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Cold shock in *Bacillus subtilis*: different effects of benzyl alcohol and ethanol on the membrane organisation and cell adaptation

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Abstract

A temperature shift-down of *Bacillus subtilis* from 40 to 20°C induces an 80 min growth lag. Benzyl alcohol reduced this period to 51 min, whereas ethanol prolonged it up to 102 min. The effect of the two alcohols on the membrane state was investigated by measuring the steady-state fluorescence anisotropy and analysing the lifetime distribution of diphenylhexatriene (DPH) and its polar derivative, TMA-DPH. As followed from the fluorescence anisotropy, the two alcohols exerted similar (fluidising) effects on the cytoplasmic membranes of *B. subtilis*. However, benzyl alcohol significantly shortened the main DPH lifetime component and widened its distribution, while ethanol had no effect. The benzyl alcohol activity was interpreted in terms of an increased membrane hydration due to disordering of the membrane structure. Such an effect imitates the cold shock induced synthesis of unsaturated fatty acids in *B. subtilis*. The fatty acid analysis revealed that ethanol hindered this adaptive synthesis of fatty acids. At the same time, its effect on the membrane state (membrane order) was very low and could not substitute the physiological response as was the case with benzyl alcohol. It can thus be concluded that the adaptation of the membrane physical state contributes significantly to the cold shock response of *B. subtilis*.

Keywords: Bacillus; Cold shock; Ethanol; Benzyl alcohol; DPH; Fluorescence lifetime

1. Introduction

Bacteria have developed various adaptive mechanisms to survive within a wide range of growth temperatures. In the bacterial cell, an abrupt decrease in the temperature (a temperature shift-down) induces a cold shock response characterised by a co-ordinate expression of a specific gene subset. The products of these stress-induced genes protect the cell structures, such as DNA, the membrane or ribosomes, against dysfunction induced by the low temperature and maintain the efficiency of the metabolism at an optimum level [1].

The physiology of bacteria is severely affected by the cold shock [2]. In *Bacillus subtilis* transferred from 40 to 20°C, an 80-min growth lag is induced as a consequence of inhibition of RNA, DNA and of the protein synthesis [3]. These processes are gradu-

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene; T_c20 or T_c40, temperature of cultivation of 20 or 40°C, respectively; r_{ss}, steady-state fluorescence anisotropy; W, width at half-height of the Lorentzian lifetime distribution; τ, centre of the Lorentzian lifetime distribution; f, fractional intensity of fluorescence; χ^2_R, reduced χ^2

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ally recovered attaining a new metabolic state equilibrium which corresponds to the low temperature. An important part of the cold adaptation mechanism occurs at the level of the cytoplasmic membrane. During the cold shock-induced growth lag, the spectrum of the fatty acids and lipid polar head groups varies continuously. Within 20 min since the onset of the cold shock, the major branched fatty acids (over 75% of the total fatty acids determined at 40°C) are replaced by straight-chain acids. Moreover, the synthesis of low melting, unsaturated fatty acids is induced, and the content of these acids attains a value of 17% of the total [4–6]. Furthermore, the ratio of phosphatidylethanolamine/phosphatidylserine increases from 3.5 to 6.0 after 30 min [4]. We have demonstrated that a biophysical parallel to these changes in the lipid composition is the maintenance of an optimal lipid order in the cytoplasmic membrane [7].

The alcohols are known as membrane-perturbing agents. We have shown that the growth lag induced in *B. subtilis* by a cold shock can be affected by benzyl alcohol and ethanol in an opposite manner, i.e. it is shortened by the former and prolonged by the latter [8]. This observation correlates with both the strong fluidising effect of benzyl alcohol [9,10] and a more complex mechanism of the ethanol influence [11,12]. While higher alcohols, such as benzyl alcohol, perturb the membrane structure in the membrane core only [13], the effect of ethanol is not so straightforward. Ethanol has been found to exhibit an ordering effect on the neuronal membrane surface while a disordering effect has been found in the membrane interior [12]. Ethanol also affects the membrane organisation in pure phospholipids, but only very small effects have been observed in natural membranes even at a 0.5 M concentration of ethanol. However, even lower concentrations of ethanol inhibit the activities of the membrane enzymes [14]. The present article verifies our hypothesis that benzyl alcohol promotes the recovery of *Bacillus subtilis* after a cold shock via simulation of the membrane 'fluidisation'. We have also analysed the effect of ethanol which has been shown to retard the cell response to the cold stress [8]. We used the fluorescence spectroscopy and fatty acid analysis for this purpose.

