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Membrane fluidization by alcohols inhibits DesK-DesR signalling in *Bacillus subtilis*

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ABSTRACT

After cold shock, the *Bacillus subtilis* desaturase Des introduces double bonds into the fatty acids of existing membrane phospholipids. The synthesis of Des is regulated exclusively by the two-component system DesK/DesR; DesK serves as a sensor of the state of the membrane and triggers Des synthesis after a decrease in membrane fluidity. The aim of our work is to investigate the biophysical changes in the membrane that are able to affect the DesK signalling state. Using linear alcohols (ethanol, propanol, butanol, hexanol, octanol) and benzyl alcohol, we were able to suppress Des synthesis after a temperature downshift. The changes in the biophysical properties of the membrane caused by alcohol addition were followed using membrane fluorescent probes and differential scanning calorimetry.

We found that the membrane fluidization induced by alcohols was reflected in an increased hydration at the lipid-water interface. This is associated with a decrease in DesK activity. The addition of alcohol mimics a temperature increase, which can be measured isothermically by fluorescence anisotropy. The effect of alcohols on the membrane periphery is in line with the concept of the mechanism by which two hydrophilic motifs located at opposite ends of the transmembrane region of DesK, which work as a molecular caliper, sense temperature-dependent variations in membrane properties.

1. Introduction

Bacterial membranes are sensitive to changes in ambient temperature. When exposed to low temperatures, bacteria remodel their membrane lipid composition in order to overcome the undesired transition from the fluid liquid-crystalline phase to the rigid gel phase [1].

Bacillus subtilis employs two distinct mechanisms to maintain the optimal membrane fluidity when exposed to low temperatures: A long-term membrane adaptation consists of an increase in the synthesis of low-melting anteiso-branched fatty acids, which fluidize the membrane. The short-term adaptation strategy consists of the rapid desaturation of fatty acid chains already incorporated in membrane phospholipids. Introduction of the double bond into membrane phospholipids is achieved by the activity of the membrane-bound fatty acid Δ5-desaturase (Des). This enzyme is expressed shortly after a temperature downshift [2,3].

The expression of Δ5-desaturase in *B. subtilis* is stringently regulated at the transcriptional level by the two-component system DesK/DesR [4]. The sensor of membrane fluidity, DesK, is a bifunctional enzyme with both kinase and phosphatase activities. When the ambient temperature drops, dimeric DesK acts as an autokinase and phosphorylates itself at His188 [4]. Autophosphorylation is carried out as a cross phosphorylation reaction between DesK dimers [5]. Consequently, DesK phosphorylates its cognate response regulator DesR. The phosphorylation of the receiver domain of DesR is allosterically coupled to two distinct exposed surfaces of the protein, controlling di- or

Abbreviations: UFA, unsaturated fatty acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 1-(4-trimethylammonium)-6-phenyl-1,3,5-hexatriene; DSC, differential scanning calorimetry; GP, generalized polarization; FA, fatty acids; Des, fatty acid Δ5-desaturase of *Bacillus subtilis*; ETH, ethanol; PRO, propanol; BUT, butanol; ISB, isobutanol; HEX, hexanol; BA, benzyl alcohol; OCT, octanol; P*des*, *des* promoter; MS-DesK, minimal sensor of DesK; TMS, transmembrane segment of DesK

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tetramerization, cooperative activation and binding to DesK [6,7]. The phosphorylated DesR then functions as a transcription factor and triggers the expression of the *des* gene. At the optimal growth temperature (37 °C), the sensor DesK acts as the phosphatase dephosphorylating response regulator DesR, thus the expression of the *des* gene is abolished.

The idea that DesK senses the membrane fluidity was supported by two independent observations. First, exogenously added unsaturated fatty acids (UFA) inhibited the expression of the *des* gene after a temperature downshift [4]. Second, exogenously added isoleucine, the precursor of fluidizing anteiso-branched fatty acids, also decreased the expression of the *des* gene under isothermal conditions [8].

