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The many ways of coping with pressure

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Abstract:

The current paper reviews strategies employed by microorganisms from the deep biosphere, especially piezophiles (from the greek piezo = to press and philo = love), to cope with high hydrostatic pressure (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, as recent reviews have covered part of this subject in detail ([Abe, 2007], [Lauro and Bartlett, 2008], [Michiels et al., 2008] and [Simonato et al., 2006]). Rather, we illustrate here, via a few chosen examples, where we stand in our understanding of the mechanisms employed by microorganisms from the depths of our planet to cope with HHP.

Keywords: Adaptation and evolution; Archaea; Deep biosphere; High hydrostatic pressure; Piezophile; Stress response

1

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 incude 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 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, H_2 and CH_4

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. 10^{16} m^3 (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 (V) and activation ($V^{\neq}$)

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

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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 $V^{\neq}$ 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 V and $V^{\neq}$ 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 processi 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).

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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 *ole*1 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 *ino*1, *opi*3, *pst*1, *rta*1, *sed*1 and *prm*5 (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} = 70MPa) and *Moritella* sp. Strain DB21MT-5 (P_{opt} = 80MPa) 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.

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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 autochtonous vs. allochtonous 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 *tox*R mutant has no growth defect

255 at high pressure, while the over-expression of the *tox*R/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 *tox*R/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

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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-

10

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: *htp*G, *dna*K, *dna*J, and *gro*EL 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}/Km, 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 is 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-protebacteria. 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.

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,

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 (dimethylsulfoniopropionate and dimethylsulfonioacetate); small carbohydrates including monosaccharides (glucose), disaccharides (trehalose, sucrose, mannosucrose), sugar derivatives (glucosylglycerol, mannosylglycerate, glucosylglycerate), polyols (glycerol, inositol, sorbitol), cyclitols (di-*myo*-inositol-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-*myo*-inositol-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

371   accumulation was anti-correlatedd 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}=28MPa and *P.*

