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Dynamic structural changes in microbial membranes in response to high hydrostatic pressure analyzed using time-resolved fluorescence anisotropy measurement

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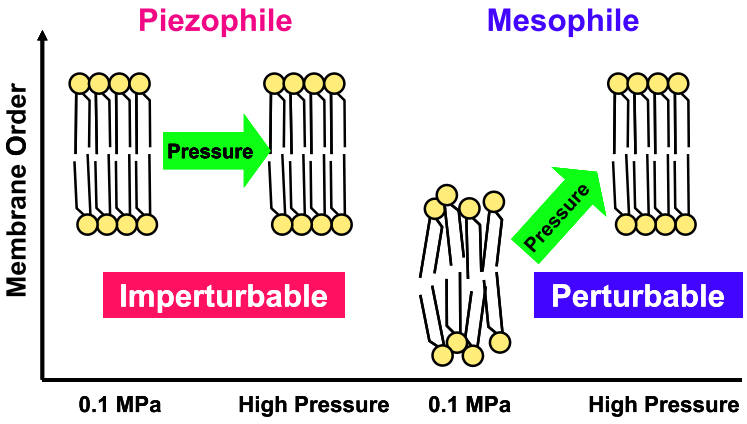
HIGHLIGHTS

- High hydrostatic pressure has a profound physiological impact on lipid membranes.
- Deep-sea organisms possess specialized cell membranes.
- This mini-review focuses on pressure-induced changes in microbial cell membranes.
- High-pressure time-resolved fluorescence anisotropy measurement is highlighted.

GRAPHICAL ABSTRACT



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ABSTRACT

High hydrostatic pressure has a profound physiological impact on lipid membranes, primarily resulting in tighter packing and restriction of acyl-chain motion. To fulfill membrane protein functions in high-pressure environments, deep-sea organisms possess specialized cell membranes. Although the effects of high-pressure on model membranes have been investigated in great detail, high-pressure-induced structural changes in living cell membranes remain to be elucidated. Of the spectroscopic techniques available to date, fluorescence anisotropy measurement is a common useful method that provides information on dynamic membrane properties. This mini-review focuses on pressure-induced changes in natural cell membranes, analyzed by means of high-pressure time-resolved fluorescence anisotropy measurement (HP-TRFAM). Specifically, the role of eicosapentaenoic acid in deep-sea piezophiles is described in terms of the structural integrity of the membrane under high pressure.

Abbreviations: HP-TRFAM, high-pressure time-resolved fluorescence anisotropy measurement; TCSPC, time-correlated single-photon counting; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-[4-(trimethylamino)pheny]-6-phenyl-1,3,5-hexatriene; *r*, fluorescence anisotropy; *S*, order parameter; D_w, rotational diffusion coefficient; SOPC, stearoyl-oleyl-phosphatidylcholine; DOPC, dioleyl-phosphatidylcholine; DPPC, dipalmitoyl-phosphatidylcholine; POPC, palmitoyl-oleoyl-phosphatidylcholine; DSPC, distearoyl-phosphatidylcholine; SOPC, stearoyl-oleyl-phosphatidylcholine; SAPC, stearoyl-arachidonoyl-phosphatidylcholine; SDPC, stearoyl-docosahexanoylphosphatidylcholine; PUFA, polyunsaturated fatty acids; EPA, eicosapentaenoic acid (C20:5); DHA, docosahexaenoic acid (C22:6).

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1. Introduction

Maintenance of structural and dynamic properties of cell membranes is required for fundamental physiological functions of organisms. High hydrostatic pressure and low temperature have a profound impact on lipid membranes, primarily resulting in tighter packing and restriction of acyl-chain motion. A number of excellent reviews of high-pressure effects on artificial lipid membranes has been published previously [1–5], and these should be consulted for a detailed biophysical basis of membranes, which will not be covered in this mini-review. Primarily, increasing hydrostatic pressure orders lipid membranes in a manner analogous to decreasing temperature. With increasing pressure, the gel-to-liquid crystalline coexistence region is shifted toward higher temperatures by approximately 22 °C/100 MPa [2]. For example, a pressure increase of 100 MPa increases the main transition (L_β/L_α) temperature of the stearoyl-oleyl-phosphatidylcholine (SOPC) and dioleylphosphatidylcholine (DOPC) membrane by 18.1 °C and 23.3 °C, respectively [6].

