Chapter 3
Adaptations of Cold- and Pressure-Loving Bacteria to the Deep-Sea Environment: Cell Envelope and Flagella

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Abstract Compared to terrestrial environments our knowledge of microorganisms inhabiting oceans, the largest ecosystem on Earth, is limited. Deep oceans contain bacteria that thrive at high pressure and low temperature. For them, as for all bacteria, the outer structures of the cell are the first point of contact with the environment, both sensing and being modified in response to it. The vast majority of studied cold- and pressure-loving bacteria are Gram-negative and so in this chapter, the adaptations of their cell envelope and flagella are presented. In deep-sea bacteria, the structure of phospholipids and lipopolysaccharides is modified in order

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to maintain membrane fluidity and enable membrane-localised proteins to perform their functions. Many of the membrane proteins involved in nutrient acquisition, transport, respiration, sensing and signalling are also specifically adapted to function at high pressure and low temperature. The ability to move towards nutrients or away from hostile environment is extremely important for bacterial survival and yet very vulnerable to increased pressure. Deep-sea bacteria are capable of swimming even at 150 MPa, which suggests their motility systems are specifically adapted to high pressure. Moreover, some bacteria have been shown to produce a second type of flagella (lateral flagella) in response to high pressure or low temperature. The findings presented in this chapter are a result of many techniques and analyses applied to whole microbial communities, single species as well as particular genes and proteins. Investigation of the adaptations to high pressure and low temperature not only expands basic knowledge but also identifies targets that could have biotechnological and industrial application. Deep-sea bacteria could be used for production of biofuels, secondary metabolites of value for drug development, and various pressure and temperature adapted enzymes.

3.1 Introduction

Oceans constitute the largest habitat on Earth, comprising over 90% of the biosphere by volume and yet, when compared to terrestrial ecosystems, the marine environment is understudied. Our knowledge of marine microorganisms is limited. Over the years, little effort has gone into the exploration of marine biodiversity (Duarte 2006). In fact, for a long time all bacteria collected from ocean water samples were regarded as terrestrial contamination (Williams 2009). Furthermore, in comparison to the number of colonies arising from studied samples with that obtained by microscopic observation it is evident that only 1% of bacteria can be cultivated (Stach and Bull 2005). Therefore, unless molecular techniques are used, a huge fraction of bacterial species present in marine samples remains undetected. Discoveries are also hindered by the fact that few countries have the equipment needed to explore ocean depths below 200 m (Duarte 2006).

The marine environment is exceptionally diverse and cannot be easily classified. The average pressure in oceans equals 38 MPa (which is 380 times more than the atmospheric pressure) and the average deep-sea temperature is 1–3 °C, but every local environment has its own characteristics (Abe and Horikoshi 2001). Hydrostatic pressure varies from 0.1 MPa on the surface to 110 MPa at the bottom of the Challenger Deep within the Mariana Trench, the deepest place in the ocean. This variety of habitats results in a diversity of bacterial adaptation to pressure. In the surface waters, pressure-sensitive (piezosensitive) microorganisms can be found, whose growth is inhibited by high pressure. Deeper waters abound in piezophiles, pressure-loving organisms that grow optimally at elevated pressures above atmospheric 0.1 MPa, and piezotolerant strains, able to grow both at atmospheric and high pressure (Abe and Horikoshi 2001). Deep-sea temperatures

can range from −2 °C in polar oceans and ∼4 °C at temperate and tropical latitudes to over 300 °C near hydrothermal vents (Deming 1998). Organisms growing optimally at low temperatures are categorised as psychrophiles (cold-loving). However, the classical definition of a psychrophilic microorganism uses arbitrary limits to define its optimal growth temperatures as being under 15 °C with ∼20 °C being the maximum temperature still allowing for growth. It has been postulated that a more relevant definition is necessary (Feller and Gerday 2003).

High hydrostatic pressure and low temperature often exert similar effect on bacterial cells. In fact a 16S rRNA-based phylogeny analysis revealed that cold-loving piezophiles appear to be descendants of polar psychrophiles (Lauro et al. 2007). However, when reactions are concerned, an increase in temperature simply accelerates the reaction, whereas pressure can accelerate, inhibit or exert no effect at all, depending on the change in reaction volume (Abe and Horikoshi 2001). A process is enhanced by elevated pressure if the system volume of its reaction decreases, and is inhibited if the associated system volume increases. The effect of pressure is especially complex as it also depends on other physicochemical factors such as temperature, pH and nutrient composition (Bartlett 2002). Moreover, when the marine environment is considered, it is noteworthy that the pressure range in the oceans varies 1000-fold, whereas temperature (excluding hydrothermal vents and cold seeps) does not change by more than 6 °C in the deep sea (Bartlett 1991). The complexity of the high pressure effect on bacterial cells has been shown in *Escherichia coli*, which increases rates of synthesis both of heat shock proteins and cold shock proteins in response to pressure (Welch et al. 1993). This unusual paradoxical response can be explained by the fact that some of the effects of elevated pressure resemble those of high temperature (e.g. destabilisation of protein quaternary structure) whilst others resemble those of cold temperature (decreased protein synthesis and membrane fluidity) (Bartlett 2002).

Most of the isolated piezophiles are psychrophilic Gram-negative bacteria that belong to the *Gammaproteobacteria* class and include species from the genera *Shewanella*, *Psychromonas*, *Photobacterium*, *Colwellia*, *Thioprofundum* and *Moritella* (Zhang et al. 2015). A few piezophilic bacteria have been obtained which belong to the *Alphaproteobacteria* and *Deltaproteobacteria* classes. Gram-positive psychropiezophiles are rare and the two isolated species belong to family *Carnobacteriaceae* (Lauro et al. 2007). Adaptations to life in the ocean depths, namely to the high pressure and low temperature, have been mostly studied in *Photobacterium* and *Shewanella* species. Investigating the adaptations of deep-sea microorganisms to their environment broadens our understanding of the processes occurring in nature, but can also have potential biotechnological and industrial applications such as production of new metabolites or low temperature- and high pressure-adapted enzymes for bioreactors and deep-sea waste disposal (Kato and Bartlett 1997). It has also been suggested that in the future obligate piezophiles could be used as a containment system for production of toxins, virulence factors and virus particles without posing a threat to the surrounding environment at atmospheric pressure (Zhang et al. 2015).

The cell envelope is the first point of contact of bacteria with their environment (Silhavy et al. 2010). This sophisticated and complex structure protects cells, but also allows selective passage of nutrients from the outside and waste products from the inside. Spanning all the layers of cell envelope and extending into the extracellular space is the flagellum, responsible for bacterial motility (Erhardt et al. 2010). Both the cell envelope and the flagella enable the deep-sea bacteria to adapt to their environment and are crucial for the survival at high pressure and low temperature. Deep-sea microorganisms and their adaptations to environmental conditions found in oceans are relatively recent research subjects. In contrast, the effect of high pressure and cold temperature on mesophilic bacteria with midrange temperature optima, such as *E. coli*, has been well documented over the years although not always understood.

Growth of mesophilic microorganisms is impeded within the range of several dozen MPa, is completely inhibited at approximately 50 MPa and pressures greater than 200 MPa kill most microorganisms (Abe 2007; Bartlett 2002). Motility is affected at 10 MPa, nutrient uptake at 15–20 MPa, membrane protein function at 25–50 MPa and protein oligomerization at 50–100 MPa. Pressure affects both lipids and proteins present in the membrane. The compression of phospholipid acyl chains causes a decrease in membrane fluidity and permeability (Abe 2007). Additionally high pressure can affect the functioning of membrane-bound enzymes. Pressure changes the conformation and activity of proteins, especially multimers, since these are stabilised by weak chemical bonds (Aertsen et al. 2009). Bacteria also become less motile, possibly due to perturbations of the cell membrane and flagellum apparatus (Meganathan and Marquis 1973). Low temperatures influence solute diffusion rates, enzyme kinetics, membrane fluidity and conformation as well as topology and interactions of proteins (Rodrigues and Tiedje 2008). The loss of membrane fluidity is the primary signal perceived by bacteria when exposed to low temperature. Although studying the response of mesophilic bacteria to high pressure and low temperature provides substantial knowledge, it is not sufficient to understand how psychropiezophiles adapt to the extreme environment.