The fluorescent probes DPH and TMA-DPH are widely used to study the physical state of biological membranes [15]. DPH penetrates into the membrane interior and its polar derivative, TMA-DPH, remains at the membrane surface in the lipid polar headgroup region [15]. The most common technique for estimation of the membrane state is the steady-state fluorescence anisotropy [16]. However, this technique may sometimes yield misleading results if the anisotropy is measured in membrane samples of different phospholipid composition [7]. The time-resolved fluorescence spectroscopy (lifetime analysis, order parameters, etc.) provides more detailed information on the membrane state and on its changes resulting from the membrane perturbation. We have reported that the fluorescence lifetime of DPH is lowered when the level of the 'fluidising' fatty acids is increased during cold adaptation [7]. This phenomenon reflects a less ordered state with a greater extent of membrane hydration that influences the fluorescence lifetime of DPH [17].

The present work is aimed at defining the role of membrane adaptation in the physiology of *B. subtilis* after a cold shock. We performed steady-state as well as time-resolved measurements of the DPH and TMA-DPH fluorescence to study the direct effect of ethanol and benzyl alcohol on the cytoplasmic membrane state of the *B. subtilis*.

2. Materials and methods

DPH and TMA-DPH were purchased from Molecular Probes (USA). POPOP was obtained from Sigma (USA). All the other chemicals used were of analytical grade.

2.1. Growth of bacteria

B. subtilis 168 strain trp_{2-} was grown aerobically in a complex medium containing (g/l): Bacto beef extract (Difco) 1.5, yeast extract (Difco) 1.5, NaCl 3.5, KH_2PO_4 1.32, K_2HPO_4 3.5, Bactopepton (Imuna, Slovak Republic) 5, glucose 5 (pH 7.0) at 40 or 20°C. The temperature was shifted down from 40 to 20°C during the mid-exponential phase. The growth was estimated turbidimetrically at 450 nm. The cells were harvested by rapidly filtering through a Synpor No. 5 filter (Synthesia, Czech Republic).

2.2. Effect of alcohols on growth

An overnight culture grown at 40 or 20°C was diluted into a fresh medium to an optical density (450 nm) of 0.01 (40°C) or 0.04 (20°C). For studying the effect of alcohols on the doubling time, benzyl or ethyl alcohol were added within the mid-exponential phase. For fluorescence measurements, the alcohols were added 2 min (40°C) or 20 min (20°C) before the measurement. Alcohols were always added 2 min before the temperature shift-down.

2.3. Membrane isolation

Cytoplasmic membranes from *B. subtilis* were isolated as described before [4,18,19], washing the cells remaining on the filter once with a phosphate buffer (0.1 mol/l, pH 7.3) and resuspending in the same buffer plus sucrose (0.5 mol/l). The cell suspension (about 12 μg wet mass per ml) was further incubated with lysozyme (500 μg/ml) without mixing. The formation of protoplasts was complete within 20 min at 40°C, or within 50 min at 20°C. The protoplast suspension was centrifuged for 30 min at $9000 \times g$ and the sediment was resuspended by homogenisation in a small volume of the phosphate buffer at 1 g wet mass per ml. RNase and DNase were then added (0.5 mg per g wet mass) together with $MgSO_4$ (10 mmol/l). The cell lysis was carried out by diluting 100-times with a hypotonic phosphate buffer (50 mmol/l, pH 6.6), mixing for 15 min (40°C), adding of EDTA (10 mmol/l), mixing again for 45 min and finally adding $MgSO_4$ (10 mmol/l). The incubation periods with EDTA or $MgSO_4$ at 20°C were 30 and 60 min, respectively.

The cell lysate was centrifuged for 90 min at $30\,000 \times g$ and the membrane sediment washed once in a phosphate buffer (50 mmol/l, pH 6.6) with KCl (1 mol/l) and EDTA (10 mmol/l) at 0°C. The final sediment was rehomogenised in the phosphate+KCl buffer (without EDTA) and stored at −60°C with 15–20 mg protein per ml. The membrane preparations were thawed only once, and used for the fluorescence measurements. The protein concentration was determined by the Lowry method [20].