In organisms that live at atmospheric pressure, most physiological processes employed by membrane proteins such as respiration, nutrient import, ion flux, and signaling are compromised by high pressure and low temperature, at least in some cases, due to perturbation of the membranes [7–10]. It is commonly believed that deep-sea organisms have developed their membranes and membrane proteins to adapt to such extreme conditions. However, there are still a limited number of examples that have been proven experimentally. In a broad range of organisms, the packing effects of the membrane can be compensated by modifying the fatty acid compositions. Cold adaptation is often associated with the incorporation of unsaturated bonds within the acyl chains [11–13]. Membrane acyl chains containing unsaturated bond(s) assume a more bulky conformation than their saturated counterparts, allowing greater conformational freedom and less packing of the membrane. Consequently, the membrane becomes more fluid. This adaptation, employed by organisms inhabiting cold and high-pressure environments, has been termed "homeoviscous adaptation" [8,14,15]. Whether homeoviscous adaptation generally occurs in deep-sea organisms remains unknown because natural membrane properties in response to high pressure have not been sufficiently analyzed. In addition, the variability and complexity of natural cell membranes make it difficult to characterize how the physicochemical properties of the membranes respond to high-pressure conditions. Membrane fluidity and phase transitions can be analyzed by a variety of methods including differential scanning calorimetry, nuclear magnetic resonance, electron spin resonance, X-ray diffraction and fluorescence anisotropy measurement. Among these spectroscopic techniques, fluorescence anisotropy measurement using rod-like probes whose direction of absorption and emission transition moments coincide with the long molecular axis is a highly sensitive, simple method that provides information on lipid order and rotational motion of acyl chains [16–18]. This mini-review focuses on the effects of high hydrostatic pressure on the dynamic structure of the microbial membrane as analyzed by fluorescence anisotropy measurements.

2. Fluorescence anisotropy measurement of membranes under high pressure

Fluorescence anisotropy measurement has been widely employed for the study of model membranes. The lipophilic fluorescent probes, 1,6-diphenyl-1,3,5-hexatriene (DPH) and its cationic derivative 1-[4-(trimethylamino)pheny]-6-phenyl-1,3,5-hexatriene (TMA-DPH), are commonly used for such analyses. When added to model membranes or natural membranes, DPH primarily distributes perpendicular to the bilayer plane near the center of the membrane, but partially distributes parallel to it within the acyl chain tails [17]. The use of DPH in natural membranes requires special care because it can form granules in some cells even at low concentrations, making interpretation of the results difficult [19]. TMA-DPH is distributed to the lipid–water interface due to its charged moiety and thereby reflects only the interfacial region of the membrane. These probes should not disturb membrane dynamics and hence they are typically incorporated into model lipid bilayers in a molar ratio of 1/1000–1/500, or are used for labeling natural cell membranes at 0.5–5 μM.

Time-resolved fluorescence anisotropy measurement based on time-correlated single-photon counting (TCSPC) provides quantitative information on membrane order and rotational motion of acyl chains in a single measurement. Fluorescence anisotropy is described by Eq. (1), where I_{VV} and I_{VH} are the fluorescence intensities (the two subscripts indicate the orientation of the excitation and emission polarizer, respectively, with H indicating horizontal and *V* indicating vertical) and $G = I_{HV} / I_{HH}$ is the instrumental factor.

$$r(t) = [I_{VV}(t) - G\, I_{VH}(t)]/[I_{VV}(t) + 2G I_{VH}(t)]. \quad (1)$$

The simplest model of the restricted motion of fluorochromes in the membrane, based on the Brownian diffusion of the label in a cone with a wobbling diffusion constant, leads to the following single exponential approximation of the anisotropy decay with time, $r(t)$ [20]:

$$r(t) = (r_0 - r_\infty)\cdot \exp(-t/\theta) + r_\infty \quad (2)$$

where r_∞ stands for limiting anisotropy, and θ (ns) for rotational correlation time. The order parameter (S) is calculated to obtain structural information on the membrane according to the following equation:

$$S = (r_\infty / r_0)^{1/2}. \quad (3)$$

The rotational (wobbling) diffusion coefficient (D_w) is calculated to obtain the dynamic nature of the membrane according to the following equation:

$$D_w = (r_0 - r_\infty)/6\theta r_0. \quad (4)$$

3. Effects of high pressure on model membranes

In the pioneering study by Bernsdorff et al., time-resolved fluorescence anisotropy measurement on model membranes, dipalmitoylphosphatidylcholine (DPPC) and palmitoyl-oleoyl-phosphatidylcholine (POPC), was performed under high pressure using a high-pressure optical chamber [21]. At temperatures where the membranes remain in the liquid-crystalline phase, the order parameter *S* for the TMA-DPH-labeled DPPC and POPC membrane shows a sharp increase at around 75 MPa at 58 °C and 95 MPa at 15 °C, respectively. These increases in *S* correspond to the liquid-crystalline-to-gel phase transition. Addition of 30 mol% cholesterol to the membrane systems results in an increase in *S* and concomitant loss of the pressure-induced sharp phase transition in both DPPC and POPC membranes [21]. This behavior is attributable to the formation of homogeneous liquid ordered phase in the systems.

In our recent study, we also created a system that enabled *h*igh-*p*ressure *t*ime-*r*esolved *f*luorescence *a*nisotropy *m*easurement, namely HP-TRFAM, under high pressure, aimed at understanding the effects of high pressure on the cell membrane in deep-sea piezophiles (referred to hereafter) [22]. The HP-TRFAM system comprises of a high-pressure optical cell, a high-pressure pump, and a TCSPC device (Fig. 1A). The strain birefringence of the quartz windows is simply corrected by determining the r_0 values for DPH in mineral oil under various hydrostatic pressures. This is based on the fact that the r_0 values do not change by applying pressures at the experimental pressure range (<200 MPa). The measured r_0 of DPH is almost unchanged at pressures up to 50 MPa, but the birefringence of the window becomes considerable at pressures greater than 50 MPa (Fig. 1B). Thus, measured r_0 and r_∞ values are multiplied by the correction coefficients to determine the correct r_0 and r_∞ values at each pressure. Fig. 2 shows a typical example

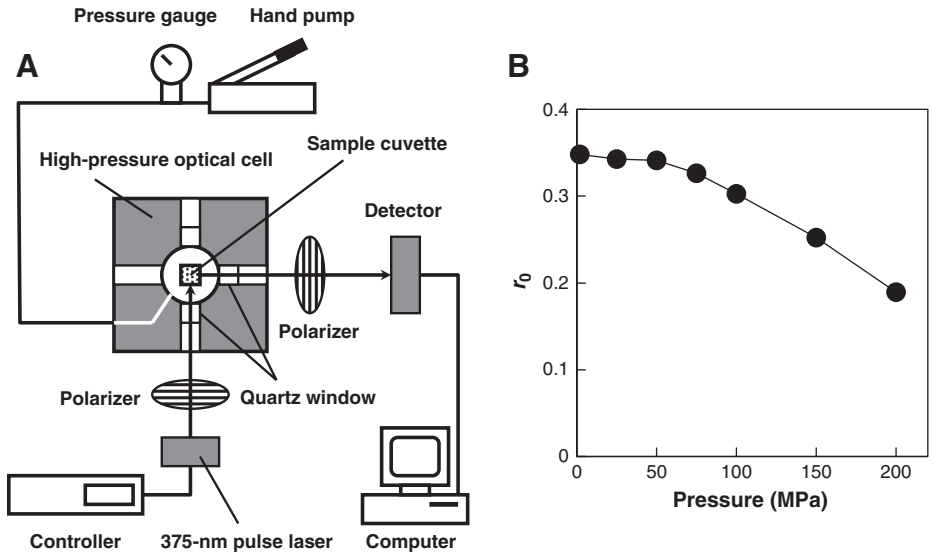

Fig. 1. Experimental design of the HP-TRFAM system. (A) A DPH- or TMA-DPH-labeled sample is placed in a high-pressure optical cell which is mounted in a TCSPC device with polarizers. The sample is excited by a 375-nm laser diode operated with a pulse frequency of 1 MHz. (B) A calibration curve determined by measuring r_0 values for 1 μM DPH in mineral oil under various hydrostatic pressures. The strain birefringence of the quartz windows becomes significant at pressures greater than 50 MPa.
The figure is reproduced from Ref. [22] with permission of the publisher.