3.2 The Effect of High Pressure and Low Temperature on the Membranes of Psychropiezophiles

The cell envelope of Gram-negative bacteria contains two membranes (Silhavy et al. 2010). The inner membrane is a phospholipid bilayer, while in the outer membrane phospholipids are confined to the inner leaflet. The outer layer of the outer membrane consists of lipopolysaccharide molecules. In addition to lipids, biological membranes contain also various proteins. In this section, the adaptation of membrane components to high pressure and low temperature in deep-sea bacteria will be discussed.

3.2.1 Phospholipids

Microorganisms need to maintain the structural and dynamic properties of their cell membranes at all times. Both high pressure and low temperature exert similar effects, leading to tighter packing and restricted motion of acyl chains (Winter and Dzwolak 2005). To counteract these changes and restore the membrane fluidity, many organisms adapt their membrane phospholipid unsaturated fatty acid content in a process known as homeoviscous adaptation (Sinensky 1974). It has been suggested that homeophasic adaptation is more physiologically relevant than the homeoviscous adaptation (McElhaney 1982). Homeophasic adaptation requires a certain percentage of lipid membrane to remain in the liquid crystalline phase rather than gel phase (a solid state). In response to high pressure and low temperature bacteria increase the ratio of unsaturated to saturated, *cis* to *trans* and short to long fatty acids and alter the size and charge of the polar groups (Shivaji and Prakash 2010). Hence, monounsaturated fatty acids (MUFAs), branched chain fatty acids (BCFAs) and polyunsaturated fatty acids (PUFAs) all play a role in bacterial adaptation to the deep sea environment. Unsaturated fatty acids pack less compactly and adopt a more expanded conformation due to the 30° bend introduced by the double bond (Hazel and Williams 1990). They also possess lower melting temperatures than their saturated equivalents, allowing for their less orderly alignment within membrane phospholipids when the temperature decreases.

Marine organisms are the major producers of omega-3 PUFAs with 20:5 (all-*cis*-5,8,11,14,17-eicosapentaenoic acid, EPA) and 22:6 (all-*cis*-4,7,10,13,16,19-docosahexaenoic acid, DHA) acids being the most common. It has been shown that genes required for the synthesis of long chain PUFAs are often present in the genome even if bacteria do not produce PUFAs (Wang et al. 2009). Production of DHA is a characteristic trait of genus *Moritella* and *Colwellia* (Bowman et al. 1998; DeLong et al. 1997; Kato et al. 1998; Nogi et al. 1998b). Approximately 70% of *Moritella yayanosi* membrane lipids are unsaturated fatty acids, which is consistent with its adaptation to grow at high pressure (optimal 80 MPa) (Kato et al. 1998). The importance of PUFAs for low temperature- and high pressure-adapted growth is different among bacterial species. EPA-deficient strains have been analysed more thoroughly and it has been found that depending on the strain, the growth of the EPA-deficient mutant can be affected by low temperature or high pressure, by both or not affected at all (Allen et al. 1999; Kawamoto et al. 2009, 2011; Wang et al. 2009).

When *Photobacterium profundum* SS9 was grown at high pressure (28–50 MPa, 15 °C) or at low temperature (4 °C, 0.1 MPa) the amount of monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs), specifically eicosapentaenoic acid EPA 20:5, was increased compared to growth at atmospheric pressure and 15 °C (Allen et al. 1999). However, elevated pressure and low temperature growth conditions resulted in the greatest increase in levels of different MUFAs, 18:1 (*cis*-11-octadecanoic acid; *cis*-vaccenic acid) and 16:1 (*cis*-9-hexadecanoic acid; palmitoleic acid) respectively. Additionally, MUFAs seem to be more effective in maintaining optimum membrane fluidity, as the EPA-deficient *P. profundum* SS9

mutant was not affected by high pressure or low temperature (Allen et al. 1999). Despite the unperturbed growth of *P. profundum* SS9 in its absence, EPA constitutes a significant proportion of fatty acids present in piezophilic *P. profundum* strains (9% in SS9 and 13% in DSJ4) while it is absent in the piezosensitive *Photobacterium* species (Nogi et al. 1998a). Interestingly, the EPA biosynthesis operon is also present in the piezosensitive *P. profundum* 3TCK (Campanaro et al. 2005), but the fatty acid content of this strain has not been analysed. The *pfa* operon responsible for EPA biosynthesis appears as alien DNA present in all three isolates of *P. profundum* (SS9, DSJ4, 3TCK) (Campanaro et al. 2005). Although high pressure and low temperature exert similar effects on bacterial membranes, in *P. profundum* SS9 they seem to be perceived and responded to in a different manner. FabF, a β-ketoacyl-acyl carrier protein synthase II, was found important for the increase in 18:1 levels at both high pressure and low temperature but only essential for the piezophilic growth of *P. profundum* SS9 (Allen and Bartlett 2000). Based on the comparison of *P. profundum* SS9 gene expression at 0.1, 28 and 45 MPa, a previously unrecognised gene, encoding a putative delta-9 fatty acid desaturase (PBPRB0742), was found up-regulated with increasing pressure (Campanaro et al. 2005).

Shewanella violacea DSS12 phospholipid fatty acid content also changes with the increasing pressure. The shift from atmospheric pressure to 30 MPa affects the two major fatty acids present in the outer membrane of *S. violacea* differently, with 16:1 increasing and 20:5 decreasing in content (Kawamoto et al. 2011). Despite its decrease at high pressure, eicosapentaenoic acid is important for growth at 30 MPa and cannot be compensated for by the increased levels of 16:1 MUFA or iso-15:0 BCFA (iso-pentadecanoic acid). The EPA-deficient strain exhibits defects in the late division steps at 30 MPa (Kawamoto et al. 2011). Similarly to *P. profundum* SS9, the growth of EPA-deficient mutant of *S. violacea* DSS12 was not affected by low temperature. This is not always the case as in the psychrophilic *Shewanella livingstonensis* Ac10 EPA is required for growth at low temperatures (Kawamoto et al. 2009). However, it has been suggested that in this bacterium EPA plays a role in membrane organization and cell division, not maintenance of membrane fluidity.

In *Shewanella piezotolerans* WP3, EPA is necessary for growth at both, high pressure (20 °C, 20 MPa) and low temperature (4 °C, 0.1 MPa) (Wang et al. 2009). Contrary to *P. profundum* SS9, in *S. piezotolerans* WP3 MUFAs cannot complement for the absence of EPA. The dissimilarity between *P. profundum* SS9 and *S. piezotolerans* WP3 possibly stems from the different content of MUFAs in the cells. In the *P. profundum* SS9 EPA-deficient strain almost 70% of fatty acids are MUFAs, whereas in *S. piezotolerans* WP3, the MUFAs levels reach only 30%, which might not be sufficient to compensate for the absence of EPA. The differences between these genera could also reflect differences in physiology, i.e., fermentative versus respiratory growth.

Along with the unsaturated fatty acids, branched chain fatty acids also play a role in adaptation to low temperatures. *S. piezotolerans* WP3 was found to increase the BCFA content at low temperature mainly by importing precursors of BCFAs from the environment using the branched-chain amino acid ABC transporter LIV-I (Wang et al. 2009). The LIV-I transporter was identified only in the cold-adapted

Shewanella species and was assumed to supply an important strategy for the cold- but not pressure-adaptation.

Generally it is accepted that organisms inhabiting cold and high-pressure environments need to maintain greater membrane fluidity. However, a recent study suggests that retaining a certain level of membrane stability under a wide range of environmental conditions is more important. *S. violacea* DSS12 attains the required membrane rigidity at its optimal growth temperature of 10 °C due to the production of substantial levels of EPA that prevents the membrane from becoming hyperfluid (Usui et al. 2012). As a result, the membrane properties do not change drastically over a wide range of hydrostatic pressures (Abe 2013; Usui et al. 2012). Moreover, EPA and DHA have been found to possess an antioxidative function, possibly shielding cells against reactive oxygen species in vivo (Okuyama et al. 2008).

Research into the fatty acids produced in marine bacteria is not limited to understanding their role in adaptation to the deep-sea environment. Fatty acids produced by bacteria could be used as a renewable source for the production of commercially available biofuels. Currently, one of the main steps towards this goal is the increase in the fatty acid production by microbes. An active dehydratase tetradomain protein fragment from the PUFA synthase enzyme complex from *P. profundum* SS9 was shown to increase the production of fatty acids in *E. coli* as much as 5-fold (Oyola-Robles et al. 2014). This enhancement in fatty acid production was more pronounced at lower temperatures. Several other enzymes encoded in the *pfa* operon of *P. profundum* SS9 have also been studied. The solved structure of tandem acyl carrier protein (ACP) domains suggests that artificial linking of multiple ACP domains could increase the yield of fatty acids in bacterial cultures (Trujillo et al. 2013). Omega-3 fatty acids have beneficial effects on human health and in the future could be produced by microbes, since currently the fish-derived PUFAs cannot sustainably meet the human consumption demands. Cloning of the DHA synthesis operon into *E. coli* and optimisation of DHA production in *Moritella marina* MP-1 have already been carried out (Amiri-Jami and Griffiths 2010; Kautharapu et al. 2013; Orikasa et al. 2006).

