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1 **The many ways of coping with pressure**

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24 **Abstract**

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

31 **Introduction**

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

42 **The deep-biosphere**

43 The deep biosphere was first defined by Jannasch as the oceanic waters below 1000m, e.g. under a pressure of 100
44 atmosphere or 10MPa (Jannasch and Taylor, 1984). It has been since extended to include all biotopes over 10MPa in an
45 oceanic, sub-seafloor or continental setting (Figure 1). The different deep-biotopes share at least one characteristic
46 which is HHP. They are otherwise very diverse in terms of physico-chemical characteristics, as is briefly summarized
47 below. In the ocean, temperature decreases with depth until an almost constant 3°C is reached below the thermocline
48 (30-100 m), although in some oceanic context, the temperatures be as low as -1.5°C. The deep ocean is characterized by
49 the lack of sunlight, only about 1% of the organic carbon produced photosynthetically at its surface eventually reaches
50 the deep-sea floor. Thus, the deep-ocean is cold and oligotrophe, to the exception of hydrothermal vent systems. In the

51 continental or sub-seafloor systems, on the contrary, the average geothermal gradient is ca. 25°C km⁻¹. The deep-
52 underground biosphere is variable with depth in terms of temperature and in terms composition as a function of the host
53 rock. It lacks oxygen and light. Potential energy sources include geothermally produced reduced minerals, H₂ and CH₄
54 (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
55 fluid fraction contained in the rock within cracks, fractures, and the intrinsic rock porosity. Amongst deep-biosphere
56 biotopes, the hydrothermal vents may be the most intriguing. The deep-sea vents were discovered in 1977 as the result
57 of a systematic search for active volcanism at submarine spreading centers (Corliss et al., 1979). They were shown to
58 harbor abundant primary productivity and diversity based essentially on the chemical harvest of the energy of the
59 geological fluids seeping through the ocean floor. Because of this, they are the only ecosystems on Earth which are not
60 linked to photosynthesis, or photosynthesis-derived products such as oxygen (Baross and Hoffman, 1985; Erauso et al.,
61 1993). As such they may well represent windows to our pre-photosynthesis metabolic past (Deming and Baross, 1993).
62 Their location at the bottom of the ocean would have protected them from harmful radiation. Geological fluids would
63 have supplied the energy and small organic molecules, while the high temperature would have facilitated chemical
64 reactions (Daniel et al., 2006).

65 Considering the actual temperature limit for life, e.g. 122°C for *Methanopyrus kandlerii* (Takai et al., 2008), and a ca.
66 25 to 30°C thermal gradient in the subsurface, the temperature limit for the putative continental biosphere ca. 5 km
67 below ground on average, with the notable exceptions of subduction zones, mid-ocean spreading ridges and volcanic
68 plumes (Figure 1). Considering the actual pressure limit for life, e.g. ca. 130-150 MPa for *Pyrococcus yayanosii* (Zheng
69 et al., 2009), the pressure limit for the deep-biosphere would thus occur ca. 4 km below ground in continental settings.
70 Pressure and temperature are limiting in specific zones. Based on a 3% average porosity of surface rock and a 5 km
71 average thickness, we estimate its total volume to be ca. 10¹⁶ m³ (Whitman et al., 1998). Even though the maximal
72 productivity of the high pressure continental or marine biosphere is orders of magnitude lower than that of the surface
73 biotopes, due to their extremely large volume, these high pressure biotopes contribute significantly to the production
74 and recycling of organic carbon on Earth (Whitman et al., 1998). The deep biosphere could represent up to 70% of all
75 cells on Earth, as well as 50% of the primary production of biomass.

76 The effect of high hydrostatic pressure on biological systems

77 Pressure affects chemical equilibria and reaction rates, depending on the reaction (ΔV) and activation (ΔV^\ddagger)
78 volumes involved. The behavior of all systems under high pressure is governed by Le Châtelier's principle, which
79 predicts that the application of pressure shifts an equilibrium towards the state that occupies a smaller volume, and

80 accelerates processes for which the transition state has a smaller volume than the ground state (Smeller, 2002). For
81 example, if a reaction is accompanied by a ΔV^\ddagger value of -50 ml mol^{-1} , it is enhanced more than 3000-fold by applying
82 a pressure of 400MPa at ambient temperature. With the knowledge of ΔV and ΔV^\ddagger values, one can draw valuable
83 conclusions about the nature of the reaction and its mechanism. Pressures encountered by living organisms on Earth
84 range from 0.1MPa to less than 200MPa. Such pressures only change intermolecular distances and affect
85 conformations, but do not change covalent bond distances or bond angles. The covalent structure of low molecular mass
86 biomolecules (peptides, lipids, saccharides), as well as the primary structure of macromolecules (proteins, nucleic acids
87 and polysaccharides), is not perturbed by pressures up to about 2 GPa. Pressure acts predominantly on the conformation
88 and supramolecular structures of biomolecular systems, thus on their functionality in the cells (Balny et al., 2002)
89 (Figure 2).

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

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

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

105 Cells : Submitted to increasing hydrostatic pressure, organisms will experience the failure of several of their cellular
106 functions (Figure 2) : (1) loss of membrane fluidity which will lead to reduced transmembrane transport, loss of
107 flagellar motility, (2) loss of protein and nucleic acids synthesis, (3) loss of enzymatic function and metabolism, (4)

108 alteration of cellular architecture, etc. which will eventually lead to the cell death, although the absolute P values for
109 each step may differ from one organism to another. An example of such values are given here for the piezosensitive
110 bacterium *E. coli* (table 1) and the piezotolerant microeucaryote *Saccharomyces cerevisiae* (Abe, 2007; Bartlett, 2002).

