also characterized by the up-regulation of genes involved in DNA repair and the transcriptional induction of the glycolytic pathway and trehalose phosphotransferase system, involved in the synthesis of well-known osmolytes. This stress response in *P. profundum* strain SS9 at low pressure (0.1MPa) mirrors that of *Escherichia coli* at high pressure (45MPa) further confirming that strain SS9 is a true piezophile.

To date, proofs of the molecular adaptation to HHP of the proteome in piezophiles mostly derive from indirect evidence such as the transcriptome data presented above. The ability to perform under HHP has been studied in many proteins isolated from mesophilic strains. Several of these proteins have been shown to perform better under increased pressure in comparison to atmospheric pressure, although they originate from piezosensitive organisms. Several showed a piezosensitive behavior (Eisenmenger and Reyes de Corcuera, 2009). To date, only a few studies have focussed on enzymes from piezophiles. In *Methanocaldococcus jannaschii*, a moderate hyperthermophilic piezophile, methanogenesis is enhanced 3.4 times under HHP, but this increase has not been correlated to protein levels inside the cells, and may reflect the adjustment of the proteome rather than the piezophilic nature of the pathway (Michels and Clark, 1997). The 3-isopropylmalate dehydrogenase from the obligate piezophile *Shewanella benthica* was proven slightly more efficient at 100MPa than that of the piezosensitive strain MR1 of *Shewanella oneidensis* (Kasahara et al., 2009). Similarly, the $K_{cat}/K_{m}$, e.g. the activity of the enzyme, of the dihydrofolate reductase increases with pressure for the enzymes isolated from obligate piezophiles, while it decreases for those isolated from piezosensitive strains (Murakami et al., 2010). Unfortunately, none of the above-mentioned studies could identify a molecular signature associated with the piezophilic phenotype.

The best described system which verges on the identification of a putative molecular signature in proteins of piezophiles may be found in a study performed on the *Shewanella* cluster of strains which comprises piezosensitive, piezotolerant and obligately piezophiles (Chilukuri and Bartlett, 1997). This study identified 1) a trend of volume change associated with the dissociation of the oligomers of the SSB protein from 253 ml mol$^{-1}$ for SSB from the piezophilic strain PT 99 of *S. benthica* to 402 ml mol$^{-1}$ for SSB from the piezosensitive *S. hanedai* strain, 2) a trend in $P_{1/2}$, the pressure at which half of the oligomers are dissociated, from 520bar for SSB from the piezosensitive strain *S. hanedai* to 75 MPa for SSB from the extremely
piezophilic strain S. PT99 and 3) a reduction in glycine and proline composition. It was proposed that the reduction in the
helix-breaking (proline) and helix-destabilizing (glycine) residues reduces the flexibility of SSB from *Shewanella* PT99
increasing its stability under HHP (Chilukuri and Bartlett, 1997). This hypothesis is supported by the observation that a
proline to glycine substitution is in a staphylococcal nuclease increases the stability of the protein to HHP (Royer et al.,
1993).

One of the structure that one would expect to have evolved to adapt to HHP is the ribosome. Indeed, in *E. coli* the
ribosome, and as a direct consequence, protein synthesis is one of the first metabolic activity which is completely
abolished by HHP, at ca. 60MPa, while DNA synthesis appear to be completely blocked a few MPa above at ca. 50 MPa
(Yayans and Pollard, 1969). The ribosome is a very complex structure composed of several protein and RNA molecules.
Lauro and colleagues have observed what may be the first evidence of a genetic adaptation of ribosomes to HHP (Lauro
et al., 2007). Indeed, they showed a correlation between the presence of sequence extensions in loops 8, 11 and 49 of
the 16S molecule and the growth optima of γ-proteobacteria. These authors have proposed that these extended loops play
a role in the stabilization of the ribosomal structure under HHP. The specificity of this feature needs to be further tested
to determine whether this is specific of psychrophilic γ-proteobacteria or more generalized amongst piezophiles.