3.2.2 Lipopolysaccharide

In Gram-negative bacteria, lipopolysaccharide (LPS) forms the outermost layer of the outer membrane with the hydrophobic lipid A situated in the interior of the outer membrane and hydrophilic O-antigen facing the outside of the cell. LPS is a fundamental component of the outer membrane and as such plays an important structural role. The phosphate groups in lipid A and the core region bind divalent cations and stabilise the outer layer of the outer membrane (Kumar et al. 2002). In marine bacteria, the O-antigen is very often anionic and also binds cations, which provides further stability towards external stressors such as high pressure (Nazarenko et al. 2011). Bonds forming between O-antigen moieties and cations stabilise the membrane but also play a role in interactions with other bacterial cells,

like in *V. cholerae* O139, where the O-antigen is required for the formation of Ca^{2+}-dependent biofilms (Kierek and Watnick 2003). O-antigen moieties help both with the aggregation of cells and with their attachment to surfaces. Atomic force microscopic analysis showed that LPS facilitates adhesion to negatively charged surfaces and longer O-antigen results in a higher force of adhesion, most likely due to formation of increased numbers of hydrogen bonds (Abu-Lail and Camesano 2003; Strauss et al. 2009). Additionally, the O-antigen acts as a surfactant and increases wettability of surfaces, promoting bacterial swarming (Toguchi et al. 2000). It is also required for successful colonisation of the host by symbiotic and pathogenic bacteria. The O-antigen ligase mutant of *Vibrio fischeri* has a motility defect and is delayed in colonisation of the light organ of the Hawaiian bobtail squid (Post et al. 2012). The O-antigen mutant of the pathogenic *E. coli* O157:H7 is cleared faster from the murine intestine and does not establish an as effective bovine intestine colonisation as the parent strain (Sheng et al. 2008). Additionally, O-antigen molecules also protect bacteria from recognition by the host's immune system and bacteriophages, since they can mask the bacteria's conserved epitopes and receptors (Whitfield et al. 1997).

When the temperature decreases or pressure increases, bacteria adapt their membranes to counteract the rigidification (Bartlett 1999). This is usually achieved by modifying the phospholipids, as discussed in the previous section. However, it has also been found that fluidity of the outer membrane increases when the concentration of LPS or the length of the polysaccharide side chain is reduced (Rottem and Leive 1977). Bacteria grown at low temperatures change the fatty acids present in lipid A or even produce new kinds of LPS. For example *Salmonella* species incorporate an unsaturated fatty acid into their lipid A when grown at low temperatures (Wollenweber et al. 1983). *Yersinia pestis* cells produce a new type of LPS at 6 °C with a number of significant structural modifications to the core and lipid A (Knirel et al. 2005). The surface polysaccharides of the Antarctic psychrophilic bacteria *Pseudoalteromonas haloplanktis* TAC 125 and *Pseudomonas syringae* Lz4W are less phosphorylated at low temperatures (Corsaro et al. 2004; Ray et al. 1994). Moreover, the hydroxylated fatty acid content of the *P. syringae* LPS increases at low temperatures (Kumar et al. 2002), but a role for these LPS changes in the cold-adapted growth of the bacterium has not been studied.

The LPS has not been studied extensively in psychropiezophiles. It is known that *P. profundum* SS9 contains both types of LPS, rough (R-LPS, consisting of lipid A and the core) and smooth (S-LPS, consisting of rough LPS and the O-antigen) (El-Hajj et al. 2009). Interestingly, the LPS of three *P. profundum* strains, two piezophilic (SS9 and DSJ4) and one piezosensitive (3TCK) differ significantly (Fig. 3.1a) (Myka 2013). All strains were grown at 15 °C, atmospheric pressure, because it has been shown before that *P. profundum* SS9 LPS PAGE profile does not change with temperature (Allcock 2009). The high molecular weight S-LPS of SS9 and 3TCK was primarily hydrophilic as it extracted into the aqueous phase, while the DSJ4 S-LPS partitioned into the phenol phase, which indicates its hydrophobicity. Moreover, the LPS profile of the piezosensitive 3TCK resembled the "ladder-like" LPS found in *Salmonella* species. The "ladder" LPS profile results

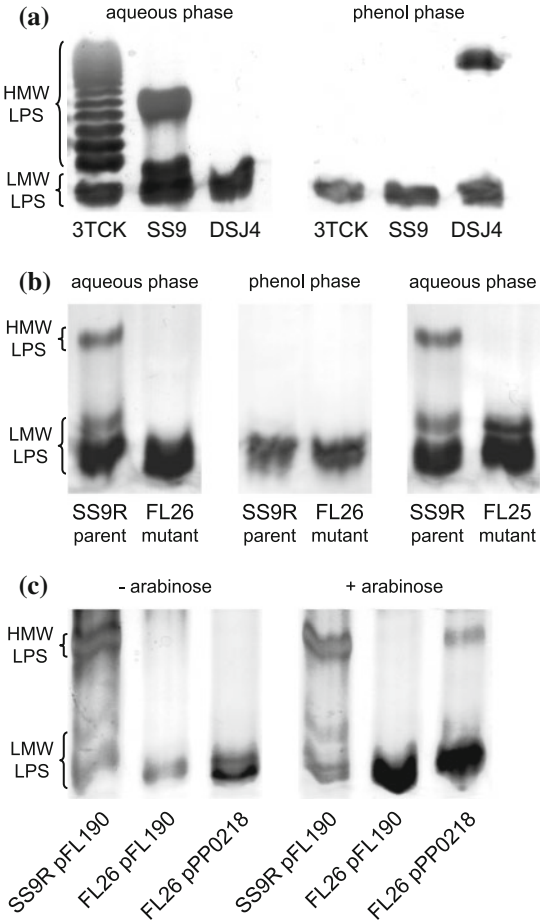

Fig. 3.1 The LPS of *Photobacterium profundum*. **a** LPS species from three *P. profundum* strains with different pressure and temperature growth optima were purified using the hot phenol-water extraction method (Carlson et al. 1978). Both aqueous and phenol phases were analysed by DOC-PAGE followed by silver staining (Tsai and Frasch 1982). SS9 grows optimally at 28 MPa, 15 °C; DSJ4 at 10 MPa with very little change up to 50 MPa, 10 °C; 3TCK is piezosensitive and grows optimally at atmospheric pressure, 20 °C (Campanaro et al. 2005; DeLong 1986; Nogi et al. 1998a). **b** The FL26 and FL25 mutants lack high molecular weight smooth LPS (HMW S-LPS) and produce only low molecular weight rough LPS (LMW R-LPS). FL26 and FL25 LPS species were purified as in (a) and analysed by DOC-PAGE followed by alcian blue-silver staining. (c) Plasmid encoded putative O-antigen ligase restores smooth LPS to the FL26 mutant. LPS species were analysed as in (b). Since S-LPS does not extract into the phenol phase, the phenol phases R-LPS species are not shown. The *P. profundum SS9 pbpra0218* gene was amplified using primers 5-ATGAATTCAGAGAATATTTTTAAACTACTGATGT-3 and 5-ATTCTAGATTA GCAATTGTTCTTCTTTGAAGTT-3 and cloned into pFL190 (Lauro et al. 2005) using EcoRI and XbaI to produce pPP0218

from the heterogeneity of LPS molecules containing O-antigens of a different chain length (Raetz and Whitfield 2002). Each band "up the ladder" in the PAGE LPS profile corresponds to the lipid A-core with an O-antigen with an additional O-unit. It is not known why only the 3TCK would have such variation in O-antigen polysaccharide length and more experiments would be necessary to test if this phenotype has a connection to its piezosensitivity and/or relatively high optimal temperature of growth.