111 Piezophily, or the need for high hydrostatic pressure

112 In the deep-biosphere, the hydrostatic pressure conditions often exceed that inhibitory to surface organisms such as *E.*
113 *coli* or *Saccharomyces cerevisiae*. Indeed, microorganisms isolated from these biotopes are often able to grow more
114 efficiently under elevated hydrostatic pressure than under atmospheric pressure. These have been called piezophiles
115 (from the greek piezo = to press and philo = love). Piezophiles have optimal growth rates at pressures greater than 1
116 atmosphere or 0.1MPa. Inhibitory pressures for piezophiles are higher than that of surface organism, often exceeding
117 the 100MPa observed in the deepest parts of the ocean. Piezophilic organisms have been isolated in pure culture from
118 several high pressure environments, including the deep-ocean, hydrothermal vents, the sub-seafloor and the continental
119 underground. They belong to a wide variety of bacterial and archeal genera (Abe and Horikoshi, 2001). In 1981, the
120 group of Pr. Yayanos isolated the first obligate piezophile organism strain MT41 of *Colwellia* sp., a psychrophilic
121 bacterium isolated from a decaying amphipod fished at the bottom of the Mariana Trench (Yayanos et al., 1981). In
122 2009, the first non bacterial, non psychrophile obligate piezophile, *Pyrococcus yayanosi* strain CH1 was isolated from
123 the Ashadze site, the deepest hydrothermal vent field explored so far (Zeng et al., 2009). Strain MT-41 and CH1 have
124 optimal growth pressures ca. 70MPa at 2°C and 52MPa at 98°C respectively. Neither strain can grow at pressures below
125 or equal to 20MPa, while both can grow at pressures exceeding 100MPa. Since the isolation of MT-41 several other
126 obligate piezophilic bacteria have been isolated (Table 2). To the exception of strain CH1, all isolates are psychrophilic,
127 belong to the γ -proteobacteria (*Shewanella* and *Colwellia*), and are closely related to *E. coli*. The ability of piezophiles
128 to grow under HHP inhibitory to surface organisms, and more so, the inability of the obligate piezophiles to grow at
129 atmospheric pressure are proof that piezophiles have adapted to HHP in the course of their evolution. Three main
130 mechanisms have been proposed to explain the ability to grow best under HHP in piezophiles : 1) Finely tuning the
131 global gene expression to compensate for loss of biological activity (Campanaro et al., 2005); 2) Expressing HHP
132 specific genes (Kato and Qureshi, 1999); 3) Adapting the structure of biomolecules to sustain HHP, e.g rendering the
133 structures piezophilic or piezotolerant (Chilukuri and Bartlett, 1997).

134

135 **Adapting to HHP through the fine tuning of the transcriptome**

136 In the pressure range relevant to HHP biotopes, most cellular structures are not profoundly affected, but their biological
137 activity might be substantially diminished. As mentioned above membranes and proteins are amongst the most pressure
138 sensitive compartments of the cells. Lipid bi-layer tend to lose their functionality (permeability, fluidity, protein
139 movement) due to the increased packing of its constituting lipids (Winter and Jeworrek, 2009). Enzymatic activities
140 may be increased by low pressures. Such is the case with *E. coli*'s aspartase activity, ethanol production from glucose in
141 yeast or the methanotrophy in *Methanocaldococcus jannaschii* (Eisenmenger and Reyes de Corcuera, 2009), but in
142 most systems enzymatic activities will be reduced under pressure, depending on the ground state and the activation
143 volume of the reactants. After 4h at 60MPa, Morita and ZoBell showed that most enzymatic were inhibited by HHP in
144 *E. coli*, but that the extent of this inhibition was protein and metabolic pathway dependent (Morita and Zobell, 1956;
145 Zobell and Morita, 1957). In these conditions, the succinic dehydrogenase of *E. coli* had already lost 50% of its activity.
146 This inhibition was reversible if the applied pressure did not exceed 100MPa. Transporters are also very pressure
147 sensitive in *E. coli*. Up to 90% inhibition has been observed for the transport of amino acids at 50MPa (Paul and Morita,
148 1971). Since the overall effect of pressure on these systems is a reduction, but not a complete inhibition of the activity,
149 one may overcome this decrease by slightly increasing the concentration of certain components, which can be obtained
150 by finely tuning the expression of a pool of genes that would be common to the piezophile and the piezosensitive
151 strains. Fine tuning of gene expression is expected to play an important role in low pressure environments, e.g. below
152 40MPa, at which most surface organisms are able to survive and grow and to respond to pressure variations.