of the corrected anisotropy decay of TMA-DPH in the POPC membrane measured at 0.1 MPa and 100 MPa, where the membrane is in the liquid-crystalline and the gel phase, respectively. Fluorescence of TMA-DPH is significantly depolarized at 0.1 MPa while the depolarization is limited at 100 MPa, reflecting the phases of the POPC membranes [22]. Pressure-induced phase transition obtained using the HP-TRFAM system is in agreement with previous spectroscopic and thermodynamic studies [21,23]. HP-TRFAM characterizes the phase transition of the POPC membrane accompanying steep increases in S at the expected hydrostatic pressures at 10 and 20 °C (Fig. 2). The rotational diffusion coefficient D_w for TMA-DPH is greater at 20 °C than at 10 °C and decreases with pressure in concert with the gradual increase in S in the L_α phase. In the L_β phase, at pressures above 75 MPa (10 °C) and 100 MPa (20 °C), an exponential approximation of the anisotropy decay with time fails to determine the rotational correlation time for TMA-DPH motion within the highly ordered POPC gel (Fig. 2).

4. Effects of high pressure on cell membranes

Macdonald et al. characterized the membranes of a number of fish that inhabit a series of water depths using steady-state anisotropy measurement. Anisotropy of DPH in the brain myelin was distinctly lower in deep-sea species, indicating a lower order of the membrane [24]. In liver mitochondria, the ratio of unsaturated fatty acids (UFAs) to saturated fatty acids showed a significant increase with depth of capture, implying the acclimation of fish membranes to habitat pressures by incorporation of unsaturated bonds [8,25].

In our recent study, we analyzed the effect of high pressure on the membrane properties of enteric bacterium *Escherichia coli* using HP-TRFAM [26]. The order parameter S of the TMA-DPH-labeled *E. coli* membrane was much higher at a lower temperature (15 °C) than at a higher temperature (37 °C) (Fig. 3). S linearly increased with increasing pressure at both temperatures. Correspondingly, the rotational diffusion coefficient D_w was much higher at 37 °C than at 15 °C, and it linearly decreased with increasing pressure at both temperatures. The changes in S and D_w in *E. coli* cell membrane with increasing pressure is analogous to those observed in the POPC membrane in the liquid-crystalline phase (Figs. 2 and 3). Based on the wobbling-in-a-cone model, the angle of rotational motion of TMA-DPH is assumed to be reduced by 2.7° with a pressure increase of 50 MPa [26]. This value is comparable to that obtained in the POPC membrane in the L_α phase corresponding to a change in the rotational angle, with a reduction of 2.4° by 50 MPa. Therefore, although the magnitudes of S are mutually different between the *E. coli* cell membrane and the POPC membrane, the degree of change in S equivalently depends on hydrostatic pressure. The same is true of the decrease in D_w upon pressurization. In the *E. coli* cell membrane, a pressure of 50 MPa reduces the rotational motion of TMA-DPH in the *E. coli* cell membrane and the POPC membrane to 69.4% and 68.4%, respectively, to the values obtained at 0.1 MPa (Figs. 2 and 3) [26]. In this regard, the pressure-dependence of the dynamic behavior is similar between the two entirely different membranes as far as the POPC membrane is in the liquid-crystalline phase.

High hydrostatic pressures greater than 200 MPa have been used for sterilizing microorganisms for preserving food stuffs as a non-thermal processing alternative [27–29]. Exponentially growing *E. coli* cells are much more pressure-sensitive than stationary-phase *E. coli*, and the loss of viability is accompanied by irreversible disruption of the cell membrane, as measured by uptake of propidium iodide and loss of osmotic response [30,31]. Casadei et al. showed that the heating profiles of differential scanning calorimetry for lipids extracted from *E. coli* cells clearly correlated with membrane fluidity index, expressed by the ratio of (unsaturated fatty acids + cyclopropane fatty acid)/saturated fatty acid, and that cells with fluid cell membranes are more resistant to high pressures of 200–400 MPa [32]. Contrary to this observation, Ulmer et al. demonstrated that *Lactobacillus plantarum* living cells with a liquid-crystalline (fluid) membrane are more sensitive to a pressure of 200 MPa than those with a gel-phase membrane, in terms of reduction in HorA multidrug resistant transporter activity and the loss of viability [33]. In their analysis, the phase state of the membrane was determined by means of Fourier transform-infrared spectroscopy and Laudan fluorescence spectroscopy. The authors showed that irreversible pressure denaturation of HorA is faster if pressure is applied to proteins embedded in a liquid-crystalline membrane compared to denaturation in a gel-phase membrane. This is in agreement with our results showing that the cell membrane of deep-sea piezophiles is rigid and imperturbable to high pressure [22] (see below). Accordingly, how the membrane phase or fluidity affects membrane protein functions and cell viability may differ with respect to different organisms and experimental systems, and hence there is no straightforward direction.