A study of 16 *Shewanella* strains including at least 8 different species found that half possessed only R-LPS while the other half contained also S-LPS (Korenevsky et al. 2002). Most *Shewanella* species tested did not alter their LPS in response to temperature (Korenevsky et al. 2002). However, the LPS of *Shewanella frigidimarina* displayed a ladder-like pattern when visualised using SDS-PAGE only when it was extracted from a strain grown at 15 °C, which was not observed at higher or lower temperatures (Korenevsky et al. 2002). The change in acyl moieties of lipid A with temperature was tested in an obligatory psychrophile *Colwellia hornerae* and psychropiezophile *Colwellia piezophila* and compared with a facultative psychrophile, *Psychrobacter cryohalolentis* (Sweet et al. 2015). Both *Colwellia* strains did not display alterations in lipid A based on growth temperature, while *P. cryohalolentis* did. Lipid A of *Colwellia* species contains shorter acyl units compared to *E. coli* and *Vibrio*. It seems that in these obligatory psychrophiles the constitutive change of lipid A is the evolutionary adaptation to cold environments. On the other hand, the facultative psychrophile (psychrotolerant) *P. cryohalolentis* relies on metabolic responses to tune the fluidity of the outer membrane to a wider range of temperatures.

Bacterial survival in the deep sea very often relies on the attachment to surfaces, either mineral or of other organisms. The outer layer of the outer membrane has the most influence on the bacterium-surface interaction. The adhesion of *Shewanella* species is mostly governed by electrostatic interactions and depends primarily on the LPS, with membrane proteins and capsular polysaccharides playing minor role (Korenevsky and Beveridge 2007). LPS is the most abundant molecule present on the Gram-negative surface (Beveridge 1999) and its O-antigen not only extends considerable distances from the cell surface but can also possess more exposed electrostatic sites than proteins (Korenevsky and Beveridge 2007). On the other hand, it was suggested that the short LPS and high potential charge of the piezosensitive *Shewanella putrefaciens* CN32 cell surface allow for a good contact of the putative outer membrane iron reductase with the iron oxide present in the environment (Korenevsky et al. 2002).

The importance of LPS in the attachment to surfaces of *P. profundum* SS9 could also be inferred from the study performed by Lauro et al. (2008). Eight out of thirty-one transposon mutants of *P. profundum* SS9 were found to possess insertions in genes predicted to encode proteins involved in the cell envelope biogenesis (Lauro et al. 2008). All of these mutants were cold-sensitive, confirming the requirement for a correctly assembled cell envelope for the survival at low temperature. Three of the genes inactivated in cold-sensitive transposon mutants are located in a cluster involved in the cell envelope biogenesis that also contains genes

regulated by temperature or pressure at the transcription level (Campanaro et al. 2005, 2012) and at the level of translation (Le Bihan et al. 2013).

Interestingly, two of the cell envelope biogenesis mutants identified by Lauro and colleagues displayed the cold-sensitive phenotype only when grown on agar (Lauro et al. 2008). The *P. profundum* SS9 mutant, FL26, has a transposon insertion in the gene encoding a putative O-antigen ligase, *pbpra0218* (Lauro et al. 2008). O-antigen ligases are responsible for attaching the O-antigen sugar polymer to the rough lipopolysaccharide (R-LPS) consisting of lipid A and the sugar core, thereby creating a complete LPS molecule, called smooth LPS (S-LPS) (Raetz and Whitfield 2002). The second cold-sensitive mutant, FL25, has a disruption in a putative glycosyltransferase, *pbpra2700* (Lauro et al. 2008). Glycosyltransferases are essential for the biosynthesis of the LPS core oligosaccharide and the O-antigen (Raetz and Whitfield 2002). It is worth noting that the expression of the *pbpra0218* gene was shown to be temperature dependent and down-regulated at 4 °C compared to 16 °C in liquid cultures (Lauro et al. 2008). Dr. Gail P. Ferguson's group analysed the LPS present in the FL26 and FL25 mutants and found that both mutants lack the high molecular weight species corresponding to the S-LPS (Fig. 3.1b) (Myka 2013). The S-LPS of the FL26 mutant could be restored by a plasmid encoded putative O-antigen ligase (PBPRA0218) (Fig. 3.1c). Sequence analysis revealed that PBPRA0218 is 48% similar (30% identical) to an O-antigen ligase from *V. cholerae* O1 (GenBank accession no. AAL76923.1) and contains characteristic for the O-antigen ligase pfam04932 domain and two conserved motifs RX_3L and $HX_{10}G$ (Myka 2013; Schild et al. 2005). However, the complementation of the cold-sensitive phenotype of FL26 could not be achieved by the plasmid-encoded *pbpra0218*, as the presence of a plasmid conferring resistance to streptomycin masked the cold-sensitive phenotype of FL26 (Myka 2013). Chi and Bartlett observed that streptomycin exerted an inhibitory effect on the growth of *P. profundum* SS9, even in the presence of a plasmid encoding streptomycin resistance (Chi and Bartlett 1995). This inhibition was enhanced by elevated pressure and low temperature. Due to these technical difficulties, it cannot be definitely stated that the lack of S-LPS is directly responsible for the cold-sensitive phenotype of the *P. profundum* SS9 mutant. Nevertheless, the discovery of mutants that are cold-sensitive only on agar calls for research that would investigate adaptations of the deep-sea bacteria to both the temperature and the mode of growth (single cell/colony/biofilm). S-LPS might protect *P. profundum* SS9 against the effects of the low temperature during colony growth by promoting the attachment of cells to the marine agar. It has been shown that surface association is important for Arctic microbes activity at sub-zero temperatures (Junge et al. 2004). The putative selective advantage of surface association that we observe for *P. profundum* SS9 in the laboratory could also extend to the environment. *P. profundum* SS9 was isolated from an amphipod homogenate (DeLong 1986) and in the cold deep sea the attachment to surfaces of marine organisms could increase its environmental fitness and enhance its survival.

Studies of the LPS in marine bacteria not only provide information about the strategies that bacteria adopt to survive in the deep sea, but could also have

biotechnological applications. The LPS of marine bacteria often differs from the well-studied mesophilic organisms. Marine bacteria were found to possess a substantial heterogeneity in their lipopolysaccharides, which contain uncommon monosaccharides, higher sugars and derivatives with non-sugar substituents (Nazarenko et al. 2003). *Shewanella oneidensis* (a piezosensitive strain) contains an unusual modification to its LPS core sugar, Kdo, which is phosphorylated and with one hydroxyl group converted to a primary amine (Kdo8N) (Gattis et al. 2013). Deletions of the biosynthetic genes led to the sensitivity or *S. oneidensis* to compounds perturbing the outer membrane, suggesting that Kdo8N increases membrane integrity. It is hypothesised that marine bacteria from high pressure and low temperature environments produce lipid A structures of possible pharmacological interest (Solov'eva et al. 2013). LPS and lipid A obtained from several marine bacteria display low toxicity and exhibit properties of endotoxin antagonists, suggesting they could be promising candidates for treating Gram-negative sepsis.

3.2.3 Membrane Proteins

High pressure and low temperature affect membrane proteins in several ways. Changes in the fluidity and physical state of the membrane lipids can affect the activity of membrane enzymes and transport systems (McElhaney 1982). Pressures up to 100 MPa induce a reversible change in the structure of transmembrane proteins, 100–220 MPa cause dissociation and conformational changes in protein subunits, and pressure higher than 220 MPa causes irreversible protein unfolding and interface separation (Kato et al. 2002). Additionally it has been shown that protein synthesis is inhibited by pressure in the range of 50 MPa (Abe 2007; Bartlett 2002). High pressure affects porins, transport proteins, membrane proteins involved in sensing and signalling and respiratory system and other proteins, as discussed below. Most of the research has been performed in *P. profundum* SS9 and the advance of transcriptomics and proteomics allowed for more insight into the high pressure and low temperature induced changes in gene expression and protein abundance (Campanaro et al. 2005, 2012; Le Bihan et al. 2013). However, future proteomic studies could benefit from a membrane enrichment strategy, as in the available analysis most differentially expressed proteins were found in the cytoplasm with membrane proteins being largely underrepresented (Le Bihan et al. 2013).