153 One of the best example of how microorganisms counteract the effects of high pressure may be found in the membrane.
154 Low temperature and high hydrostatic pressure have related and synergistic effects on biological membranes (Winter
155 and Jeworrek, 2009), reducing their fluidity by increasing the packing of fatty acyl chains. The felt impact of combined
156 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
157 atmospheric pressure. Deep-sea microbes are thought to preserve membrane functionality at high pressure and low
158 temperature by increasing the proportion of unsaturated fatty acids in their lipids. In fact, DeLong and Yayanos
159 observed a positive correlation between the proportion of mono and polyunsaturated fatty acids in the membrane of
160 deep-sea microorganisms and the depth of isolation (DeLong and Yayanos, 1985, 1986). Mono- and poly-unsaturated
161 fatty acid increase membrane fluidity by increasing membrane disorder, thus reducing the pressure-dependent packing
162 of the lipid by-layer. Substitution of saturated with unsaturated fatty acids is also observed as a response to increased
163 pressure in yeast or *E. coli*. After a 30 min 200MPa treatment *Saccharomyces cerevisiae* up-regulates the expression of
164 the *ole1* gene (stearoyl- CoA desaturase), which activity could increase the proportion of unsaturated fatty acids
165 (Fernandes et al., 2004). Yeast exposure to sub lethal pressure (30MPa) also results in an up-regulation of genes

166 involved in the response to membrane structure stresses such as *ino1*, *opi3*, *pst1*, *rta1*, *sed1* and *prm5* (Iwahashi et al.,
167 2005). In the piezophile *Photobacterium profundum* SS9, Allen and colleagues observed an increased proportion of
168 both mono- and poly-unsaturated fatty acids, correlated with an up-regulation of the corresponding synthesis genes,
169 when grown at elevated pressure, although only mono-unsaturated fatty acids are required for HHP growth (Allen and
170 Bartlett, 2002; Allen et al., 1999; Vezzi et al., 2005). A similar trend is also observed in obligate piezophiles.
171 *Shewanella* sp. strain DB21MT- 2 ($P_{opt} = 70\text{MPa}$) and *Moritella* sp. Strain DB21MT-5 ($P_{opt} = 80\text{MPa}$) contain high
172 proportions of the mono-unsaturated fatty acid C18:1 and tetradecenoic acid (14:1), respectively, compared with the
173 type strain of *Shewanella benthica* and the type strain of *Moritella marina* (Kato et al., 1998; Nogi et al., 1998). Thus,
174 far only modifications of the relative proportions of membrane lipids between high and low pressures have been
175 reported. No novel fatty acid synthesis genes have been described in piezophiles. The concentration variations always
176 resulted from the differential expression of the the cell's fatty acid synthesis genes. While evidence is accumulating
177 about the role of mono-unsaturated fatty acids in membrane piezoadaptation, the role of poly-unsaturated fatty acids
178 remain to be confirmed. It should be noted that due to the protocols employed for their isolation, it remains to
179 demonstrate that these lipids are integral part of the membrane. In absence of mutants with a HHP-sensitive phenotype
180 their biological and ecological importance can only be inferred by their prevalence in deep-sea bacteria (Delong and
181 Yayanos, 1986). The absence of a direct link with HHP adaptation would rather support a physiological role for these
182 lipids, for example as a reserve molecule.

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

196 variations, whether increases or decreases, in hydrostatic pressure, other mechanisms may be required.

197

198

199 **Adapting to HHP through the expression of piezophile specific genes**

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

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

221 One of the most deeply studied example of HHP specific gene expression may be found in the study of the respiratory
222 chains of the piezophilic *Shewanella violacea* strain DSS12 and *Shewanella benthica* DB6705 (Kato et al., 1995).
223 DSS12, is a psychrophilic facultative piezophilic bacterium that was isolated from the mud of the Ryukyu Trench (5110
224 m depth). DSS12 displays optimal growth at a temperature of 8 °C and a pressure of 30MPa, and it can grow from 0.1

225 to 70MPa. DB6705 is an obligate piezophile displaying an optimal growth at 70MPa and 8°C. It is well known that
226 many bacteria change their respiratory systems in order to adapt to a particular environment. In piezophilic *Shewanella*,
227 the external growth pressure significantly alters the respiratory chain components, leading to the presence of two kinds
228 of respiratory chains regulated in response to pressure (Kato and Qureshi, 1999).

229 Two HHP-regulated promoters have been isolated and characterized from the two strains. The second operon encodes
230 the CydD protein, which was shown to be required in *E. coli* for the assembly of the cytochrome bd complex one of the
231 components of the aerobic respiratory chain (Kato et al., 1996b). The cytochrome bd protein complex of strain DSS12
232 is observed only under HHP growth conditions (Tamegai et al., 1998). Two additional c-types cytochromes, namely c-
233 551 and c-552, are expressed constitutively or only at 0.1MPa respectively (Qureshi et al., 1998; Yamada et al., 2000).
234 Piezophilic *Shewanella* also code for a novel membrane bound ccb-type quinol oxidase which is expressed only under
235 HHP conditions, while cytochrome c oxidase activity only is present at 0.1MPa (Qureshi et al., 1998). The
236 reconstruction of the complete respiratory chains of *Shewanella* under low or high hydrostatic pressure drawn from
237 these observations are schematized in figure 3 (Kato and Qureshi, 1999). At low pressure, three respiratory chain
238 enzyme complexes are present, NADH-dehydrogenase, bc1-complex and terminal cytochrome c oxidase, which is the
239 archetypical respiratory chain in mitochondria and mesophilic bacteria. At high pressure, the three respiratory chain
240 enzyme complexes include the NADH-dehydrogenase, the membrane-bound cytochrome c-551 and the terminal
241 oxidase enzyme is the quinol oxidase. Whether the pressure regulation of these respiratory systems in piezophilic
242 *Shewanella* plays a significant role in cell growth under HHP conditions raises little doubts. However, whether this is a
243 response to pressure-induced membrane modifications remains to be elucidated.