The sensor DesK is composed of a cytosolic kinase domain connected by a short linker to the membrane domain consisting of five transmembrane segments [9]. A truncated DesK sensor which lacks its transmembrane domain is locked in the kinase activity state [10]. This leads to permanent activation of DesR and constitutive expression of the *des* gene. Therefore, the membrane domain is essential to sensing membrane fluidity and to controlling the conformational changes required by DesK to alternate between its kinase and phosphatase conformations [10,11]. The structures of the cytoplasmic domain of DesK associated with the kinase and phosphatase conformations were described in detail [12].

The mechanism of membrane fluidity perception by the transmembrane domain of DesK has been characterized at the molecular level using a DesK construct in which the cytoplasmic catalytic domain is fused to a chimerical single-membrane spanning segment [9]. The transmembrane segment of DesK has two hydrophilic motifs at the N- and C-terminus that work together as a molecular caliper. This molecular caliper senses changes in membrane thickness that occurs as a consequence of temperature changes and not temperature itself [9,13,14]. At lower temperature, the membrane is thicker due to a more ordered packing of lipids, and these motifs are buried in the lipid phase, triggering the kinase conformation of DesK. At higher temperatures, the membrane becomes thinner with more disordered lipids, and hydrophilic residues are pushed to the cytoplasm, promoting the required conformational changes to switch to the phosphatase conformation [15].

Furthermore, recent results strongly support the theory that DesK regulation is linked to changes in membrane thickness. An increase in short chain fatty acids (FA) by treating *B. subtilis* with cerulenin alters the lipid-protein interaction required for DesK activation at low temperature. In addition, increased synthesis and incorporation of long-chain FA into *B. subtilis* membranes results in the constitutive expression of *des* at high temperature [16].

In order to elucidate the nature of the signals that modulate the activity of DesK, we employed a series of alcohols, which are widely used because these compounds affect lipid packing, fluidity and hydration. Considering the great complexity of biological membranes, it is obvious that the interaction of alcohols with membrane enzymes is not a simple process. Incorporation of a particular alcohol into a specific region of a bilayer depends on the length and hydrophobicity of a given alcohol. A direct correlation between alcohol chain length and its potency to be solubilized by lipids is provided by octanol/water partition coefficients. For each additional CH_2 group, the concentration required to produce the same effect in the physical properties of the membrane was reduced by approximately a factor of 3 [17,18].

Further, localization of an alcohol in the membrane depends on the hydrophobicity of the alcohol. Short-chain alcohols occupy the region of polar lipid headgroups, as was proved for ethanol [19,20], while long-chain alcohols localize deeper in the membrane [21]. Alcohols interact with the polar lipid headgroup region via their polar −OH groups; therefore the effect of the alcohol on the hydrophobic region is influenced by its chain length [22]. This was confirmed by the observation that alcohols had the most pronounced effect on membrane disorder at intermediate depths (2–9 carbon atoms). In contrast, little or no increase in fluidity was determined in the deep hydrophobic region close to the bilayer centre [23,24].

The presence of alcohols in the membrane has a disordering effect on lipid hydrocarbon chains, which results in an increase in the area per lipid and the overall fluidity of the membrane. This is accompanied by a drop in the membrane thickness and by a reduction in tension at the membrane/water interface [25]. Short-chain alcohols have a strong effect on membrane properties, which depends on both the length of the hydrophobic part of the alcohol and its concentration [26], as the headgroup area per lipid molecule increases with alcohol concentration [21].