393   *abyssi* strain GE5, P_{opt}=20MPa) 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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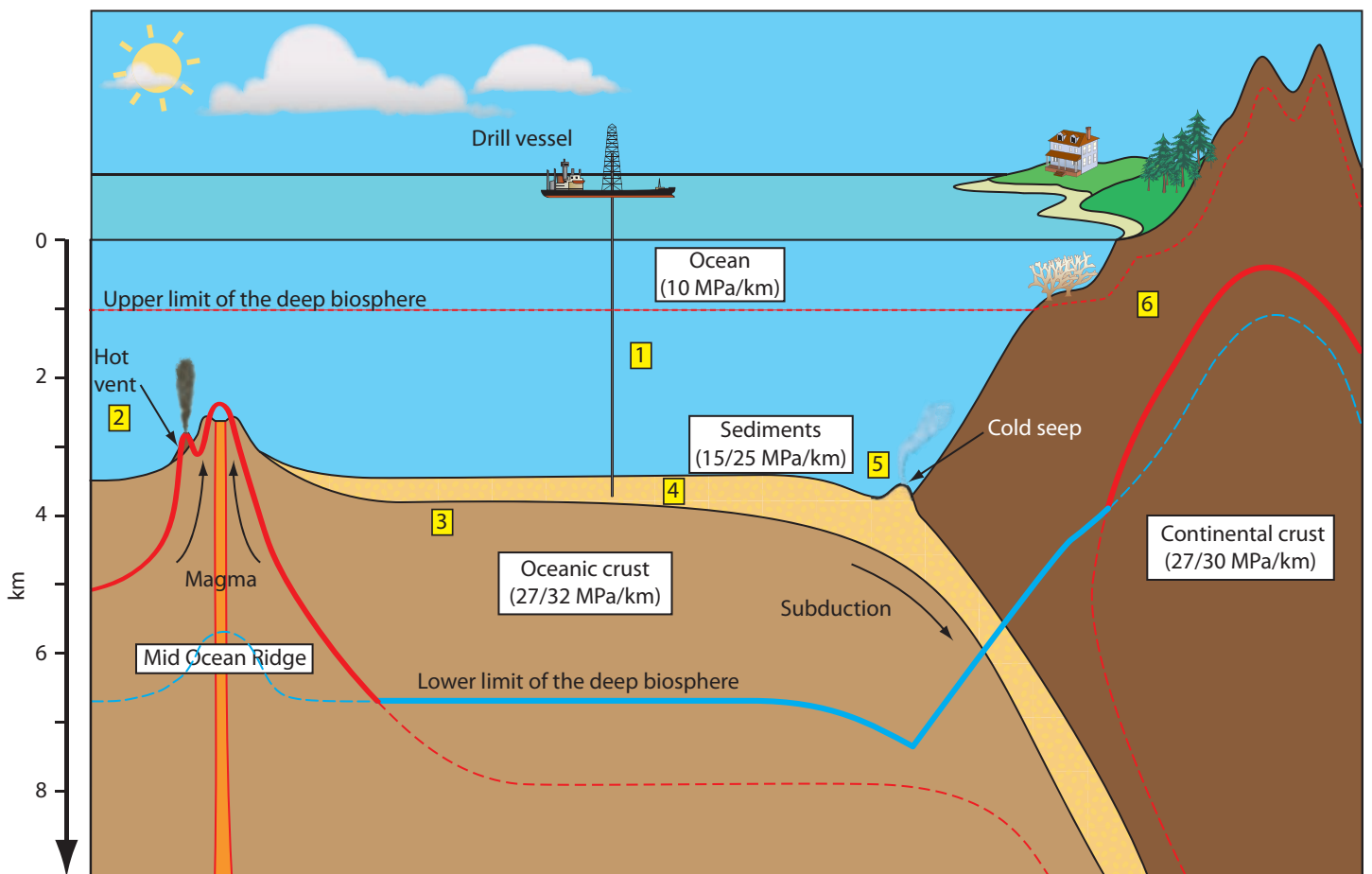
Figure 1 : Schematic transversal section of the earth highlighting the different deep-biosphere settings. 1 : deep-sea; 2 : deep-sea hydrothermal vents; 3 : deep-oceanic crust; 4 : sedimentary sub-seafloor; 5 : deep-sea cold seep; 6 : continental deep-biosphere. The red and blue lines represent the temperature 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.
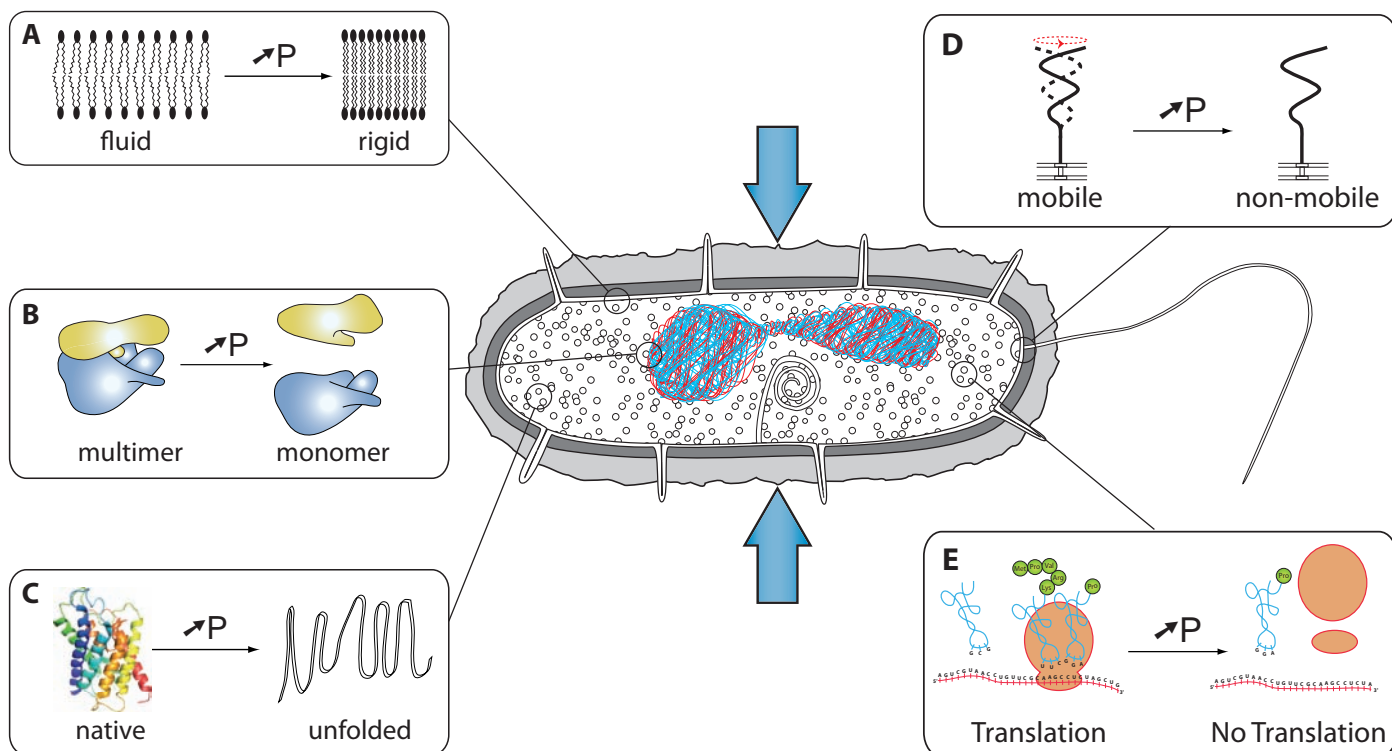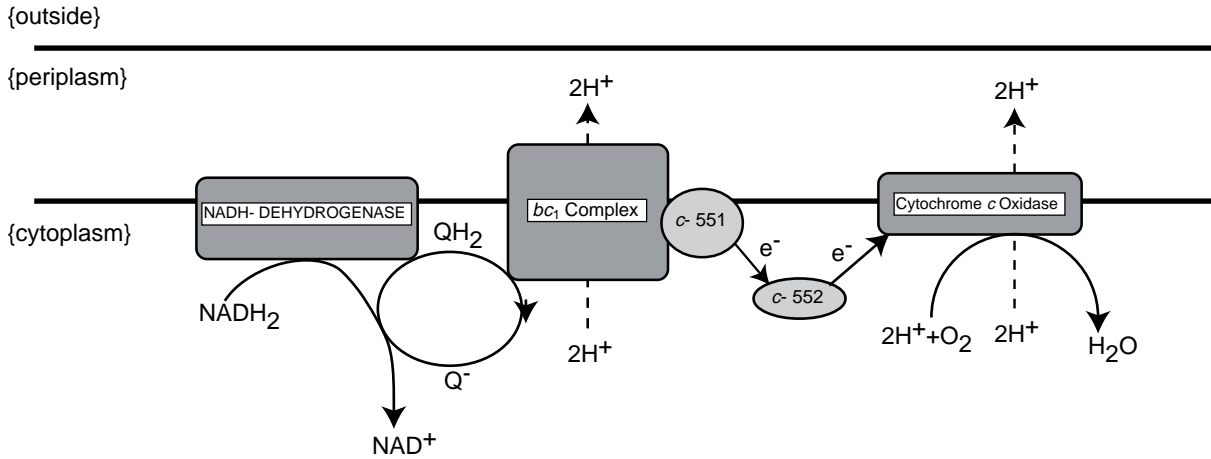
Figure 2 : Examples of the effects of high hydrostatic pressure on cells and cellular components.
A : lipids in membranes; B : multimeric protein assemblages. C : Protein structure; D : cellular motility;
E : protein translation by ribosomes.