244 The second example of well-documented pressure regulated set of genes has been described in the *P. profundum* strain
245 SS9, in which the first pressure-regulated gene was described (Bartlett et al., 1989). This gene was shown to encode a
246 porin named OmpH for outer membrane protein high pressure. A second porin, named ompL was expressed at low
247 pressure. OmpH is maximally expressed at 28MPa, the optimum SS9 growth pressure, whereas OmpL is preferentially
248 expressed at 0.1MPa (Welch and Bartlett, 1996). OmpH is thought to function as a nutrient transporter in nutrient-
249 limited environments such as the deep sea, which makes its expression at high pressure relevant in the current context.
250 Subsequent studies identified transmembrane proteins bearing similarity to the ToxR and ToxS proteins as the
251 transcriptional regulator involved in pressure sensing and OmpH/OmpL regulation. Members of this family of proteins
252 are involved in responses to temperature, pH or osmolarity. ToxR is a multimeric transmembrane protein which activity
253 is modulated by ToxS and binds directly to genes under its control via a cytoplasmic DNA binding domain. To date, the
254 ToxR/S two-component system is the only pressure sensor that has been described. A *toxR* mutant has no growth defect

255 at high pressure, while the over-expression of the *toxR/S* sensing system leads to pressure-sensitive growth. It should be
256 noted that the *toxR* mutants have not been tested in nutrient limited environment such as would be found in the deep-
257 sea. Thus, if OmpH and other ToxR regulated genes are required for life in these nutrient limited conditions, the
258 putative pressure sensitive phenotype of the *toxR* mutant might not express properly under laboratory conditions. It is
259 also interesting to note that transferring the *toxR/S* system from strain SS9 to another bacterium does not confer high
260 pressure adaptation. Thus, if the system centralizes the response to pressure, the putative adaptation to HHP is encoded
261 by ToxR/S regulated genes not by the sensor itself.

262

263

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

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

274 The best proof that a structural adaptation exists stems from the expression data of the *Photobacterium* strain cluster
275 (Campanaro et al., 2005; Simonato et al., 2006; Vezzi et al., 2005). If the proteome of a given microorganism was
276 adapted to HHP, one would expect the pressure optimum of its activity to coincide with its pressure optimum for
277 growth. Increasing or decreasing the pressure from that optimum should lead to a reduced proteome activity. Reduced
278 activity could be compensated for through the fine regulation of the proteome expression, in the same way fine tuning is
279 proposed to help maintain protein activity in piezosensitives. In the moderate piezophile *Photobacterium* strain SS9,
280 which is able to grow efficiently at 28MPa, but also at atmospheric pressure, this may be the case. Indeed, decreasing
281 the pressure from 28MPa to 0.1MPa leads to the up-regulation of the largest number of genes in that strain, when
282 compared to an increase in pressure from 28MPa to 45MPa (Campanaro et al., 2005). The majority of these up-

283 regulated genes are transporter or metabolic enzymes, stressing the sensibility and importance of metabolism and
284 transport to high pressure resistance. In addition, four stress-response genes involved in protein folding and in response
285 to stress conditions: *htpG*, *dnaK*, *dnaJ*, and *groEL* are up-regulated at atmospheric pressure, indicating that the proteins
286 of this piezophilic bacterium require the help of these chaperones to fold correctly at 0.1MPa , and thus may be
287 optimized for high pressure. The proteome expression of SS9 at 0.1MPa is also characterized by the up-regulation of
288 genes involved in DNA repair and the transcriptional induction of the glycolytic pathway and trehalose
289 phosphotransferase system, involved in the synthesis of well-known osmolytes. This stress response in *P. profundum*
290 strain SS9 at low pressure (0.1MPa) mirrors that of *Escherichia coli* at high pressure (45MPa) further confirming that
291 strain SS9 is a true piezophile.

292 To date, proofs of the molecular adaptation to HHP of the proteome in piezophiles mostly derive from indirect evidence
293 such as the transcriptome data presented above. The ability to perform under HHP has been studied in many proteins
294 isolated from mesophilic strains. Several of these proteins have been shown to perform better under increased pressure
295 in comparison to atmospheric pressure, although they originate from piezosensitive organisms. Several showed a
296 piezosensitive behavior (Eisenmenger and Reyes de Corcuera, 2009). To date, only a few studies have focussed on
297 enzymes from piezophiles. In *Methanocaldococcus jannaschii*, a moderate hyperthermophilic piezophile,
298 methanogenesis is enhanced 3.4 times under HHP, but this increase has not been correlated to protein levels inside the
299 cells, and may reflect the adjustment of the proteome rather than the piezophilic nature of the pathway (Michels and
300 Clark, 1997). The 3-isopropylmalate dehydrogenase from the obligate piezophile *Shewanella benthica* was proven
301 slightly more efficient at 100MPa than that of the piezosensitive strain MR1 of *Shewanella oneidensis* (Kasahara et al.,
302 2009). Similarly, the K_{CAT}/K_m , e. g. the activity of the enzyme, of the dihydrofolate reductase increases with pressure for the
303 enzymes isolated from obligate piezophiles, while it decreases for those isolated from piezosensitive strains (Murakami et
304 al., 2010). Unfortunately, none of the above-mentioned studies could identify a molecular signature associated with the
305 piezophilic phenotype.