The thickness of the plasma membrane has been shown to directly affect membrane protein function. The highest protein activity occurs when the thickness of the transmembrane domains of intrinsic membrane proteins and the hydrophobic thickness of the membrane itself match [27]. Alcohol molecules are able to enter a membrane and influence its structure, thus inducing structural changes to the environment of a membrane protein. The sensitivity of membrane proteins to membrane thickness is thought to be caused by the large energy penalty generated by the hydrophobic mismatch [28,29]. In order to characterize which physical parameters of the membrane influence the DesK signalling state, we decided to study the effects of membrane-perturbing agents on the DesK sensor. We used a set of linear alcohols with varying lengths (2–8 carbon atoms) together with the widely-used fluidizing agent benzyl alcohol. The physical changes induced by alcohols in the membrane periphery and at the membrane-lipid interface were assessed by measuring the fluorescence anisotropy of the fluorescent probes DPH and by the general polarization of the probe Laurdan. The effect of alcohols on the phase transition of the membrane lipids was determined using differential scanning calorimetry. Our results showed that a fluidization induced by most of the alcohols was effective at changing the DesK signalling state. We compared the isothermal membrane perturbation imposed by alcohols with the concept of the sensing of the membrane thickness by hydrophobic amino acids in the sequence of the linker region of DesK. We concluded that alcohols are able to induce the same effects as those resulting from either temperature change or from modifying the fatty acid length. Various alcohols can therefore be used in order to modify the signalling state of the temperature sensor, and the fluorescence anisotropy of DPH can be used for a rough prediction of such an effect.

2. Materials and methods

2.1. Bacterial strains

Two bacterial strains were used in this work: *Bacillus subtilis* strain M19 bearing the *lacZ* gene under the control of the *des* promoter in the *amyE* locus (168 amyE::P*des*-lacZ) constructed previously [30], and the DesK null mutant strain LCK 20 overexpressing the response regulator DesR gene (168 desK::Km^R pKm-desR amyE::P*des*-lacZ) constructed in this work. The LCK20 strain was constructed by transforming the parental strain *Bacillus subtilis* 168 with the plasmid pFG1765KAN, giving rise to the LCK19 strain. Briefly, a 1765 bp fragment coding for *desK* was cloned in pBluescript SKII. The resulting plasmid was digested with *Eco*RI and *Hin*dIII, and a Km^R cassette without its transcriptional terminator was inserted, interrupting the *desK* gene and leaving *desR* under the strong pKm promoter [4]. The LCK19 strain was then transformed with the pAR11 plasmid containing a transcriptional fusion between the *des* promoter and the reporter gene *lacZ* [4] to give the LCK20 strain. Integration of the plasmids at the correct loci was confirmed by differential PCR. The LCK20 strain overproduces the DesR response regulator, which can bind to the desaturase promoter but also activates transcription even in the absence of DesK-mediated phosphorylation. Analysis of the β-galactosidase activity of the LCK20 strain showed overexpression of the reporter gene at both 25 and 37 °C compared to the M19 strain (wild-type *B. subtilis* 168 transformed with pAR11).

2.2. Cultivation of bacteria

B. subtilis was grown aerobically in Erlenmeyer flasks in a shaken incubator at 170 rpm. A complex cultivation medium was used containing: 1.5 g/l beef extract (Difco), 1.5 g/l yeast extract (Oxoid), 5 g/l bactopepton (Oxoid), 3.5 g/l NaCl, 3.5 g/l K2HPO4, 1.32 g/l KH2PO4 and 5 g/l glucose (pH 7.0). Antibiotics were added to the cultivation medium as follows: chloramphenicol (5 μg/ml) for strain M19 cultivation and chloramphenicol (5 μg/ml) with kanamycin (5 μg/ml) for strain LCK20 cultivation. The optical density of the growing culture at the wavelength 450 nm (OD_{450}) was measured turbidimetrically (spectrophotometer Beckman DU530). The cold shock was performed as a rapid transfer of bacteria from 40 °C to 20 °C, respectively, when the bacterial culture reached an OD_{450} of 0.15–0.20.

2.3. Effect of alcohols on growth rate

Ethanol, propanol, butanol, hexanol, benzyl alcohol and octanol were tested for their effect on the doubling time of the bacterial culture after a cold shock. Alcohols were added when the culture reached an OD_{450} of 0.15–0.20. Each alcohol was diluted to the appropriate concentration in 1.5 ml of fresh medium, mixed well and then added to 60 ml of growing bacterial culture in a 500 ml Erlenmeyer flask 2 min before the temperature downshift.