**Are there non-genetic components to HHP adaptation?**

The efforts to understand the adaptation of piezophiles to HHP have been focussed on various aspects of genome
structure and expression. In the light of our partial inability to isolate relevant signatures of this adaptation in the genetic
material, one may wonder whether the adaptation of the proteome to sustain HHP might not as well require additional,
non structural components. As mentioned above, one of the first adaptation exemplified in piezophile and piezosensitive
microorganisms is a modification of membrane composition to maintain its function despite the fluctuations in pressure.
One of the main consequences of HHP is the loss of efficiency of the proteome due to HHP-induced conformational
changes, which resemble that observed for organisms living under high or low temperature, or high salinity. Eukaryote
and prokaryote organisms thriving under such harsh T or salinity conditions have evolved adaptive mechanisms. High
salinity and high and low temperatures have in common a cell dehydration effect and a loss of internal water that
compromise cell ability to survive. An increase in hydrostatic pressure does not result in changes in the pressure
differential across the cell membrane, while increased salinity, trigger an increase in osmotic pressure outside the cell
that trigger a change in turgor pressure. To maintain the appropriate cell turgor and to restore the cell volume, all
organisms accumulate low-molecular-weight osmolytes that are mainly organic solutes in response to cold, heat, pH and
HHP stresses (Yancey, 2005). These solutes are amino acids and derivatives, polyols, sugars and derivatives,
methylamines, and methylsulfonium compounds. The organic osmolytes fall into a few chemical categories: amino acids (glycine, alanine, proline, α-glutamate, β-glutamate, and N-acetyl-β-lysine), and derivatives N-methyl-substituted amino acids (i.e., glycine betaine, homobetaine, carnitine, proline betaine, trimethylamine oxide [TMAO]), ectoine and hydroxyectoine, methylsulfonium solutes (dimethylsulfiniopropionate and dimethylsulfonioacetate); small carbohydrates including monosaccharides (glucose), disaccharides (trehalose, sucrose, mannosucrose), sugar derivatives (glucosylglycerol, mannosylglycerate, glucosylglycerate), polyols (glycerol, inositol, sorbitol), cyclitols (di-myoinositol-phosphate) (Empadinhas and da Costa, 2006; Wood et al., 2001). Some solutes are widespread. For example, glycine betaine is found in all three domains of the tree of life and carbohydrate osmolytes occur in bacteria, archaea, fungi, algae, plants and mammalian kidneys and possibly deep-sea invertebrates. Other solutes are restricted to a small number of organisms like those thriving in hot environments (Empadinhas and da Costa, 2006). Most organic osmolytes are neutral (either zwitterionic or lacking charges) at physiological pH, although some organic osmolytes (i.e. mannosylglycerate and di-myoinositol-phosphate in hyperthermophilic prokaryotes) are negatively charged and are paired with potassium to achieve neutrality. These solutes are often called “compatible solutes”, a term that refers to compounds that can accumulate to very high concentrations without perturbing cell metabolism and enzyme activity (Brown, 1976). Many of these solutes have protective properties, such as cell metabolic protection by serving as antioxidants that scavenge free radicals and reactive oxygen species generated under stresses treatments (Yancey, 2005).

More importantly, these types of solutes can help stabilize macromolecular structures (proteins, membranes) and are accumulated when these stresses directly destabilize these cell components (Empadinhas and da Costa, 2006; Santos and da Costa, 2002; Singer and Lindquist, 1998). Since HHP destabilizes the structure of macromolecules, one may expect compatible solutes to play a role in HHP tolerance.

We have very little data on the counteraction by compatible solutes of the destabilizing effect of high hydrostatic pressure on biological systems and macromolecules, especially in prokaryotic cells. Yancey and coworkers have shown that the organic osmolytes TMAO and taurine increase in concentration in cells with depth in many deep sea animals in comparison to related shallow water species (Fiess et al., 2002; Gillett et al., 1997; Kelly and Yancey, 1999). Since P is the only parameter increasing with depth, Yancey and his coworkers suggested that TMAO and taurine might counteract HHP effects. In P. profundum strain SS9, cells accumulate preferentially glutamate and glycine betaine at atmospheric pressure (0.1MPa), while at optimal growth pressure (28MPa), cells preferentially accumulate β-hydroxybutyrate and β-hydroxybutyrate oligomers. Hence, these have been named “piezolytes” for solutes that are accumulated at HHP (Martin et al., 2002). Recently, we have observed that cells of Thermococcus barophilus, a hyperthermophilic piezophile archaea, accumulate mannosylglycerate in response to high salinity and high temperature stress, and that this
accumulation was anti-correlated with HHP (Cario, Jebbar and Oger, unpublished data). Whether other osmolytes are accumulated under HHP, e.g. piezolytes, in this strain remains to be elucidated for that strain. No experiments have been performed yet to test the ability of these “piezolytes” to counteract in vitro or in vivo the effects of HHP on proteins or membranes. We also lack information on putative piezolytes accumulated in other piezophiles. Given their capacity to protect protein unfolding under stress conditions, it is probable that osmolytes will play a role in the adaptation to HHP. In the future, it will be crucial to pursue our genome-wide efforts to isolate the genetic signatures scattered along the genomes of piezophiles, but it will also be crucial to devote some efforts to the exploration of osmolytes and their protective effects to HHP as well.