306 The best described system which verges on the identification of a putative molecular signature in proteins of piezophiles
307 may be found in a study performed on the *Shewanella* cluster of strains which comprises piezosensitive, piezotolerant and
308 obligately piezophiles (Chilukuri and Bartlett, 1997). This study identified 1) a trend of volume change associated with the
309 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
310 402 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
311 are dissociated, from 520bar for SSB from the piezosensitive strain *S. hanedai* to 75 MPa for SSB from the extremely

312 piezophilic strain *S. PT99* and 3) a reduction in glycine and proline composition. It was proposed that the reduction in the
313 helix-breaking (proline) and helix-destabilizing (glycine) residues reduces the flexibility of SSB from *Shewanella* PT99
314 increasing its stability under HHP (Chilukuri and Bartlett, 1997). This hypothesis is supported by the observation that a
315 proline to glycine substitution in a staphylococcal nuclease increases the stability of the protein to HHP (Royer et al.,
316 1993).

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

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

327 The efforts to understand the adaptation of piezophiles to HHP have been focussed on various aspects of genome
328 structure and expression. In the light of our partial inability to isolate relevant signatures of this adaptation in the genetic
329 material, one may wonder whether the adaptation of the proteome to sustain HHP might not as well require additional,
330 non structural components. As mentioned above, one of the first adaptation exemplified in piezophile and piezosensitive
331 microorganisms is a modification of membrane composition to maintain its function despite the fluctuations in pressure.
332 One of the main consequences of HHP is the loss of efficiency of the proteome due to HHP-induced conformational
333 changes, which resemble that observed for organisms living under high or low temperature, or high salinity. Eukaryote
334 and prokaryote organisms thriving under such harsh T or salinity conditions have evolved adaptive mechanisms. High
335 salinity and high and low temperatures have in common a cell dehydration effect and a loss of internal water that
336 compromise cell ability to survive. An increase in hydrostatic pressure does not result in changes in the pressure
337 differential across the cell membrane, while increased salinity, trigger an increase in osmotic pressure outside the cell
338 that trigger a change in turgor pressure. To maintain the appropriate cell turgor and to restore the cell volume, all
339 organisms accumulate low-molecular-weight osmolytes that are mainly organic solutes in response to cold, heat, pH and
340 HHP stresses (Yancey, 2005). These solutes are amino acids and derivatives, polyols, sugars and derivatives,

341 methylamines, and methylsulfonium compounds. The organic osmolytes fall into a few chemical categories: amino
342 acids (glycine, alanine, proline, α -glutamate, β -glutamate, and N-acetyl- β -lysine), and derivatives N-methyl-substituted
343 amino acids (i.e., glycine betaine, homobetaine, carnitine, proline betaine, trimethylamine oxide [TMAO]), ectoine and
344 hydroxyectoine, methylsulfonium solutes (dimethylsulfoniopropionate and dimethylsulfonioacetate); small
345 carbohydrates including monosaccharides (glucose), disaccharides (trehalose, sucrose, mannosucrose), sugar derivatives
346 (glucosylglycerol, mannosylglycerate, glucosylglycerate), polyols (glycerol, inositol, sorbitol), cyclitols (di-*myo*-
347 inositol-phosphate) (Empadinhas and da Costa, 2006; Wood et al., 2001). Some solutes are widespread. For example
348 glycine betaine is found in all three domains of the tree of life and carbohydrate osmolytes occur in bacteria, archaea,
349 fungi, algae, plants and mammalian kidneys and possibly deep-sea invertebrates. Other solutes are restricted to a small
350 number of organisms like those thriving in hot environments (Empadinhas and da Costa, 2006). Most organic osmolytes
351 are neutral (either zwitterionic or lacking charges) at physiological pH, although some organic osmolytes (i.e.
352 mannosylglycerate and di-*myo*-inositol-phosphate in hyperthermophilic prokaryotes) are negatively charged and are
353 paired with potassium to achieve neutrality. These solutes are often called “compatible solutes”, a term that refers to
354 compounds that can accumulate to very high concentrations without perturbing cell metabolism and enzyme activity
355 (Brown, 1976). Many of these solutes have protective properties, such as cell metabolic protection by serving as
356 antioxidants that scavenge free radicals and reactive oxygen species generated under stresses treatments (Yancey, 2005).
357 More importantly, these types of solutes can help stabilize macromolecular structures (proteins, membranes) and are
358 accumulated when these stresses directly destabilize these cell components (Empadinhas and da Costa, 2006; Santos
359 and da Costa, 2002; Singer and Lindquist, 1998). Since HHP destabilizes the structure of macromolecules, one may
360 expect compatible solutes to play a role in HHP tolerance.

361 We have very little data on the counteraction by compatible solutes of the destabilizing effect of high hydrostatic
362 pressure on biological systems and macromolecules, especially in prokaryotic cells. Yancey and coworkers have shown
363 that the organic osmolytes TMAO and taurine increase in concentration in cells with depth in many deep sea animals in
364 comparison to related shallow water species (Fiess et al., 2002; Gillett et al., 1997; Kelly and Yancey, 1999). Since P is
365 the only parameter increasing with depth, Yancey and his coworkers suggested that TMAO and taurine might counteract
366 HHP effects. In *P. profundum* strain SS9, cells accumulate preferentially glutamate and glycine betaine at atmospheric
367 pressure (0.1MPa), while at optimal growth pressure (28MPa), cells preferentially accumulate β -hydroxybutyrate and β -
368 hydroxybutyrate oligomers. Hence, these have been named “piezolytes” for solutes that are accumulated at HHP
369 (Martin et al., 2002). Recently, we have observed that cells of *Thermococcus barophilus*, a hyperthermophilic
370 piezophile archaea, accumulate mannosylglycerate in response to high salinity and high temperature stress, and that this