2.4. Labelling of B. subtilis cells and cytoplasmic membranes with fluorescent probes

DPH was directly added to the exponentially growing cells at a final concentration of 10^{-6} M and the incorporation proceeded for 30 min at 40°C. The DPH-labelled cells were harvested (after the alcohol treatment and a temperature shift-down) by rapid filtering through a Synpor filter, the bacteria remaining on the filter were washed once and suspended in a phosphate buffer (0.1 mol/l, pH 7.1) [21]. DPH or TMA-DPH was incorporated into the isolated bacterial membranes in vitro at a lipid/probe molar ratio of 1:500 as described previously [22]. Under these conditions, the alcohols were directly added to the spectrofluorimeter cuvette containing the DPH-labelled membrane suspension.

2.5. Steady-state fluorescence anisotropy measurements

Steady-state anisotropy measurements were performed with a SLM 4800S fluorometer (SLM Instruments, Urbana, IL), equipped with the standard polarisation accessory. The excitation wavelength was set to 360 nm. Fluorescence was detected through a cut-off emission filter (Schott, 50% transmittance at 405 nm) and the emission monochromator was set to 430 nm. The correction for the fluorescence intensity of non-labelled cells (10–20% intensity of the DPH-labelled cells) was calculated according to [23]. The background fluorescence of non-labelled membranes did not exceed 1% of the experimental values. The steady-state anisotropy was calculated according to [24]. The steady-state anisotropy values presented below correspond to the average of at least three determinations performed with the independent membrane preparations.

2.6. Dynamic fluorescence measurements and data analysis

Lifetime measurements were performed with a ISS K2 multifrequency phase fluorimeter (ISS Fluorescence Instrumentation, Champaign, IL) interfaced with a PC 486 for data collection and analysis. The 300-W xenon arc lamp was used as an excitation light source. The excitation wavelength was 360

nm, the emission was measured at 430 ± 10 nm. Ten modulation frequencies were used between 5 and 200 MHz. All the measurements were carried out with POPOP in the reference cell [24]. The temperature of the samples was maintained at 20°C with an external bath circulator. As the T_c20 and T_c40 membranes provided the same results, only T_c20 membranes were used for measurements involving alcohol treatment.

The measurements and data analysis were carried out according to [23]. A program provided by ISS (La Spezia, Italy) was used for the lifetime analysis. This program minimises the reduced χ^2 (χ^2_R) defined by an equation reported elsewhere [26]. The data were analysed employing either the sum of the exponentials or a continuous distribution of lifetime values [27]. The fit was improved (the χ^2_R value decreased) by about 20% when using a bimodal Lorentzian distribution instead of a two-component exponential model. The distribution was characterised by a centre τ and a width at half-maximum, W.

2.7. Lipid extraction and fatty acid analysis

Bacterial phospholipids were isolated as described by [4]. To determine the fatty acid composition, the methyl esters were prepared from 20 mg of the isolated phospholipids by transesterification with sodium methoxide and HCl in methanol according to [28]. Gas chromatography was performed on a Chrom 4 apparatus (Laboratorni pristroje, Prague, Czech Republic), equipped with FID, Dual Gas Flow Controller (Brooks Instruments) and DP 88 Computing Integrator (Pye Unicam).

3. Results

3.1. Effect of alcohols on growth of B. subtilis

The effect of benzyl alcohol and ethanol on the growth of *B. subtilis* was first followed under steady-state conditions, i.e. during the cultivation at a constant temperature of 40 or 20°C. Both alcohols increased the doubling time when added to the exponentially growing cells (Table 1). This inhibitory effect was stronger at the higher temperature (40°C) and was directly proportional to the alcohol concentration. Concentrations of 0.005 M for benzyl alcohol and 0.35 M for ethanol increased the doubling time by approximately 10% and were selected for further studies.

Table 1
The effect of benzyl alcohol and ethanol on the doubling time t at 40 and 20°C and on the growth lag after temperature shift-down from 40 to 20°C

	t (min) (40°C)	t (min) (20°C)	Growth lag (min)
Benzyl alcohol (M)			
0	15	120	80
0.001	16	120	75
0.005	18	120	51
0.010	20	133	51
0.050	22	0.0	lysis
Ethanol (M)			
0	15	120	80
0.17	15	120	80
0.35	17	130	102
0.52	25	130	122
0.87	67	171	144

The data correspond to the average of at least three determinations.