Conclusion

The above data fit nicely together to support the existence in piezophilic organisms of a genetic adaptation to the high hydrostatic pressures encountered in the deep-biosphere. At this time it is still not yet clear whether HHP adaptation requires just a change of one or a few genes in a few pathways, a global alteration of many genes in a genome, mainly regulatory modulations or if it also requires non-genetic components. However, we now know that it requires at least these three adaptive strategies. The increasing number of complete genome sequences from piezophilic organisms will allow to better compare the different genomes for their adaptation to HHP. The three comparative genomic studies performed so far have not allowed to identify a signature of this adaptation in protein and gene sequences. Several reasons may explain our inability to identify these HHP-specific signatures in silico. First, as mentioned before pressures in the physiological relevant range, e.g. less than 200MPa, will not affect protein tertiary but only quaternary structures by weakening the low energy bonds between or within monomers of proteins. To date, we have very little knowledge about the structure of the protein dimerization, protein/protein, protein/RNA or protein/DNA interaction domains. Hence, it is very conceivable that modifications in these structures did elude analysis. Second, to date, only piezosensitive (E. coli, S. cerevisiae) or moderately piezophile strains (P. profundum strain SS9, P$_{op}$=28MPa and P. abyssi strain GE5, P$_{op}$=20MPa) have been studied extensively. It is expected that the structural modifications required to adapt the proteome of a procaryote to ca. 20MPa, is far less extended than that required to adapt that of strains MT41 or CH1 to sustain pressures above 80MPa. Third, it is conceivable that the overall approach employed so far to pinpoint molecular adaptations in the genomes of piezophiles does not have the sensitivity necessary in part due to the limited set of genomes analyzed, regardless of their pressure optima. Fourth, P. profundum strain SS9 is a psychrotolerant bacterium. As mentioned above, pressure and cold have comparable and synergistic effects at least on some biomolecules. All results converge to demonstrate a large overlap between cold and HHP regulation. Indeed, HHP shock
in *E. coli* and yeast triggers part of the cold-shock response. It is thus possible that the adaptation to HHP in strain SS9 may be masked by its psychrophily. Similarly, the adaptation of *P. abyssi* to HHP may be masked by its adaptation to very high temperatures.

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Kato, C., Tamegai, H., Ikegami, A., Usami, R., Horikoshi, K., 1996b. Open reading frame 3 of the barotolerant bacterium strain DSS12 is complementary with cydD in *Escherichia coli*: cydD functions are required for cell stability at high pressure. J. Biochem. 120:301-305


Figure 1: Schematic transversal section of the earth highlighting the different deep-biosphere settings. 1: deep-sea; 2: deep-sea hydrothermal vents; 3: deep-oceanic crust; 4: sedimentary sub-seafloor; 5: deep-sea cold seep; 6: continental deep-biosphere. The red and blue lines represent the temperature and pressure limits for life respectively. Solid lines highlight which parameter is limiting the depth of the deep-biosphere. The upper dashed red line symbolizes the 10MPa arbitrary upper limit of the deep-biosphere.
Figure 2: Examples of the effects of high hydrostatic pressure on cells and cellular components. A: lipids in membranes; B: multimeric protein assemblages. C: Protein structure; D: cellular motility; E: protein translation by ribosomes.
Figure 3: Structure of the low- and high-pressure respiratory chains in S. benthica (redrawn from Kato and Qureshi, 1999)
Table 1: Cellular processes/structures impaired by high hydrostatic pressure in *E. coli*