2.4. Membrane isolation

The bacterial culture was harvested in the mid-exponential phase (OD_{450} of 0.5) by filtration (filters Pragopor, no. 5, Pragochema, Czech Rep.). The isolation of membranes was performed as described in our previous work [30]. The membrane pellet was resuspended in 50 mM Tris-HCl buffer, pH 7.0 and stored at − 80 °C. The Pierce BCA Protein assay (Pierce Biotechnology) was used for determining the protein concentration in the sample.

For fluorescence anisotropy and generalized polarization measurements, the samples were thawed only once and diluted in the same buffer to a final protein concentration of 10 μg/ml.

2.5. Lipid isolation

Lipid isolation and the preparation of unilamellar liposomes were performed as described previously [30]. For the suspension of unilamellar liposomes, 50 mM Tris-HCl (pH 7.0) buffer was used. For DSC measurements, the final concentration of lipids was 1 mg/ml.

2.6. DSC measurement

Lipid samples for DSC measurements were prepared as described above. Alcohols at the appropriate concentrations were added to the suspension of unilamellar liposomes. The samples were incubated for 10 min at 40 °C, then cooled down to room temperature and used for DSC measurement. DSC measurement was performed as published previously [30]. Data were collected in the range 5–55 °C. The scan rate was 1 °C/min. The addition of alcohols to the samples induced pronounced changes in thermograms that could not be ascribed to the effect of alcohols on the thermal behaviour of lipids, but to the heat absorbed by the alcohols themselves. In control samples without alcohols, the molar heat capacity Cp was constant from 55 °C to about 25 °C, where an increase in Cp began due to the phase transition. However, in samples with alcohols the Cp exhibited a linear increase from 55 °C that was dependent on the alcohol concentration. Moreover, the descending part of the thermogram in the low-temperature region was affected by a steep decrease in Cp due to the inherent artefacts with the end of the measured interval. This non-symmetric thermogram was analysed using the program Fityk (freeware, http://fityk.nieto.pl/, version 0.8.3) as follows:

A linear "background" slope was found in the interval from 35 to 25 °C, and the resulting function was subtracted from the data. The T_m values were found by fitting the data to a Gaussian function in the interval from 6 to 17 °C.

2.7. Fluorescence anisotropy and generalized polarization measurement

A Fluoromax-3 spectrofluorometer (Horriba Jobin Yvon) was used for fluorescence measurements. For measuring the steady-state fluorescence anisotropy (r_{ss}) of DPH, the polarization accessory was employed as described previously [30] with excitation and emission wavelengths of 360 nm and 425 nm, respectively. The generalized polarization of Laurdan (GP) was measured without the polarization accessory. The excitation wavelength was 365 nm, and the intensity of emission was recorded at wavelengths of 435 nm and 510 nm. The temperature of the samples was controlled with an accuracy of 0.1 °C using a thermometer placed in a cuvette and a thermostated water bath. The samples of membrane vesicles (2.5 ml) were labelled with the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) dissolved in acetone. The final concentration of the probe in the sample was 1 μM. After labelling, samples were incubated in the dark at 37 °C for 30 min. For GP measurement, the fluorescent probe 2-dimethylamino-6-lauroylnaftalen (Laurdan) was dissolved in dimethylsulfoxide and used at a final concentration of 1 μM. Labelled samples were incubated in the dark at 37 °C for 90 min. After sample labelling, the tested alcohol was added using a Hamilton syringe. To ensure proper distribution of the alcohol throughout the sample, the samples were heated to 40 °C.

After stabilization of the fluorescence signal, the sample was cooled down slowly while fluorescence data were gathered. The temperature interval from 40 to 20 °C was used for r_{ss} measurements and 40 to 15 °C for GP measurements. The r_{ss} and GP values were calculated according to Lakowicz [31]. The background fluorescence of non-labelled samples did not exceed 2% of the experimental values.