3.2.3.1 Outer Membrane Porins

P. profundum SS9 was shown to modulate its outer membrane protein content in response to pressure. OmpH increases its abundance with increasing pressure

(10–100 times) and is maximally expressed at 28 MPa, optimal for SS9 growth (Bartlett et al. 1989; Chi and Bartlett 1993). Conversely, OmpL, encoded by one of the first pressure-regulated genes identified in SS9, is preferentially expressed at 0.1 MPa and decreases with increasing pressure (Le Bihan et al. 2013; Welch and Bartlett 1996, 1998). Both OmpL and OmpH encode outer membrane porins, but it has been suggested that the OmpH provides a larger diffusion channel (Bartlett and Chi 1994). *ompH* and *ompL* are each the fourth most expressed protein-encoding gene at high and atmospheric pressure, respectively (when genes coding for rRNA transcripts are not considered) (Campanaro et al. 2012). Despite the fact that OmpH is one of the most abundant proteins in the outer membrane of *P. profundum* SS9 at elevated pressure, an *ompH* mutant is not impaired in its growth at high pressure (Chi and Bartlett 1993). Moreover, the expression of the *ompH* gene can also be induced under low pressure under the conditions of increasing cell density and energy starvation (Bartlett and Welch 1995; Chi and Bartlett 1993).

The *P. profundum* SS9 genome contains 9 additional porin genes (Campanaro et al. 2012). Other porins influenced by high pressure include OmpC (PBPRB1639) and two hypothetical maltoporins PBPRB2004 and PBPRB0413 (Campanaro et al. 2012). The PBPRB2004 maltoporin transcript was found to be more abundant at 28 MPa, while another gene belonging to a porin superfamily, *pbpra2139*, was expressed more at atmospheric pressure. The various expression levels of genes encoding porins in response to high pressure are a clear example of the complexity of high-pressure adaptation. In a nutrient-scarce environment such as the deep sea, an increase in abundance of porins involved in diffusion of large compounds could enhance piezophile growth and survival.

3.2.3.2 Regulation by ToxR

The *P. profundum* SS9 *ompH* and *ompL* genes are transcriptionally regulated by the inner membrane proteins ToxR and ToxS (Welch and Bartlett 1996, 1998). ToxR has been mostly studied in *V. cholerae* and is an oligomeric transmembrane protein localised in the inner membrane (Dziejman and Mekalanos 1994; Ottemann and Mekalanos 1995). It functions together with ToxS, a membrane bound periplasmic effector protein (DiRita and Mekalanos 1991). ToxR is an environmental sensor that regulates gene expression in response to changes in osmolarity, pH, temperature and levels of certain extracellular amino acids (Gardel and Mekalanos 1994; Miller and Mekalanos 1988). In *V. cholerae* ToxR controls the expression of over 150 genes, including those responsible for virulence (Bina et al. 2003). It binds directly to genes under its control via a cytoplasmic DNA binding domain (Miller and Mekalanos 1988). ToxS modulates ToxR activity and protects ToxR from premature proteolysis at late stationary phase, under starvation conditions and at alkaline pH (Almagro-Moreno et al. 2015a, b; DiRita and Mekalanos 1991). All of these are associated with the entry of *V. cholerae* into a dormant state, which enables its survival in the aquatic environment.

In *P. profundum* SS9, ToxR protein levels and its activity decrease at high pressure (Le Bihan et al. 2013; Welch and Bartlett 1998), while the ToxS level of expression in relation to pressure could not be significantly evaluated (Le Bihan et al. 2013). ToxR is required for *ompL* expression and *ompH* repression, since the *toxR* mutant does not possess OmpL, but OmpH is maintained constitutively at a high level (Le Bihan et al. 2013; Welch and Bartlett 1998). The *toxR* mutant has no growth defects at a high pressure, which suggests that ToxR/S is not required for high pressure adaptation. However, the overexpression of the *toxRS* genes leads to pressure-sensitive growth, either because genes necessary for survival at high pressure are repressed or genes deleterious to high pressure growth are activated (Simonato et al. 2006). ToxR functions only as a pressure sensor. At increased temperatures, the ToxR abundance drops, but its activity increases, which maintains the same level of overall ToxR activity (Bartlett 2002). Moreover, ToxR/S pressure sensing depends on the physical state of the membrane (Bartlett 1999). The increase in *P. profundum* SS9 membrane fluidity with local anaesthetics resulted in a low pressure ToxR/S signalling phenotype (high OmpL, low OmpH abundance) even when the cells were grown at high pressure (Welch and Bartlett 1998).

ToxR controls the expression of outer membrane porins, but also other genes involved in membrane structure and starvation response (Bidle and Bartlett 2001; Campanaro et al. 2012). It is possible that its primary function is to homeostatically control membrane structure and energy flow under diverse environmental conditions as well as to cope with various nutrient stresses (Bartlett et al. 2008). Genes regulated by ToxR are just a fraction of those influenced by high pressure (Campanaro et al. 2012). An independent transcriptional regulator, the OmpR-2 protein, was recently found to be strongly up-regulated at 28 MPa (Campanaro et al. 2012). The comparison of the putative ToxR regulon in *V. cholerae* and *P. profundum* SS9 revealed that only four genes were shared between these two microorganisms, which suggests that there is not much overlap between adaptations to pathogenesis (*V. cholerae*) and deep-sea conditions (*P. profundum* SS9).

3.2.3.3 Membrane Transport

Transport is one of the processes in the bacterial cell most influenced by hydrostatic pressure (Vezzi et al. 2005). Changes to the fluidity of the membrane at high pressure most likely affect the efficiency of transporters embedded in the membrane (Campanaro et al. 2005). High pressure inhibits reactions that are accompanied by an increase in volume and so transport of amino acids such as tryptophan, lysine, histidine and leucine is reduced at high pressure due to the volume change of activation of the transport process (Abe and Horikoshi 2000). In marine bacteria the rates of uptake of many substrates, including glutamate and acetate, were shown to be greater at atmospheric pressure than at increased pressure (Jannasch and Taylor 1984). Somewhat counterintuitively, in *P. profundum* SS9 genes for amino acid and ion transport were up-regulated at 0.1 MPa and not at high pressure (Vezzi et al. 2005). This might reflect the adaptation of *P. profundum* SS9 transporters to high

pressure. A number of iron, phosphate, amino acids and sugar ABC transporters were found to have different isoforms that function at different pressure and temperature conditions, one being up-regulated at 0.1 MPa and the other at 28 MPa (Campanaro et al. 2005; Le Bihan et al. 2013). The differently regulated transporters could be an adaptation of *P. profundum* SS9 to grow over a large range of pressures. The sensing of hydrostatic pressure might allow *P. profundum* SS9 to detect its position (depth) in the ocean and adapt to the particular nutrient limitations. Transport was also identified as the most notable process that underwent positive selection based on amino acid substitution rates calculated between two deep-sea microorganisms, *P. profundum* SS9 and *Shewanella benthica* KT99, and their respective shallow water relatives (Campanaro et al. 2008). Higher frequencies of substitution occurred preferentially in extracellular regions of membrane proteins.

3.2.3.4 Other Membrane Proteins

Insertions in genes localised in the *rpoE* locus of *P. profundum* SS9 also lead to high pressure and cold sensitivity (Chi and Bartlett 1995). *E. coli rpoE* encodes the alternative RNA polymerase sigma factor σ^E that responds to extracytoplasmic stimuli and controls the expression of genes involved in the extracytoplasmic stress response (Missiakas and Raina 1998). It is maintained in its inactive state in the cytoplasmic membrane by the products of adjacent genes, *rseA* and *rseB* (Hayden and Ades 2008). *P. profundum* SS9 *rseB* mutants have been isolated from pressure and cold sensitivity screens (Chi and Bartlett 1995; Lauro et al. 2008). However, complementation experiments demonstrated that their sensitive phenotype occurs due to polar effects on the downstream gene, *rseC* (Chi and Bartlett 1995). *rseB* insertion mutants synthesised very low levels of numerous OMPs, including OmpL, but they were able to induce OmpH (Chi and Bartlett 1995). It seems that *rseB* is necessary for proper regulation of outer membrane proteins while *rseC* is necessary for psychro- and piezoadaptation. RseB in *E. coli* has been suggested to detect mislocalised lipoproteins in the cell envelope and induce the σ^E response (Wollmann and Zeth 2007). Moreover, many genes under the control of σ^E encode proteins involved in the biosynthesis, refolding and degradation of outer membrane proteins. The importance of genes in the *rpoE*-locus for growth at elevated pressure suggests that high pressure might ultimately lead to an increase in the accumulation of misfolded proteins in the periplasm, which is toxic for bacterial growth (El-Hajj et al. 2010). Hence, activation of the *rpoE*-locus might be important for maintaining the cell envelope integrity at high pressure. RseC in *E. coli* is believed to have a negligible effect on RpoE activity but functions as an inner transmembrane protein affecting electron transport (Beck et al. 1997). Perhaps the *P. profundum* SS9 *rseC* mutants are high pressure and cold-sensitive because of the effects of those growth conditions on membrane-based electron transport (Bartlett et al. 2008).