<table>
<thead>
<tr>
<th>Process</th>
<th>Pressure abolishing process (MPa)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motility</td>
<td>10</td>
<td>(Meganath and Marquis, 1973)</td>
</tr>
<tr>
<td>Substrate transport (Isopropylthiogalactopyranoside)</td>
<td>26</td>
<td>(Landau, 1967)</td>
</tr>
<tr>
<td>Cell division</td>
<td>20-50</td>
<td>(Zobell and Cobet, 1962, 1963)</td>
</tr>
<tr>
<td>Growth</td>
<td>50</td>
<td>(Yayanos and Pollard, 1969)</td>
</tr>
<tr>
<td>DNA replication</td>
<td>50</td>
<td>(Yayanos and Pollard, 1969)</td>
</tr>
<tr>
<td>Translation</td>
<td>60</td>
<td>(Yayanos and Pollard, 1969)</td>
</tr>
<tr>
<td>Transcription</td>
<td>77</td>
<td>(Yayanos and Pollard, 1969)</td>
</tr>
<tr>
<td>Viability</td>
<td>200</td>
<td>(Pagan and Mackey, 2000)</td>
</tr>
</tbody>
</table>
Table 2: Known cultivable obligate piezophiles.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Location of isolation</th>
<th>Growth pressure range</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Colwellia sp.</em></td>
<td>MT41</td>
<td>Mariana Trench (Amphipod) 10476m</td>
<td>30 - 120 (opt=103)</td>
<td>(Yayanos, 1986)</td>
</tr>
<tr>
<td><em>Colwellia hadalensis</em></td>
<td>BNL1</td>
<td></td>
<td>&gt;50 - ??</td>
<td>(Demin, 1988)</td>
</tr>
<tr>
<td><em>Colwellia piezophila</em></td>
<td>Y223G</td>
<td>Japan Trench 6278 m</td>
<td>40 - 80 (opt=60)</td>
<td>(Nogi et al., 2004)</td>
</tr>
<tr>
<td><em>Piezobacter thermophilus</em></td>
<td>108</td>
<td>TAG field Mid Atlantic Ridge 3660m</td>
<td>5 - &gt;70 (opt=35)</td>
<td>(Takai et al., 2009)</td>
</tr>
<tr>
<td><em>Psychromonas hadalis</em></td>
<td>K41GT (Type)</td>
<td>Japan Trench (Sediment) 7542 m</td>
<td>30 - 90 (opt=60)</td>
<td>(Nogi et al., 2007)</td>
</tr>
<tr>
<td><em>Psychromonas kaikoe</em></td>
<td>JT7304 (Type)</td>
<td>Japan Trench (Sediment) 7434m</td>
<td>20 - &gt;70* (opt=50)</td>
<td>(Nogi et al., 2002)</td>
</tr>
<tr>
<td><em>Moritella yayanosii</em></td>
<td>DB21MT-5</td>
<td>Challenger Deep 10898m</td>
<td>50 - 100 (opt=80)</td>
<td>(Kato et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>DB6101</td>
<td>Ryuku Trench 5110m</td>
<td>10 - &gt;70** (opt=50)</td>
<td>(Kato et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>DB5501</td>
<td>Suruga Bay 2485m</td>
<td>10 - &gt;70** (opt=50-60)</td>
<td>(Kato et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>DB6906</td>
<td>Japan trench (sea-side) 6269m</td>
<td>20 - &gt;70*** (opt=50-60)</td>
<td>(Kato et al., 1995)</td>
</tr>
<tr>
<td><em>Shewanella benthica</em></td>
<td>DB6705</td>
<td>Japan Trench (land-side) 6356m</td>
<td>20 - &gt;70*** (opt=60)</td>
<td>(Kato et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>DB21MT-2</td>
<td>Challenger Deep 10898m</td>
<td>50 - &gt;100 (opt=70)</td>
<td>(Kato et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>DB172R</td>
<td>Izu-Bonin Trench 6499m</td>
<td>20 - &gt;80 (opt=60)</td>
<td>(Kato et al., 1996a)</td>
</tr>
<tr>
<td></td>
<td>DB172F</td>
<td>Izu-Bonin Trench 6499m</td>
<td>20 &gt; 80 (opt=70)</td>
<td>(Kato et al., 1996a)</td>
</tr>
<tr>
<td><em>Pyrococcus yayanosii</em></td>
<td>CH1</td>
<td>Ashadze Mid Atlantic Ridge 4100m</td>
<td>20 - 120 (opt=52)</td>
<td>(Zeng et al., 2009)</td>
</tr>
</tbody>
</table>

* : Optimal growth conditions at 10°C; Optimal growth conditions at 4°C: 10 - > 70 (opt=20); Optimal growth conditions at 15°C: 50 - >70 (opt=70)

** : Strains DB6101 and DB5501 only exhibit obligate piezophilic growth at 15°C.

*** : Strains DB6705 and DB6906 only exhibit obligate piezophilic growth at 10 and 15°C.