2.8. β-Galactosidase activity assay

Culture samples (1 ml) were taken during the *B. subtilis* cultivation and assayed for β-galactosidase activity as described previously [30]. The specific activity was expressed in Miller units [32].

3. Results

3.1. Propanol, benzyl alcohol, hexanol and octanol decreased the activity of the des promoter after cold shock from 40 to 20 °C without influencing the growth rate

The excess of short- or long-chain fatty acids in the *Bacillus subtilis* membrane was shown to affect the DesK-DesR signalling [16]. In our first experiments, we investigated whether alcohols, which have already been reported to affect the membrane fluidity, are able to cause a similar effect. We searched for concentrations of alcohols capable of decreasing the *des* promoter activity after cold shock. To this end, we analysed the effect of a series of alcohols, namely ethanol (ETH), propanol (PRO), butanol (BUT), benzyl alcohol (BA), hexanol (HEX) and octanol (OCT) on *des* expression via reporter β-galactosidase activity. The *B. subtilis* M19 strain bearing a P*des*-lacZ fusion at the amyE locus [33] was cultivated at 40 °C. When the cultures reached the mid-exponential phase of growth (OD_{450} of 0.15–0.2) they were supplied with the given alcohol and rapidly transferred to 20 °C. After cold shock, the culture was assayed for β-galactosidase activity. The results shown in Fig. 1 demonstrate that all alcohols except ethanol were able to significantly reduce *des* expression after cold shock in a concentration-dependent manner. In contrast, P*des* activity increased at 428 mM ethanol, while 856 mM ethanol had an inhibiting effect on P*des*. The minimum effective concentration of a given alcohol that could change the *des* expression decreased dramatically from 428 mM (ethanol) to