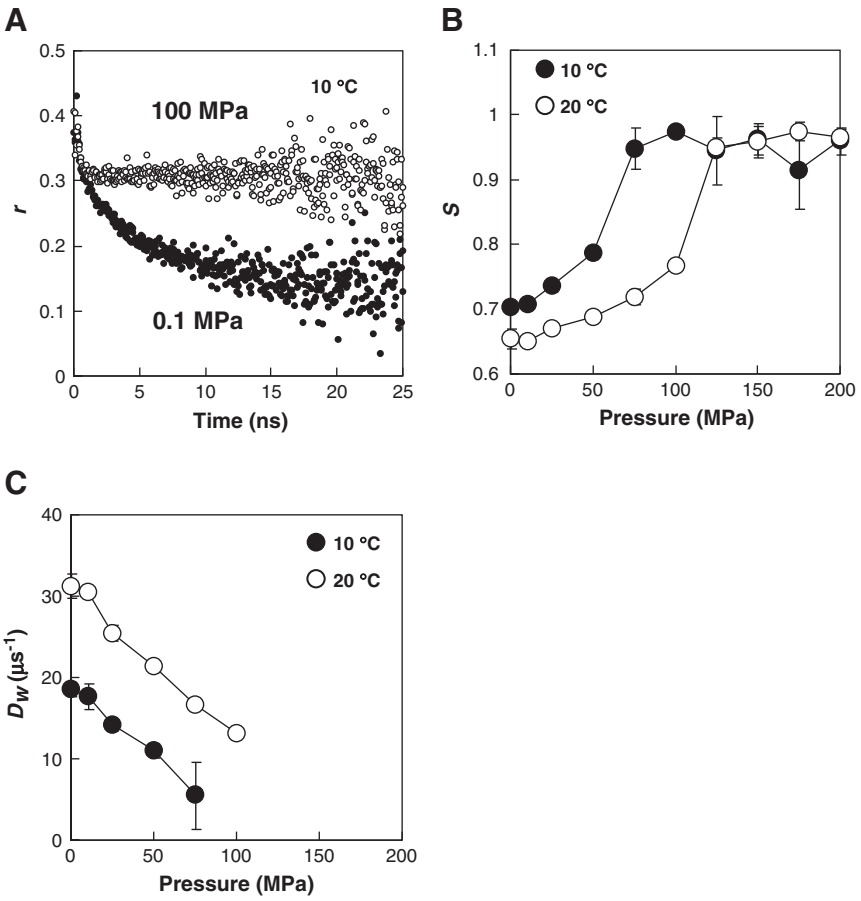

Fig. 2. Phase behavior of the POPC membrane analyzed using HP-TRFAM. The POPC membrane was labeled with TMA-DPH. (A) Anisotropy decays of TMA-DPH in the POPC membrane at 10 °C under pressure of 0.1 MPa and 100 MPa, where the membrane is in the L_α (liquid crystalline) and $L_{\beta'}$ (gel) phase, respectively. (B) Changes in the order parameter *S* as a function of pressure. (C) Changes in the rotational diffusion coefficient D_w as a function of pressure.
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5. Importance of PUFAs in deep-sea piezophiles

Trevors et al. have extensively investigated structural and compositional changes that occur in bacterial membranes exposed to varied environmental factors including temperature, ions, pH, and chemicals, and demonstrated various adaptive strategies employed by bacterial membranes [34,35]. In response to these perturbations, bacterial cells potentially alter their lipid acyl chain structure by changing the ratio of (i) saturation to unsaturation, (ii) *cis* to *trans* unsaturation, (iii) branched to unbranched structure, and (iv) acyl chain length. These alterations occur simultaneously in membranes to adjust the properties to fulfill physiological functions in hostile environments. When cells of *E. coli* and psychrophilic *Vibrio* sp. strain ABE-1 are grown at higher temperatures, membrane fluidity remained constant by altering lipid composition [14,36]. However, lipid composition is not always altered with environmental changes. For example, *Yersinia enterocolitica* can change