3.2.3.5 Respiratory Chain

Bacterial respiratory chains can be quite diverse and adaptable to environmental conditions. It has been proposed that two types of electron transport systems are present in the inner membrane of the piezophilic deep-sea bacterium *S. benthica* DB172F, depending on the growth pressure (Kato and Qureshi 1999). At atmospheric pressure complex I (NADH-dehydrogenase) oxidizes $NADH_2$ to NAD and two electrons are transferred to quinone Q, which is then reduced to quinol QH_2. Complex III (cytochrome bc_1-complex) then transfers the two electrons from quinol to the membrane-bound cytochrome *c*-551. The electrons are then passed onto the soluble cytochrome *c*-552, which transfers them to complex IV (terminal cytochrome *c* oxidase). The terminal oxidase reduces oxygen to water and pumps protons into the periplasmic space. Protons are also pumped by the bc_1-complex. The proton flow back into the cytoplasm enables the ATP synthase to produce ATP. At 60 MPa, the respiratory chain is more compact and electrons from quinol are passed onto the terminal *ccb*-type quinol oxidase, which reduces the oxygen supplied by the membrane bound cytochrome *c*-551 and pumps protons into the periplasmic space (Kato and Qureshi 1999; Qureshi et al. 1998a). The soluble cytochrome *c*-552 is not produced at high pressure (Qureshi et al. 1998b).

S. violacea DSS12 has also been proposed to utilise different electron transport systems depending on the growth conditions (Chikuma et al. 2007). The respiratory system of *S. violacea* DSS12 is branched, with 60% activity depending on the cytochrome bc_1-complex and 40% being independent, regardless of pressure. There are two types of soluble cytochrome *c* present in *S. violacea* DSS12, taking part in the bc_1-complex dependent electron transfer (Yamada et al. 2000). Cytochrome c_A (belonging to group c_5) is constitutively expressed, regardless of pressure, and c_B (group c_4) is repressed at high pressure. There are three terminal cytochrome *c* oxidases (one of cbb_3-type) and two quinol oxidases, *bo*- and *bd*-type in the *S. violacea* DSS12 genome (Ohke et al. 2013). The expression of *bd*-type quinol oxidase genes was unaffected at low oxygen and high pressure conditions, while the expression of all other four terminal oxidases genes decreased. However, the expression of genes encoding a glutathione transporter required for the assembly of the *bd*-type quinol oxidase, *cydC* and *cydD*, was up-regulated at high pressure (Ohke et al. 2013; Tamegai et al. 2005). It has been suggested that the *bd*-type quinol oxidase might be dominant at high pressure, but the cytochrome *c* oxidase also contributes to the respiration process. The respiratory terminal oxidase activity of the membrane of *S. violacea* DSS12 grown under high pressure showed piezotolerance in comparison with that of cells grown at atmospheric pressure (Ohke et al. 2013). In conclusion, *S. violacea* DSS12 has been shown to adapt its respiration system to environmental conditions.

Genomic analysis revealed that *P. profundum* SS9 encodes three putative gene sets for cytochrome *c* oxidases (including a cbb_3-type), one set for quinol oxidase and one for glutathione transporter, one gene for cytochrome c_5 and one for c_4 (Tamegai et al. 2012). The total amount of cytochromes decreased when

P. profundum SS9 cells were grown microaerobically but was not influenced by increased pressure. The expression of representative genes from each set of cytochromes was analysed by RT-PCR and shown not to be altered by increased pressure. Increased aeration elevated only expression of *pbpra0168*, encoding cytochrome *c* oxidase subunit. RT-PCR showed that the expression of ccb_3-type cytochrome *c* oxidase subunit I was not affected by pressure. However, proteomics analysis identified the putative CcoP subunit of the same oxidase as up-regulated at 28 MPa (Le Bihan et al. 2013). These contradictory results could stem from discrepancies between the mRNA levels and protein abundance. Cytochrome c oxidase ccb_3-type has a reduced proton pumping ability, but higher catalytic activity at low oxygen concentration, which supports an enhanced requirement for this protein in low oxygen environments, such as deep-sea (Buschmann et al. 2010). However, *P. profundum* SS9 can grow by both respiration and fermentation, and most likely uses the latter when oxygen is limited (Nogi et al. 1998a). The piezotolerance of the terminal oxidase activity of the membrane of aerobically-grown *P. profundum* SS9 at optimal temperature was lower than that of *S. violacea* DSS12 and increased when *P. profundum* SS9 was grown at high pressure (Tamegai et al. 2012). It seems that the pressure adaptation of the respiratory system in *P. profundum* SS9 is different than in *S. violacea* DSS12. It has been suggested that the activity of terminal oxidases could be affected by membrane lipid composition or other genes up-regulated at high pressure.

Possibly due to insufficient functioning of the membrane-based cytochrome respiratory system at 28 MPa, both the Stickland reaction (a pathway previously found only in anaerobic bacteria, responsible for amino acid fermentation using an amino acid reductase containing selenocysteine) and the TMAO (trimethylamine-*N*-oxide) reductase respiratory systems were up-regulated at 28 MPa (Le Bihan et al. 2013; Vezzi et al. 2005). Additionally, nitrate reductase and cytochrome c552, involved in the anaerobic respiration pathway were also found to be up-regulated at high pressure in a proteomic study (Le Bihan et al. 2013). In contrast, several proteins involved in the oxidative phosphorylation pathway, typical of aerobic respiration were up-regulated at low pressure. These included NADH dehydrogenase, cytochrome *d* ubiquinol oxidase subunit I and subunits of the F_0F_1 ATP synthase. Combined, these results suggest that pressure may regulate two different modes of respiration in *P. profundum* SS9, with aerobic respiration being up-regulated at atmospheric pressure and anaerobic respiration and fermentation being up-regulated at high pressure.

Bacterial membrane-bound F_0F_1 ATP synthases catalyse ATP synthesis from ADP and inorganic phosphate using the proton motive force or, in a few species, sodium motive force (Deckers-Hebestreit and Altendorf 1996). In anaerobic conditions they function as ATPases, generating a transmembrane ion gradient at the expense of ATP hydrolysis. The expression of genes encoding for F_0F_1 ATP synthase localised on the *P. profundum* SS9 chromosome II was higher at 0.1 MPa than 28 MPa (Campanaro et al. 2012). In contrast, expression of the chromosome I ATP synthase was not influenced by pressure and its absolute expression level was very high with respect to the chromosome II ATP synthase. The F_0F_1 ATP synthase

is duplicated in a small number of *Vibrionaceae* species and the two copies are different. It is possible that they play distinct roles in *P. profundum* SS9 adaptation to high pressure. The chromosome II ATP synthase possibly compensates for the reduction of functionality of the main chromosome I ATP synthase at suboptimal environmental conditions.

To summarise, high pressure and low temperature affect all elements of the cell envelope of deep-sea bacteria. The chemical structures of phospholipids and lipopolysaccharides, fundamental components of membranes, need to be modified in order to maintain membrane fluidity at high pressure and low temperature and enable membrane-localised proteins to perform their functions. Proteins present in the cell envelope allow for nutrient diffusion and active transport, respiration and take part in sensing and signalling. All of these processes are indispensable for the survival of bacterial cells and also subject to pressure and temperature adaptation.

3.3 The Role of Flagella in High Pressure- and Low Temperature-Adapted Growth of Deep-Sea Bacteria

Motility is a very important process for bacterial survival as it allows cells to escape unfavourable conditions and move towards environments abundant in nutrients. The locomotion is possible due to flagella, which are embedded in the cell envelope and extend into the extracellular space (Erhardt et al. 2010; Schuhmacher et al. 2015). Flagella of different bacterial species have a similar structure that can be divided into three parts: basal body, hook and filament. The basal body consists of the cytoplasmic C-ring, structures embedded in the membranes and the rod. The flagellum is built of approximately 25 different types of proteins and the type III secretion system is used to assemble the rod, hook and filament. The filament is constructed from 20,000 to 30,000 flagellin subunits, which are added at its distal end (Erhardt et al. 2010; Schuhmacher et al. 2015). Flagellar synthesis is complicated and requires many genes that are organised in a hierarchical manner with early, middle and late genes temporally expressed under the control of specific sigma factors and transcriptional regulators (Merino et al. 2006).