371 accumulation was anti-correlated with HHP (Cario, Jebbar and Oger, unpublished data). Whether other osmolytes are
372 accumulated under HHP, e.g. piezolytes, in this strain remains to be elucidated for that strain. No experiments have been
373 performed yet to test the ability of these “piezolytes” to counteract *in vitro* or *in vivo* the effects of HHP on proteins or
374 membranes. We also lack information on putative piezolytes accumulated in other piezophiles. Given their capacity to
375 protect protein unfolding under stress conditions, it is probable that osmolytes will play a role in the adaptation to HHP.
376 In the future, it will be crucial to pursue our genome-wide efforts to isolate the genetic signatures scattered along the
377 genomes of piezophiles, but it will also be crucial to devote some efforts to the exploration of osmolytes and their
378 protective effects to HHP as well.

379 **Conclusion**

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

400 in *E. coli* and yeast triggers part of the cold-shock response. It is thus possible that the adaptation to HHP in strain SS9
401 may be masked by its psychrophily. Similarly, the adaptation of *P. abyssi* to HHP may be masked by its adaptation to
402 very high temperatures.

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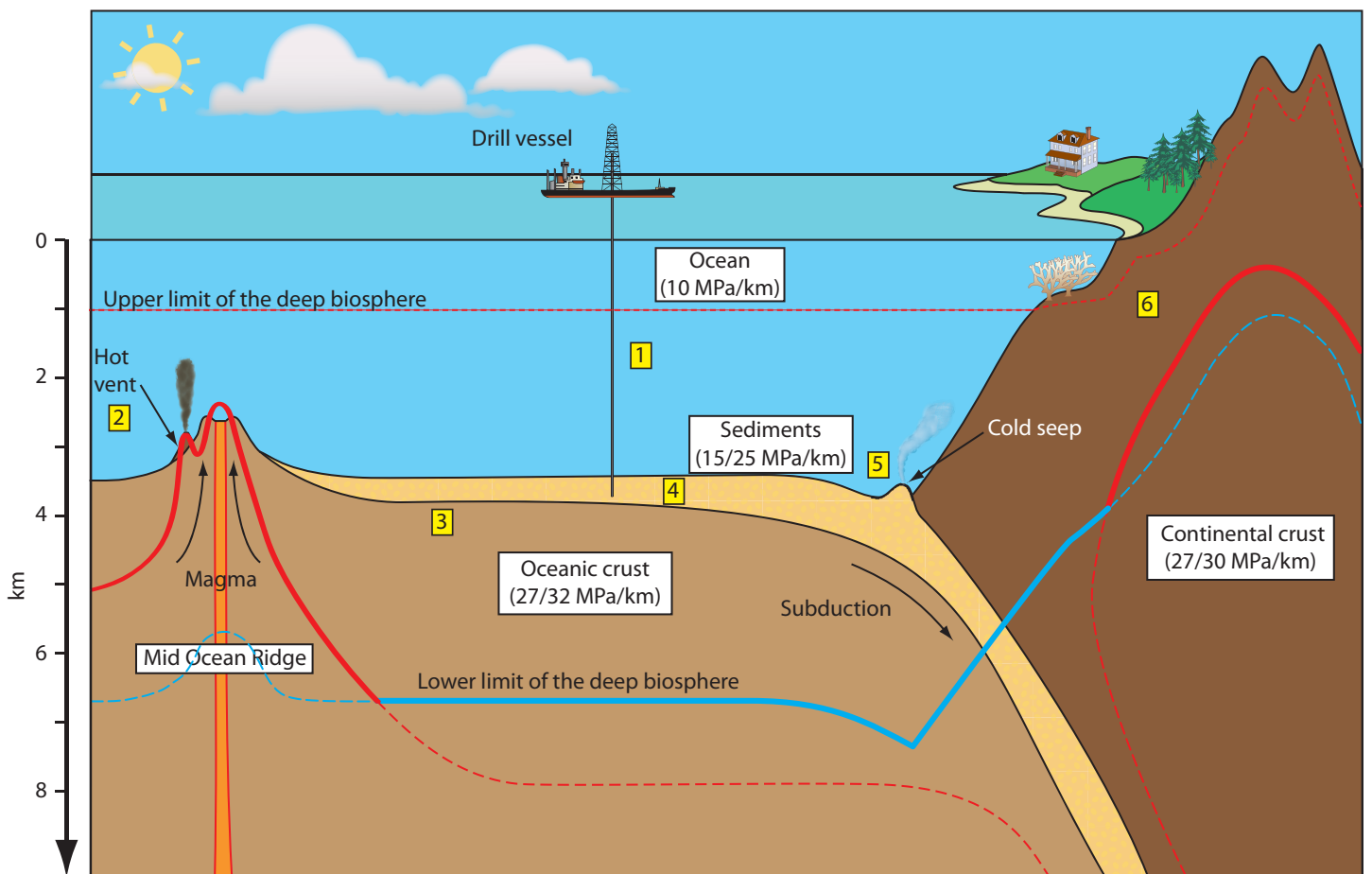


Figure 1 : Schematic transversal section of the earth highlighting the different deep-biosphere settings. 1 : deep-sea; 2 : deep-sea hydrothermal vents; 3 : deep-oceanic crust; 4 : sedimentary sub-seafloor; 5 : deep-sea cold seep; 6 : continental deep-biosphere. The red and blue lines represent the temperature 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.