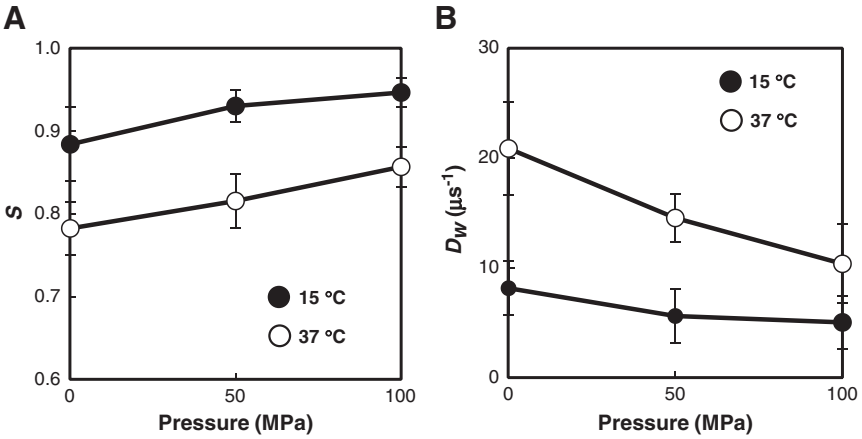

Fig. 3. Effects of high pressure on the dynamic structure of the *Escherichia coli* cell membrane. Cells were grown at 0.1 MPa and 37 °C, labeled with TMA-DPH, and analyzed using HP-TRFAM. (A) Changes in the order parameter *S* as a function of pressure. (B) Changes in the rotational diffusion coefficient D_w as a function of pressure.
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lipid properties independently of alteration in acyl chain composition [37].

Many deep-sea piezophiles contain high proportions of UFAs, particularly polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6), in their membrane lipids, which in some cases increase with increasing growth pressure [38–41]. Because of their extremely low melting temperatures, the incorporation of PUFAs is thought to significantly promote membrane fluidity. For example, the main transition temperatures of the distearoylphosphatidylcholine (DSPC), stearoyl-oleyl-phosphatidylcholine (SOPC), and stearoyl-arachidonoyl-phosphatidylcholine (SAPC) membranes are 55.6 °C, 6.7 °C, and −13.0 °C, respectively [23]. According to the thermodynamic properties of phase transitions, the volume change (ΔV) associated with the gel-to-liquid crystalline phase is 31.6 $ml \cdot mol^{-1}$, 18.9 $ml \cdot mol^{-1}$, and 10.1 $ml \cdot mol^{-1}$ in the DSPC, SOPC, and SAPC membrane, respectively [23]. The clear difference in the ΔV can be explained on the basis of the large free volumes of UFAs in the gel phase without any significant difference in their volumes in the liquid crystalline phase. In this sense, incorporation of unsaturated bonds is not always biologically relevant because natural membranes are primarily in the liquid-crystalline-like phase. In the stearoyldocosahexanoylphosphatidylcholine (SDPC) membrane, the polyunsaturated fatty acyl chains assume a helical configuration, which reduces the effective chain length and stabilizes the gel phase [42]. In addition, natural membranes are complex mixtures of various lipid species and membrane proteins, and hence the role of unsaturation of acyl chains in deep-sea organisms cannot be completely understood.

Photobacterium profundum strain SS9 is a piezophile that optimally grows at 28 MPa and 9 °C. It contains EPA at a proportion of 11% when cultured under the condition. The lack of EPA does not affect the growth of this bacterium under high pressure and low temperature, whereas the lack of *cis*-vaccenic acid (C18:1) significantly diminishes growth under the same conditions [39]. Accordingly, monounsaturated fatty acids but not PUFAs are required for growth of *P. profundum* under high pressure and low temperature. In cold-adapted bacterium *Shewanella livingstonensis* Ac10 isolated from Antarctic seawater, EPA plays a specific role in fulfilling appropriate cell division at low temperatures [43]. However, EPA may not be required for membrane fluidity, based on the finding that the loss of EPA does not affect the diffusion rate of a small lipophilic molecule, pyrene, in the membrane. In *Shewanella piezotolerans* WP3 isolated from the western Pacific Ocean, the loss of EPA results in growth defects at low temperature (4 °C, 0.1 MPa) and high pressure (20 °C, 20 MPa), indicating the requirement of EPA for growth under these conditions. *Shewanella violacea* strain DSS12 is a deep-sea bacterium isolated from the Ryukyu Trench at a depth of 5110 m and exhibits piezophily with optimal growth at 30 MPa and 8 °C [44]. In this bacterium, EPA also plays a role in appropriate cell division under high pressure [45].