The flagellum can fulfil its role in propelling the bacterium through the rotation of its filament due to the activity of the motor embedded in the membrane. Motor-generated rotation is passed onto the rod and hook and then the filament which results in cell movement (Schuhmacher et al. 2015). The energy for that rotation is derived from the transport of protons or sodium ions. Most flagellar motors can rotate clockwise (CW) and counterclockwise (CCW). In species with peritrichous flagella (multiple flagella projecting in all directions), such as *E. coli*, the CCW movement of filaments allows them to bundle and propel the cell (Erhardt et al. 2010). When a bacterium encounters unfavourable conditions the signal from the chemosensory pathway is relayed to the motor and in response, the motor changes its rotation to CW. This causes the bundle to fall apart and as a result, the cell tumbles and changes the swimming direction. In *Vibrio alginolyticus*, which possesses a

single polar flagellum, the CCW rotation of the motor drives the cell forward and the CW rotation pulls it backwards (Xie et al. 2011). When the cell resumes forward swimming, it reorients itself and the new swimming direction is chosen at random. Depending on the environment bacteria are found in, they can either display swimming or swarming. Both movements are powered by rotating flagella, but swimming occurs as individual cells move in a liquid environment, while swarming is defined as rapid multicellular movement across surfaces (Kearns 2010).

Motility could be the most pressure-sensitive process in bacteria. Swimming of *E. coli* cells is affected by pressures lower than 10 MPa (Bartlett 2002; Meganathan and Marquis 1973). Taking into account that the inhibition of cell growth and protein synthesis happens at higher pressure (50–200 MPa), it has been suggested that the flagellum assembly process is more sensitive to high pressure compared to the synthesis of the flagellum components (Abe 2007; Bartlett 2002; Meganathan and Marquis 1973). Hence, deep-sea bacteria must possess specific adaptations that allow them to swim at high pressure. The bacterial motility is not only affected by high pressure, but also changes with temperature. The rotational speed of the sodium driven flagellum motor was found to decrease with a drop in temperature (Baker et al. 2011; Yuan and Berg 2010).

The flagellum propels the cell, but also plays the role of a mechanosensor, allowing for adhesion to surfaces and formation of a biofilm, which in pathogens can contribute to virulence (Belas 2014; Merino et al. 2006). In *Vibrio parahaemolyticus* surface sensing occurs via the polar flagellum. There are less than 70 surface-responsive genes and most of them are positively regulated (Gode-Potratz et al. 2011). Almost two thirds are involved in swarming motility, several in sensing and/or transducing signals (encoding chemotaxis receptor proteins, enzymes modulating levels of secondary messengers) and some in colonisation and virulence (encoding chitin-binding proteins, type III secretion system components, proteases, collagenases, etc.). Thus, surface sensing in *V. parahaemolyticus* induces swarming but also prepares cells for the changed environmental conditions and potential encounter with a host. In the deep-sea environment, where nutrients are limited, the ability to adhere to particles or surfaces of animals may provide a more sustainable source of organic matter for bacteria.

The flagella have been studied in more detail in two piezophiles, *P. profundum* SS9 and *S. piezotolerans* WP3. Both species contain two flagellar systems, polar (PF) and lateral (LF). It is noteworthy that the LF cluster is present in the *P. profundum* SS9 and the other pressure-loving isolate, DSJ4, but missing in the piezosensitive *P. profundum* 3TCK strain (Campanaro et al. 2005). The lateral flagellum is a very complicated structure, encoded by almost 40 genes with considerably higher GC content, which suggests they were horizontally acquired (Campanaro et al. 2005; Eloe et al. 2008). The polar and lateral flagellum gene clusters of *P. profundum* SS9 and *S. piezotolerans* WP3 are very similar to the flagellum clusters found in *V. parahaemolyticus* BB22. Both *P. profundum* SS9 and *V. parahaemolyticus* strains possess two kinds of motors for the propulsion of flagella: sodium-driven for the polar and proton-driven for the lateral flagellum (Eloe et al. 2008). The LF proton-driven system was also identified in

S. piezotolerans WP3, while the polar flagellum gene cluster does not seem to contain motor protein genes (Wang et al. 2008). Two candidate genes were found away from both flagellar clusters that could be responsible for the movement of the polar or both, polar and lateral flagella. *S. piezotolerans* WP3 contains two flagellin genes (Wu et al. 2011) and *P. profundum* SS9 three. In the latter, *flaA* and *flaC* encode the flagellins of the polar flagellum and *flaB* the lateral (Eloe et al. 2008).

The polar flagellum of *P. profundum* SS9 is required for swimming at high pressure and lack of *flaA* or *flaC* or a component of the sodium-driven motor (*motA2*) leads to an inhibition of motility (Eloe et al. 2008). The lateral flagellum is necessary for swarming and is induced at high pressure and increased viscosity conditions. Mutants Δ*flaB* and Δ*motA1* (component of a proton-driven motor) are non-motile under these conditions. All tested mutants unable to swim, with non-functional polar flagellum (Δ*motA2*) or missing polar flagellum (Δ*flaA* and Δ*flaC*) were also unable to swarm. Moreover, it has been found that expression of the *flaB* gene increases under high pressure and high viscosity conditions in the parent but not in the mutants with inhibited swimming motility. Together, this suggests that the production of lateral flagella is dependent on a functional polar flagellum. The opposite has been observed in *V. parahaemolyticus*, where physical or genetic disruption of the polar flagellum results in induction of lateral flagella (McCarter et al. 1988).

The regulation of the polar and lateral flagella gene cluster in *S. piezotolerans* WP3 is different from *P. profundum* SS9 and does not depend on the pressure and viscosity but on pressure and temperature (Wang et al. 2008). As shown using microarrays and quantitative RT-PCR, the LF genes of *S. piezotolerans* WP3 are up-regulated at 4 °C compared to 20 °C and are slightly repressed by high pressure (20 MPa vs. 0.1 MPa). The genes of PF show the opposite relationship, being up-regulated at high pressure but down-regulated at low temperature. Mutants lacking the polar or lateral flagella displayed decreased swimming motility at 20 °C on 0.3% agar. The swimming motility of PF mutants was not inhibited at 4 °C, while mutants in the lateral flagellum gene cluster displayed no motility and their growth decreased. Hence, it was concluded that the lateral flagellum is required for growth and motility at low temperatures. The swarming motility seems to also be controlled by the temperature-dependent function of LexA, a protein involved in the SOS response (Jian et al. 2015).

The expression of genes belonging to the lateral flagellum gene cluster in *S. piezotolerans* WP3 is not only affected by temperature, but also by the filamentous phage SW1 present in the WP3 strain both on the chromosome and as a plasmid (Jian et al. 2013). The phage is cold-active, but does not influence the growth of the WP3 strain at 4 °C or at optimal 20 °C. The phage has been shown to affect the expression of 49 genes at 4 °C, among which 16 belonged to the lateral flagellum gene cluster. The phage has no effect on swimming motility but inhibits swarming motility at 4 °C. It is still unclear how the phage affects the lateral flagellum. SW1 regulates a variety of host's genes and clearly has an evolutionary advantage since it has not been lost. Both phage production and motility are energy consuming. 2% of total energy produced by a cell is necessary for flagellum

synthesis and functioning (Soutourina and Bertin 2003). At low temperature *S. piezotolerans* WP3 must balance the energy spent on motility and on production of the phage, which inhibits motility.

The swimming velocity of *P. profundum* strains was measured by Eloe and colleagues using a high-pressure microscopic chamber (Eloe et al. 2008). Not surprisingly, 3TCK and SS9 displayed highest swimming velocity at their optimal pressures for growth, 0.1 and 30 MPa, respectively. *P. profundum* SS9 increased the swimming velocity up to 30 MPa and was able to swim up to the maximum measured pressure of 150 MPa. On the other hand, increased pressure resulted in gradual reduction of the swimming velocity of the piezosensitive 3TCK up to 120 MPa. In comparison, the swimming velocity of *E. coli* immediately decreased at elevated pressure and was completely abolished at 50 MPa.