When transferred from 40 to 20°C (temperature shift-down), bacteria stopped their growth for about 80 min before the multiplication was established at another rate, see Table 1. The addition of the alcohols before the temperature shift-down significantly changed the length of the lag phase. Benzyl alcohol at a concentration of 0.005 M reduced the lag phase from 80 to 51 min, whereas 0.35 M ethanol prolonged this interval from 80 to 102 min. The same results, i.e. shortening of the lag phase by benzyl alcohol and its prolonging by ethanol, were obtained at higher concentrations of both the alcohols (Table 1).

To further analyse the role of benzyl alcohol and ethanol in cold adaptation of *B. subtilis*, the membrane physical state was monitored by steady-state fluorescence anisotropy measurements of the DPH-labelled cells and the cytoplasmic membranes.

3.2. Steady-state anisotropy measurements of living cells and isolated membranes

To approach the in vivo conditions as close as

possible, the steady-state anisotropy r_{ss} of the DPH fluorescence was first measured in the intact living cells. Benzyl alcohol (0.005 M) or ethanol (0.35 M) were added to the DPH-labelled cells which were grown at 40°C (T_c40). The fluorescence measurements were also carried out at 40°C. The effect of the alcohols on the doubling time was followed in parallel (by measuring the optical density). Benzyl alcohol caused an immediate decrease in the DPH anisotropy, i.e. 'fluidisation', from 0.162 to 0.143. Ethanol exerted the opposite effect, i.e. the r_{ss} value increased from 0.162 to 0.210. A substantial increase in the DPH fluorescence intensity (by 30%) was observed simultaneously. The values of both r_{ss} and the fluorescence intensity became constant 20 min after the addition of ethanol. This effect caused by ethanol may reflect a formation of a new hydrophobic environment for the DPH molecules, most probably in the area of the bacterial cell wall.

To avoid methodological artefacts inherent in work with living cells (such as a decrease in the benzyl alcohol concentration during aerobic growth due to evaporation), similar experiments were performed in the membranes isolated from the *B. subtilis* cells. Both DPH and its polar analogue TMA-DPH were used to discriminate between the inner and outer regions of the membrane.

Surprisingly, both the alcohols decreased the DPH anisotropy r_{ss} in the membranes prepared from cells cultivated at 40°C (T_c40). The decrease in the r_{ss} values induced by 0.005 M benzyl alcohol was higher (from 0.247 to 0.229) than that induced by 0.35 M ethanol (from 0.247 to 0.241). Using TMA-DPH, benzyl alcohol did not exert any significant effect, while ethanol only slightly increased the r_{ss} values (from 0.252 to 0.253). The same effects were observed in the membranes cultivated at 20°C (T_c20), (data not shown).

The steady-state anisotropy measurements were further complemented with the fluorescence lifetime measurements.