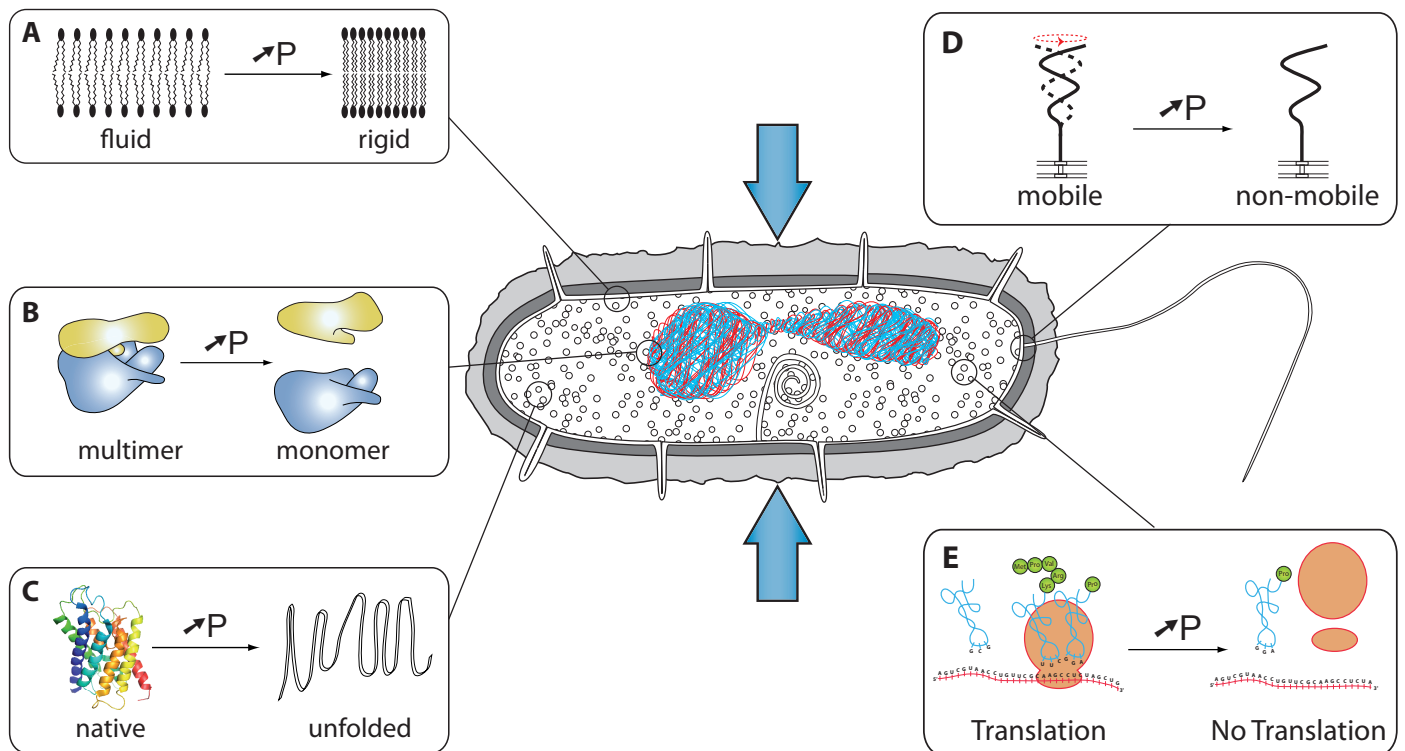
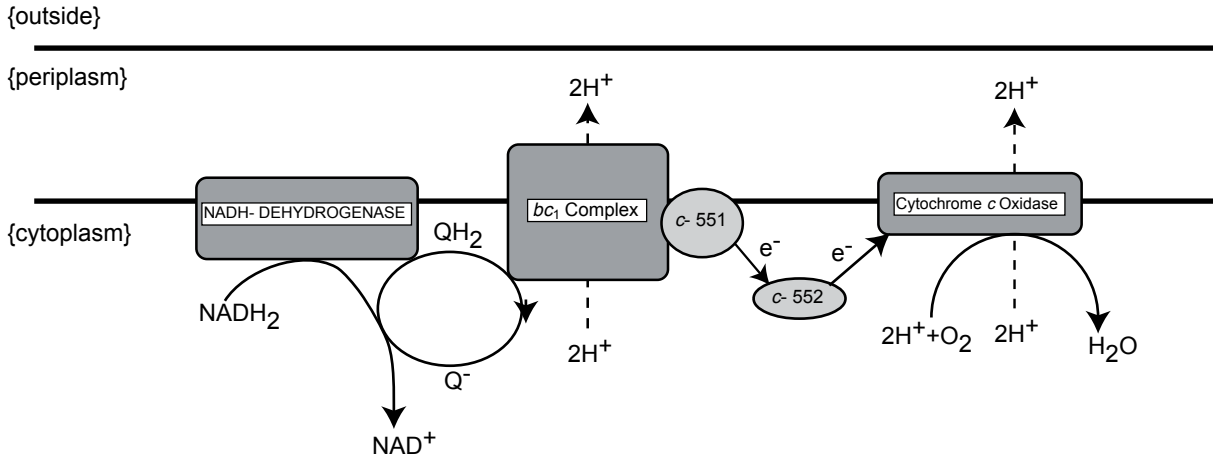


Figure 2 : Examples of the effects of high hydrostatic pressure on cells and cellular components. A : lipids in membranes; B : multimeric protein assemblages. C : Protein structure; D : cellular motility; E : protein translation by ribosomes.