6. The role of EPA in membrane integrity in deep-sea piezophiles

In our recent study using the HP-TRFAM system, we showed that the *S. violacea* membrane was extremely rigid ($S = 0.9$) and the rotational acyl chain motion was highly restricted ($D_w = 5.4\ \mu s^{-1}$) even at 0.1 MPa and 10 °C (Fig. 4) [22,26]. Importantly, *S. violacea* membrane is imperturbable over a wide range of hydrostatic pressures with respect to small effects on S and D_w. This is in sharp contrast with *E. coli* membrane, of which S and D_w are markedly influenced by increasing pressure (Fig. 3). The loss of EPA mandated a compensatory increase in palmitoleic acid (C16:1) by two-fold in the *S. violacea* cell membrane [22]. Analogous to the POPC and *E. coli* cell membrane, *S. violacea* cell membrane lacking EPA showed a lower S and a higher D_w compared with the membrane containing EPA, possibly occupying more spaces within the lipid bilayer (Fig. 4). Consequently, the EPA-less membrane undergoes considerable packing with increasing pressure. In this situation, membrane-embedded proteins in the EPA-less membrane could be more perturbed by high pressure due to the changes in membrane structure. In this analogy, high-pressure sensitivity of *E. coli* could be in part explained by enhanced packing of the membrane and reduced acyl-chain motion under high pressure that could result in impairment in membrane protein functions. Some microorganisms would increase membrane fluidity to survive in cold, high-pressure environments. In our scheme, however, maintaining membrane physical properties at a certain level, even highly rigid, under a wide range of pressures could be viewed as another way of adaptation to high-pressure environments possibly allowing the deep-sea piezophiles to stand against pressure fluctuations in vertical currents in deep sea. This is in accordance with the above-mentioned observation in *L. plantarum* that the HorA multidrug resistant transporter is more resistant to high pressure when the membrane is in the gel phase [33]. High pressure is assumed to weaken protein–lipid interactions, induce oligomer dissociation, and, in some cases, release membrane proteins. There are a number of excellent reviews that may be consulted for greater perspective [2–4]. Currently, not many membrane-embedded proteins have been investigated, with respect to the role of EPA in their functions, but it can be readily understood that high pressure differentially influences membrane proteins by

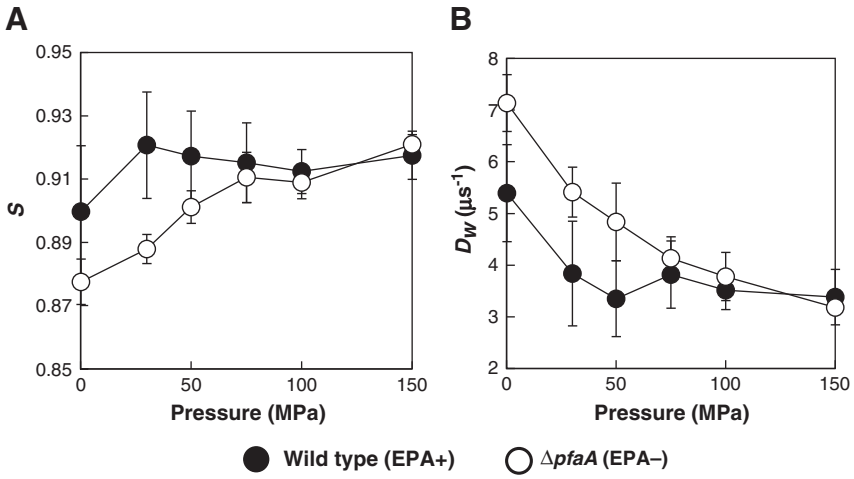

Fig. 4. Effects of high pressure on the dynamic structure of the membrane in deep-sea piezophile *Shewanella violacea*. Cells of the wild-type strain and EPA-less (Δ*pfaA*) mutant were grown at 0.1 MPa and 10 °C, labeled with TMA-DPH, and analyzed using HP-TRFAM. (A) Changes in the order parameter S as a function of pressure. (B) Changes in the rotational diffusion coefficient D_w as a function of pressure.
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changing the dynamic membrane structures and phase behavior. Although many issues remain to be elucidated, the approaches by means of HP-TRFAM may offer clues linking the structural aspects of membranes and membrane protein functions in a quantitative manner.

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