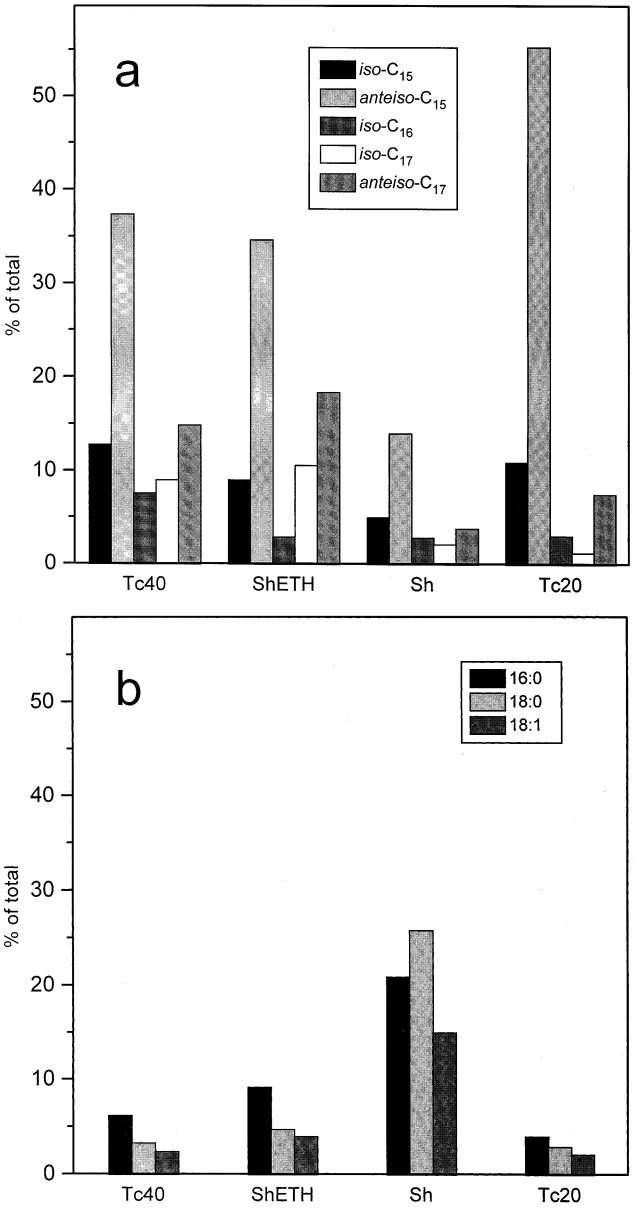

Fig. 1. Effect of the growth conditions on the composition of branched-chain fatty acids (a) and straight-chain fatty acids (b). Fatty acids isolated from the cells cultivated at 20°C (T_c20), 40°C (T_c40), 30 min after temperature shift from 40 to 20°C (Sh), 30 min after temperature shift from 40 to 20°C in the presence of 0.35 M ethanol (ShETH).

3.3. Fluorescence lifetime measurements

The DPH lifetime distribution in the bacterial membranes (T_c40) can be described in terms of two Lorentzian components (Table 2). In the membranes without any alcohol treatment, DPH has a main fluorescence lifetime component of $\tau_1 = 7.18$ ns and a wide distribution $W_1 = 1.74$ ns. This result seems to be typical for natural membranes exhibiting a large heterogeneity [25]. TMA-DPH exhibits the main lifetime component of $\tau_1 = 4.62$ ns with a narrower distribution $W_1 = 0.85$ ns, most probably because of its

location near the membrane periphery. The minor (i.e. the second) lifetime component the origin of which is discussed elsewhere [15,29], has the value of $\tau_2 = 2.3$ ns and $\tau_2 = 1.5$ ns for DPH and TMA-DPH, respectively.

Benzyl alcohol (0.005 M) induces a dramatic reduction in both the long and the short lifetime components for both the fluorescence probes (Table 2). Furthermore, benzyl alcohol widens the distribution of the long component. This effect is more pronounced for TMA-DPH (increase of W from 0.44 ns to 1.14 ns) than for DPH (W increases from 1.74 to 2.18 ns). Ethanol (at concentrations up to 0.87 M) does not exert any significant change in the lifetime values of DPH or TMA-DPH.

We performed the fatty acid analysis of the cold-shocked cells to explain why ethanol extends the growth lag without a substantial effect on the membrane state.

3.4. Fatty acid analysis

Ethanol strongly suppresses the adaptive synthesis of fatty acids after the cold shock. This is demonstrated in Fig. 1 which compares the composition of branched-chain (Fig. 1a) and straight-chain (Fig. 1b) fatty acids in the membranes of *B. subtilis* cultivated under different conditions.

The steady-state growth at a constant temperature of 40°C (T_c40) is primarily characterised by a high content of the branched-chain fatty acids (almost 80% of the total acids), with the *anteiso*-C_{15} fatty acid as the predominant component representing 39% of the total (Fig. 1a). In contrast, the transfer of bacterial cells from 40 to 20°C for 30 min (Sh) is associated with a remarkable decrease in the content of the branched-chain fatty acids. However, the branched fatty acids are fully recovered after a prolonged steady-state cultivation at 20°C (T_c20); the content of the *anteiso*-C_{15} fatty acid also increases at 20°C up to 56% of the total, at the expense of the other branched *anteiso*-C_{17}, *iso*-C_{15} and *iso*-C_{17} fatty acids. The preferential synthesis of *anteiso*-C_{15} acid with the low melting point ($T_m = 23$°C) is supposed to be responsible for the membrane fluidisation in cells growing at 20°C.