Defects in motility can affect cell growth and survival under some conditions, as shown by the phenotype of a cold-sensitive transposon mutant with insertion in the polar flagellum gene cluster (Lauro et al. 2008). The gene disrupted in the *P. profundum* SS9 FL24 mutant encodes a putative FliS protein (Lauro et al. 2008). FliS prevents the premature folding or inappropriate association of newly synthesised flagellin subunits and postpones their filamentation until they are translocated through the narrow, hollow core of the growing flagellum (Muskotal et al. 2006). FliS is the most widely conserved flagellar chaperone in bacteria. The gene disrupted in the FL24 mutant encodes a protein which is 77% identical (91% similar) to *V. cholerae* FliS (GenBank accession no. YP_129130.1 and NP_231769.1). The absence of a putative FliS protein in *P. profundum* SS9 results in a cold-sensitive phenotype, but only when the mutant strain is grown on agar (Lauro et al. 2008). The connection between surface-growth and the flagellum has been studied before in *Vibrio* species. As mentioned previously, in *V. parahaemolyticus* the polar flagellum acts as a surface sensor and induces the expression of genes encoding proteins required for swarming motility, virulence factors and sensory enzymes (Gode-Potratz et al. 2011). The role of the FliS protein has not been studied before in the context of surface sensing or adaptation to cold temperature.

Dr. Gail P. Ferguson's group found that the FL24 mutant is non-motile at high pressure (Fig. 3.2a) (Myka 2013). As expected, the FL24 mutant lacked the polar flagellum when analysed using fluorescence microscopy (Emiley Eloe, in Myka 2013). Plasmid-encoded *fliS* restored the swimming motility of the FL24 mutant (Fig. 3.2b), but the complementation of the cold sensitivity was unsuccessful (Myka 2013). The presence of the plasmid conferring resistance to streptomycin masked the cold-sensitive phenotype of FL24, similarly to the situation with the LPS mutant FL26 described above and as observed by Chi and Bartlett (Chi and Bartlett 1995). Since the *P. profundum* SS9 Δ*flaA* mutant (EAE1) lacking the polar flagellum (Eloe et al. 2008) did not display cold-sensitive phenotype on marine agar (Fig. 3.2c), we inferred that merely the absence of the polar flagellum in FL24 could not account for its cold-sensitive colony growth (Myka 2013). We hypothesise that the cold-sensitive phenotype could be caused by the intracellular accumulation of flagellin in the absence of FliS chaperone. Further experiments will be needed to assess the basis of FL24 cold sensitivity.

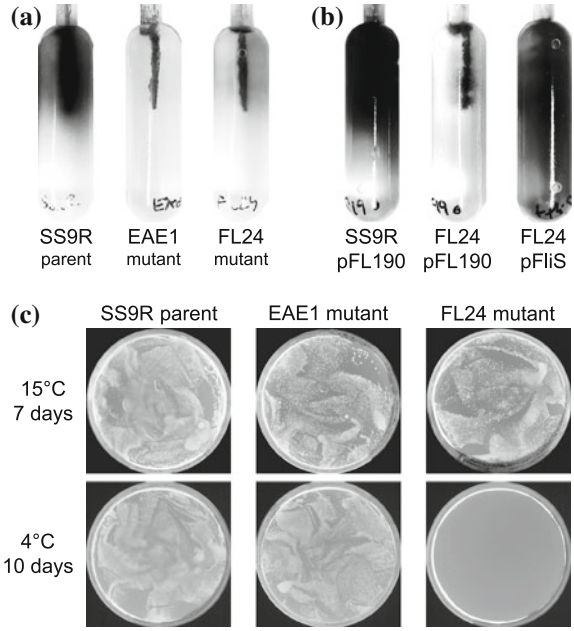

Fig. 3.2 The cold sensitivity of the *P. profundum* SS9 putative *fliS* mutant (FL24) does not result from the lack of polar flagellum. **a** The FL24 mutant displays a non-motile phenotype. The parent strain SS9R and the non-motile EAE1 mutant, lacking the FlaA flagellin (Eloe et al. 2008) were used as controls. Late exponential phase cultures (OD600 = 0.6–0.8) grown anaerobically at 15 °C, 0.1 MPa were inoculated in a straight line using a thin metal rod into the plastic Pasteur pipette bulb containing marine 0.3% agar with 0.05 mg ml^{-1} tetrazolium violet. Bulbs were incubated at 28 MPa for 48 h. **b** Plasmid-encoded putative FliS chaperone restores swimming motility of the FL24 mutant. Strains containing plasmids were pre-grown aerobically at 15 °C, 0.1 MPa and 100 μg ml^{-1} streptomycin and 1% (w/v) arabinose (for the expression of plasmid-encoded gene) were added to the media throughout the experiment. The strains containing plasmids grow slower, hence the incubation time at 28 MPa was extended to 72 h. The *P. profundum* SS9 *pbpra0917* (*fliS*) gene was amplified using primers 5-GTAGAATTCG CTCGATGCAGGCTTATAAT-3 and 5-TGCTCTAGAGATTATCTCTCGCTACACACCA-3 and cloned into pFL190 (Lauro et al. 2005) using EcoRI and XbaI to produce pPPfliS. **c** 100 μl of an early stationary phase culture adjusted to an $OD_{600} \sim 0.2$ was spread on marine agar plates. Growth was assessed at 15 and 4 °C after 7 and 10 days, respectively

To conclude, motility is extremely important for bacteria as it allows them to find a better-suited nutrient-abundant environment and escape unfavourable conditions. In mesophilic bacteria, motility is one of the processes most sensitive to increased pressure. This suggests that the motility systems of deep-sea bacteria must be specifically adapted to high pressure and low temperature. Indeed, the pressure optima for *P. profundum* SS9 and 3TCK swimming matched those for growth (Eloe et al. 2008). Interestingly, both strains were also capable of short-term swimming under pressures higher than the known growth limit of microbial life. The induction of lateral flagella required for swarming at high pressure or low temperature

(*P. profundum* SS9 and *S. piezotolerans* WP, respectively) suggests that for deep-sea marine bacteria planktonic swimming might not be the best survival strategy. Association with particles or surfaces of animals could provide a more stable nutrient source. Future studies investigating the precise role of proteins involved in polar and lateral flagella assembly are likely to reveal temperature- and pressure-sensitive assembly steps.

3.4 Conclusions

This chapter focused on the adaptations of the cell envelope and flagella of deep-sea bacteria to high pressure and low temperature. Psychropiezophiles adjust their fatty acid content to optimize membrane fluidity and many of their membrane proteins involved in nutrient acquisition, transport, respiration, sensing and signalling are specifically adapted to function at high pressure and low temperature. Deep-sea bacteria swim at elevated pressures (even up to 150 MPa), which suggests that their motility systems differ from mesophilic bacteria, since the motility of mesophiles is affected even by 10 MPa. Moreover, some deep-sea bacteria produce a second type of flagella, lateral flagella, in response to increased pressure or low temperature.

The findings presented in this chapter rely on a plethora of techniques and analyses applied to whole microbial communities, single species as well as particular genes and proteins. The use of metagenomics can shed light on the deep-sea microbial communities regardless of the ability to grow the organisms in the laboratory. However, future research into cultivation approaches will be highly beneficial to our understanding of the bacteria found in the oceans' depths. In order to fully understand the adaptations of bacteria to the deep-sea environment we need to obtain a more phylogenetically-diverse set of reference piezophiles, since all species discussed here belong to Gram-negative *Gammaproteobacteria*, mostly to the genera *Photobacterium* and *Shewanella*. Whole-genome sequencing and comparative genomics of piezophilic and piezosensitive isolates of the same bacterial species can better pinpoint genes likely to be involved in the high pressure adaptation (Campanaro et al. 2005), which can be further analysed using molecular genetics. The analysis of the bacterial transcriptome in response to different growth conditions (Vezzi et al. 2005) and the comparison of genes expressed in the parent strain and a particular mutant are also invaluable, especially when complemented by the quantitative proteomics studies (Campanaro et al. 2012; Le Bihan et al. 2013). The development of genetic tools for large scale mutagenesis, gene deletion, recombineering and protein expression in psychropiezophiles is important for the research aiming to understand the effects of pressure and temperature on biological systems, but also for possible biotechnological applications. Similarly, the biotechnology sector could greatly benefit from biophysical and biochemical characterisation of structure and function of high pressure-adapted proteins. Deep-sea bacteria could be used as a renewable source of fatty acids for the production of biofuels, PUFAs for human consumption, secondary metabolites and

chemical compounds for drug development, and various pressure and temperature adapted enzymes. Research on the adaptations of deep-sea microorganisms to high pressure and low temperature has the potential not only to broaden our understanding of the processes occurring in nature, but also identify targets that could have biotechnological and industrial application.

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