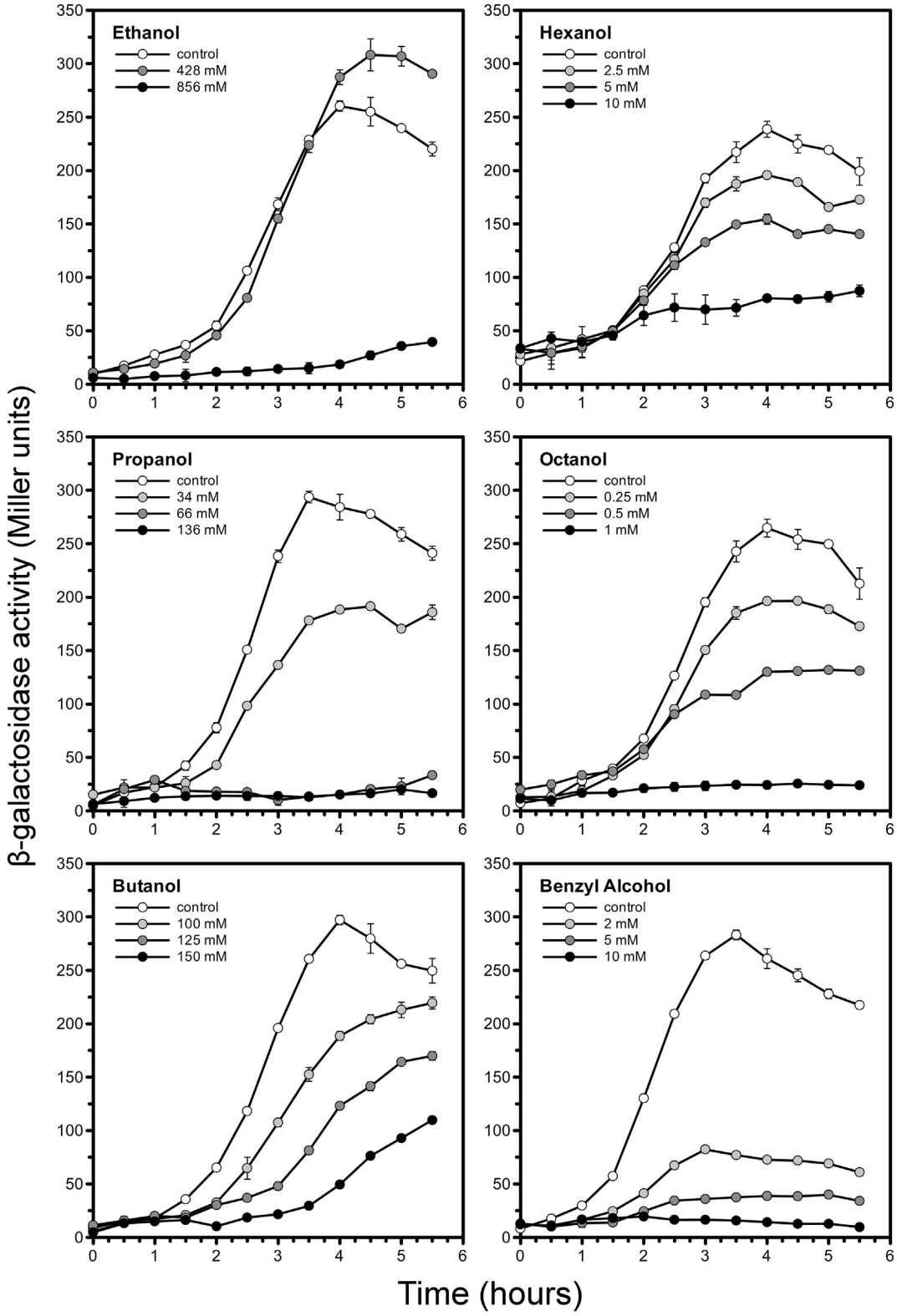

Fig. 1. Effect of alcohols on activity of Pdes-controlled β-galactosidase activity after temperature downshift from 40 to 20 °C in *B. subtilis* M19. The *B. subtilis* M19 culture, bearing a Pdes-lacZ fusion at the amyE locus, was grown overnight at 40 °C in the complex medium and then diluted into fresh medium. When the culture reached an OD_{450} of 0.15–0.2, the particular alcohol was added at its respective concentration (shown in individual panels). For the control culture no alcohol was added, see Materials and Methods section. After 2 min, bacterial cultures were transferred to 20 °C. Then the samples for the β-galactosidase assay were collected every 30 min. The data points correspond to the mean values (n = 3) and their standard errors.

Table 1
Effect of different alcohols on doubling times (T) of *B. subtilis* M19 after cold shock from 40 to 20 °C. Bacterial cultures were cultivated at 40 °C, alcohols were added 2 min before the temperature downshift, which was performed at OD_{450} = 0.15–0.2. The doubling time of the culture at 40 °C (prior to the shock) was 20 min. The standard error of T determination is ~9% for each alcohol concentration (n = 3). Concentrations of individual alcohols which significantly decreased the growth rate compared to control samples are marked with an asterisk ($p < 0.05$, using Students's *t*-test).

Alcohol	Concentration (mM)	T (min)
No alcohol (control)	0	172
Ethanol	428	220*
	856	283*
Propanol	34	175
	68	170
	136	168
Butanol	100	194*
	125	196*
	150	228*
Hexanol	2.5	182
	5	187
	10	175
Octanol	0.25	185
	0.5	176
	1	178
Benzyl alcohol	2	176
	5	180
	10	177

0.25 mM (octanol). Further, we tested the effect of the alcohols shown in Fig. 1 on the doubling time of *Bacillus subtilis* M19 in order to confirm that the decrease in *des* expression was not due to an inhibiting effect on the cellular metabolism.

The doubling times are presented in Table 1. The results indicate that the only concentrations that significantly increased the doubling time were those of ethanol (both 428 and 856 mM), together with all concentrations of butanol. These results confirm that ethanol and butanol do not only affect *des* expression by their incorporation into the membrane; therefore their effect must be more complex.