The opposite is observed for the straight-chain fatty acids (Fig. 1b), namely palmitic ($C_{16:0}$), stearic ($C_{18:0}$) and oleic ($C_{18:1}$) acid. These components represent a minor part of the membrane lipids in bacteria grown at a constant temperature (T_c40 and T_c20). However, on the transfer from 40 to 20°C (Sh) the straight-chain fatty acids prevail within 30 min in the membrane and entirely substitute the family of branched fatty acids (Fig. 1b). The synthesis of the rare monounsaturated $C_{18:1}$ fatty acid with $T_m = 0.5$°C was especially depicted to attenuate the membrane rigidisation brought about by the cold stress.

The cold-shock response characterised in the cells transferred from 40 to 20°C by the replacement of the branched-chain fatty acids by the straight-chain ones is blocked by ethanol (ShETH in Fig. 1a,b). Simultaneously, 0.35 M ethanol has almost no effect

Table 2
Lifetime distribution analysis of DPH and TMA-DPH in the cytoplasmic membranes of *B. subtilis*. The effect of benzyl alcohol (5 mM) and ethanol (0.87 M)

	$\tau_1 \pm \sigma$ (ns)	$W_1 \pm \sigma$ (ns)	$f_1 \pm \sigma$	$\tau_2 \pm \sigma$ (ns)	$W_2 \pm \sigma$ (ns)	$f_2 \pm \sigma$	χ^2_R
DPH							
Control	7.18 ± 0.07	1.74 ± 0.22	0.92 ± 0.002	2.29 ± 0.25	0.05 ± 0.03	0.08 ± 0.002	2.2
Benzyl alcohol	6.29 ± 0.06	2.18 ± 0.016	0.91 ± 0.002	1.18 ± 0.30	0.06 ± 0.04	0.09 ± 0.002	2.4
Ethanol	7.15 ± 0.08	1.69 ± 0.020	0.92 ± 0.002	2.60 ± 0.30	0.05 ± 0.06	0.08 ± 0.002	3.1
TMA-DPH							
Control	4.62 ± 0.04	0.44 ± 0.12	0.85 ± 0.003	1.51 ± 0.18	0.05 ± 0.12	0.15 ± 0.005	3.42
Benzyl alcohol	3.67 ± 0.03	1.14 ± 0.13	0.94 ± 0.003	0.40 ± 0.05	0.05 ± 0.11	0.06 ± 0.003	2.65
Ethanol	4.63 ± 0.03	0.36 ± 0.09	0.85 ± 0.002	1.54 ± 0.05	0.05 ± 0.27	0.15 ± 0.003	1.65

$\tau_{1,2}$, centres of Lorentzian distributions (ns), $W_{1,2}$, width at half maximum (ns), $f_{1,2}$, fractional intensities, χ^2_R, reduced χ^2. The data in table correspond to the average ± S.D. of at least three measurements of a representative sample of labelled membranes.

on the fatty acid composition in the cells cultivated at a constant temperature of 20 or 40°C (results not shown).

Therefore, it may be suggested that the fatty acid composition of the cell membrane is petrified in the presence of ethanol, irrespective of the environmental stimuli.

4. Discussion

The cytoplasmic membrane of *B. subtilis* undergoes dramatic changes in the phospholipid composition in response to the growth temperature [3,4,8]. It may be suggested that these changes serve to maintain the optimum membrane function. In search for an appropriate description of these thermoadaptive processes occurring in the bacterial membrane, we have followed various physical characteristics [4,18,21] and found out that the order in the inner membrane region is maintained constant at different growth temperatures [7].

The temperature downshift of *B. subtilis* (from 40 to 20°C) results in a growth lag; during this period of cell adaptation, the changes occurring in the composition of the cytoplasmic membrane represent the crucial regulatory step. The length of the lag phase can be affected in both directions by agents that perturb the membrane structure, such as benzyl alcohol (shortening of the lag) and ethanol (prolonging of the lag) [3,8].