Respiratory chain at 0.1 MPa



Respiratory chain at 60 MPa

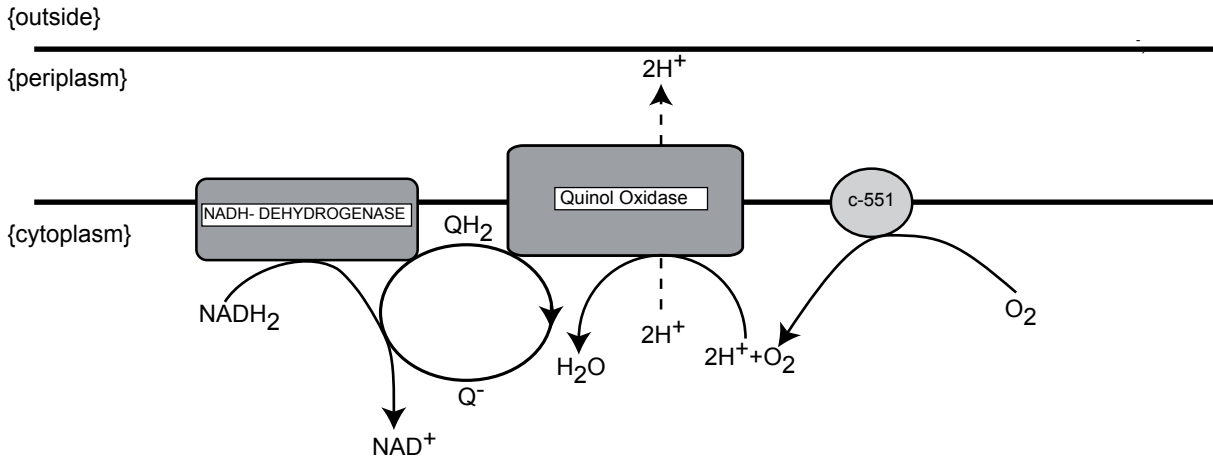


Figure 3 : Structure of the low- and high-pressure respiratory chains in *S. benthami* (redrawn from Kato and Qureshi, 1999)

433 Table 1 : Cellular processes/structures impaired by high hydrostatic pressure in *E. coli*

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Process	Pressure abolishing process (MPa)	Reference
Motility	10	(Meganath and Marquis, 1973)
Substrate transport (Isopropylthiogalactopyranoside)	26	(Landau, 1967)
Cell division	20-50	(Zobell and Cobet, 1962, 1963)
Growth	50	(Yayanos and Pollard, 1969)
DNA replication	50	(Yayanos and Pollard, 1969)
Translation	60	(Yayanos and Pollard, 1969)
Transcription	77	(Yayanos and Pollard, 1969)
Viability	200	(Pagan and Mackey, 2000)

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437 Table 2 : Known cultivable obligate piezophiles.

<i>Species</i>	Strain	Location of isolation	Growth pressure range	Reference
<i>Colwellia sp.</i>	MT41	Mariana Trench (Amphipod) 10476m	30 - 120 (opt=103)	(Yayanos, 1986)
<i>Colwellia hadalensis</i>	BNL1		>50-??	(Demin, 1988)
<i>Colwellia piezophila</i>	Y223G	Japan Trench 6278 m	40 - 80 (opt=60)	(Nogi et al., 2004)
<i>Piezobacter thermophilus</i>	108	TAG field Mid Atlantic Ridge 3660m	5 - >70 (opt=35)	(Takai et al., 2009)
<i>Psychromonas hadalis</i>	K41GT (Type)	Japan Trench (Sediment) 7542 m	30 - 9 0 (opt=60)	(Nogi et al., 2007)
<i>Psychromonas kaikoe</i>	JT7304 (Type)	Japan Trench (Sediment) 7434m	20 - >70* (opt=50)	(Nogi et al., 2002)
<i>Moritella yayanosii</i>	DB21MT-5	Challenger Deep 10898m	50 - 100 (opt=80)	(Kato et al., 1998)
	DB6101	Ryuku Trench 5110m	10 - >70** (opt=50)	(Kato et al., 1995)
	DB5501	Suruga Bay 2485m	10 - >70** (opt=50-60)	(Kato et al., 1995)
	DB6906	Japan trench (sea-side) 6269m	20 - >70*** (opt=50-60)	(Kato et al., 1995)
<i>Shewanella benthica</i>	DB6705	Japan Trench (land-side) 6356m	20 - >70*** (opt=60)	(Kato et al., 1995)
	DB21MT-2	Challenger Deep 10898m	50 - >100 (opt=70)	(Kato et al., 1998)
	DB172R	Izu-Bonin Trench 6499m	20 - >80 (opt=60)	(Kato et al., 1996a)
	DB172F	Izu-Bonin Trench 6499m	20 > 80 (opt=70)	(Kato et al., 1996a)
<i>Pyrococcus yayanosii</i>	CH1	Ashadze Mid Atlantic Ridge 4100m	20 - 120 (opt=52)	(Zeng et al., 2009)

* : 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.