Respiratory chain at 0.1 MPa

{outside}

{periplasm}

{cytoplasm}

Respiratory chain at 60 MPa

{outside}

{periplasm}

{cytoplasm}

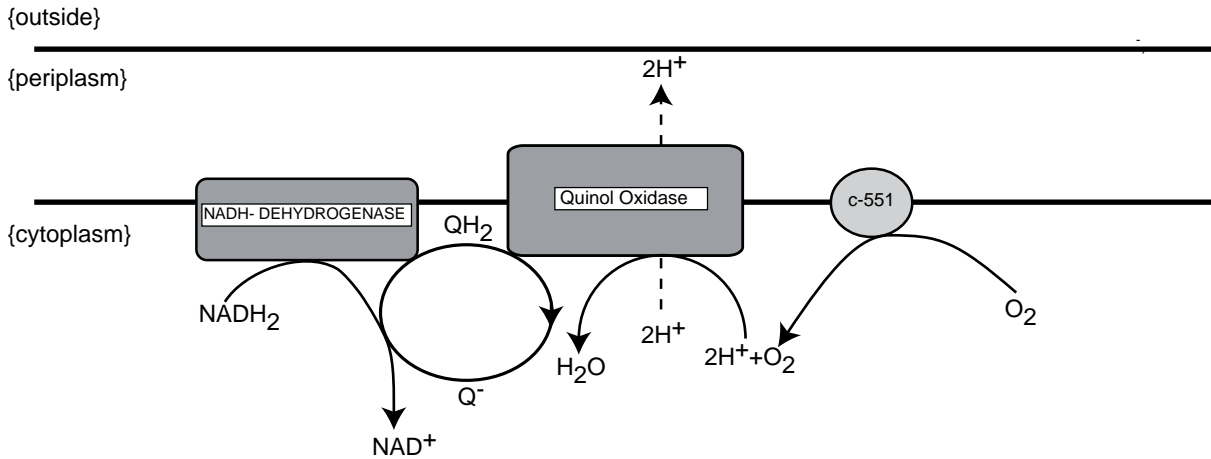Figure 3 : Structure of the low- and high-pressure respiratory chains in S. benthica (redrawn from Kato and Qureshi, 1999)

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

434

435

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.

Species	Strain	Location of isolation	Growth pressure range	Reference
Colwellia sp.	MT41	Mariana Trench (Amphipod) 10476m	30 - 120 (opt=103)	(Yayanos, 1986)
Colwelia hadalensis	BNL1		>50-??	(Demin, 1988)
Colwellia piezophila	Y223G	Japan Trench 6278 m	40 - 80 (opt=60)	(Nogi et al., 2004)
Piezobacter thermophilus	108	TAG field Mid Atlantic Ridge 3660m	5 - >70 (opt=35)	(Takai et al., 2009)
Psychromonas hadalis	K41GT (Type)	Japan Trench (Sediment) 7542 m	30 -9 0 (opt=60)	(Nogi et al., 2007)
Psychromonas kaikoe	JT7304 (Type)	Japan Trench (Sediment) 7434m	20 - >70* (opt=50)	(Nogi et al., 2002)
Moritella yayanosii	DB21MT-5	Challenger Deep 10898m	50 - 100 (opt=80)	(Kato et al., 1998)
Shewanella benthica	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)
	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)
Pyrococcus yayanosii	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.