The concentrations of ethanol and benzyl alcohol used in our study for this purpose have been chosen such as to reduce the growth of *B. subtilis* to the same extent. The difference of two orders of magnitude in the concentrations of the two alcohols (0.35 M ethanol, 0.005 M benzyl alcohol) can be explained by a difference in the membrane/buffer partition coefficient of ethanol and benzyl alcohol (0.14 or 13, respectively) [14].

The steady-state DPH anisotropy data on the living cells demonstrate membrane 'rigidisation' induced by ethanol and 'fluidisation' caused by benzyl alcohol. The cold shock induces in Bacilli the synthesis of a greater proportion of unsaturated fatty acids, i.e. the fluidisation of the membrane [4,30]. The fluorescence measurements correlate well with the growth experiments (i.e. the shortening of the lag correlates with the fluidising effect of benzyl alcohol measured by fluorescence anisotropy).

In the isolated membranes, however, both the alcohols decrease the steady-state fluorescence anisotropy of DPH which indicates a fluidisation of the bacterial membrane. To explain the difference between the data obtained on the cells and on the isolated membranes treated by the two alcohols, the more sophisticated method of the time-resolved fluorescence spectroscopy was employed.

The measurements of the DPH fluorescence lifetime and lifetime distributions are extremely helpful in investigation of the effect of alcohols. Ho et al. [31] found that ethanol and hexanol decreased the fluorescence lifetime and widened the distribution of DPH-related probes. This phenomenon was interpreted as resulting from an increased hydration at the protein–lipid interface. In our experiments, DPH and TMA-DPH lifetime analysis indicates that benzyl alcohol substantially shortens the lifetime of both the probes with simultaneous widening of the lifetime distribution, whereas ethanol has no effect. The measurements of DPH fluorescence in isotropic benzyl alcohol reveal that the fluorescence lifetime values of DPH are similar to those measured in bacterial membranes without benzyl alcohol (I. Konopásek, unpublished data). Therefore, the decrease in the DPH lifetime values caused in the membranes by benzyl alcohol, must result from an enhanced penetration of the water molecules into the hydrophobic membrane [17].

The disordering effect of benzyl alcohol presumably mimics the physiological adaptation mechanism after the cold shock (i.e. the synthesis of higher levels of unsaturated fatty acids and thus the membrane 'fluidisation' [4]. This can explain the promoting effect of benzyl alcohol on the cold adaptation of *B. subtilis*. Ethanol is much less potent in disordering the membrane structure than benzyl alcohol, as estimated on the basis of both the lifetime and steady-state anisotropy measurements.

When comparing the anisotropy and lifetime measurements, it is obvious that the steady-state fluorescence anisotropy alone cannot fully assess the changes in the membrane structure imposed by different alcohols. The ethanol-induced decrease in the fluorescence anisotropy is observed within the same fluorescence lifetime (time window) as that before the

ethanol addition. On the other hand, the changes induced by benzyl alcohol are associated with the shortening of the lifetime (time window). This means that the decrease in the anisotropy detected after adding benzyl alcohol 'underestimates' the highly disordering effect of this agent.

Bacteria, like most other organisms, change markedly their fatty acid composition in response to ambient temperature. In *B. subtilis* grown at 20°C, the synthesis of branched fatty acids with low melting points (*anteiso*-$C_{15:0}$ and *anteiso*-$C_{17:0}$) substantially increases as compared with the cultivation at 40°C [4]. On the contrary, the acute cold stress, i.e. the temperature downshift from 40 to 20°C, induces the synthesis of unsaturated fatty acids, whereas the content of the branched-chain fatty acids is reduced. The preferential synthesis of rare unsaturated fatty acids occurs as a consequence of the induction of specific desaturase after cold shock [32]. This thermal control is also operative in *Bacillus megaterium* [33] at the level of the enzyme synthesis and activity. The mechanism controlling the expression of the gene coding for desaturase in *B. subtilis* has been described, based on an increase in the DNA supercoiling associated with a temperature downshift [5]. In our experiments, the cold stress-induced synthesis of unsaturated fatty acids was strongly suppressed in the presence of ethanol. A plausible explanation for this ethanol effect may consider direct inhibition of the membrane-bound desaturase activity; alternatively, it can be caused by an ethanol-induced disordering of the phospholipid matrix [34] that can mediate the pleiotropic effect on the catalytic activity of the membrane-bound enzymes.

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