3.2. Constitutive expression of Pdes-LacZ in the presence of alcohols showed that ethanol and butanol decrease Des synthesis

The above results showed that PRO, HEX, BA and OCT decreased the activity of P*des* after the cold shock without affecting the growth rate. In contrast, the effect of ETH and BUT resulted in a different pattern of P*des* induction and in an increased doubling time of *B. subtilis* M19. In order to confirm that the effect on the *des* expression of PRO, HEX, BA and OCT was mediated by DesK and not due to an inhibiting effect on P*des* induction, we studied the effect of alcohols on P*des* activity in the *B. subtilis* strain LCK20 (168 desK::Km^R pKm-desR amyE::P*des*-lacZ). This strain lacks a functional membrane sensor DesK and overproduces the response regulator DesR, which results in a constitutive activation of P*des* (Fig. 2, control). As with the M19 strain, *des* expression in LCK20 was quantified using the *lacZ* reporter. As for the alcohol concentrations used, we chose representative concentrations of PRO, HEX, BA and OCT that decreased *des* expression after the cold shock by about 50%, (see Fig. 1) but did not affect growth.

PRO, HEX, BA and OCT did not affect P*des*-driven β-galactosidase activity after the cold shock, as in the control, suggesting that the effect of alcohols on P*des* expression is mediated by DesK, which is immersed in the lipid membrane, and not by a DesK-DesR independent pathway or by decreasing cellular metabolism.

When we tested both concentrations of ethanol (428 and 856 mM) and the lowest concentration of butanol (100 mM) there was no increase in *des* expression in cultures with 856 mM ETH and 100 mM BUT, in contrast with 428 mM ETH. We concluded that 856 mM ETH and 100 mM BUT inhibited *des* expression in a DesR-independent way, probably by decreasing cellular metabolism.

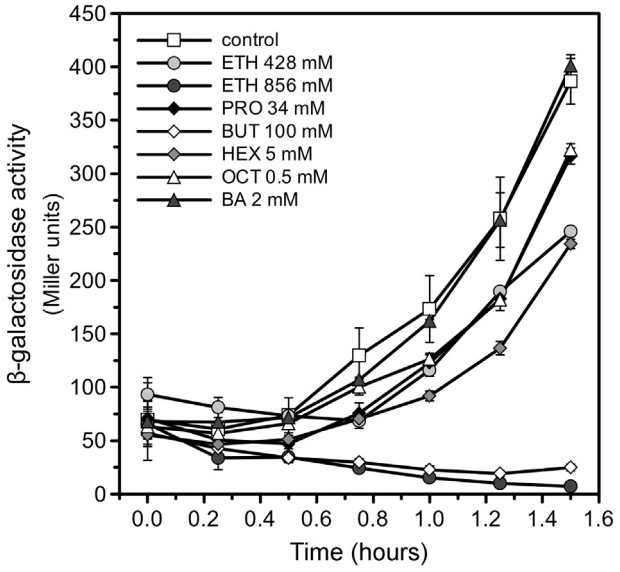

Fig. 2. Effect of alcohols on the Pdes-controlled β-galactosidase activity in *B. subtilis* strain LCK 20.
B. subtilis strain LCK20 (168 desK::Km^R pKm-desR amyE::P*des*-lacZ) with constitutive Des synthesis was grown overnight at 40 °C in the complex medium and then diluted into the medium. When the culture reached an OD_{450} of 0.15–0.20, the alcohol was added to the indicated final concentration. Then the samples for β-galactosidase assay were collected every 15 min. A bacterial culture without alcohol was used as the control. Abbreviations: ETH - ethanol, PRO - propanol, BUT - butanol, HEX - hexanol, OCT - octanol BA - benzyl alcohol, respectively. The data points correspond to the mean values (n = 3) and their standard errors.

3.3. Fluorescence anisotropy of DPH confirmed the fluidizing effect of PRO, BUT, HEX, BA and OCT on bacterial membranes

The following series of experiments was aimed at characterizing the changes in the membrane state of *Bacillus subtilis* M19 produced by a given alcohol. To this end, we employed three approaches: i) First, we used the membrane fluorescent probe DPH by analyzing its fluorescence anisotropy (r_{ss}). This fluorescence parameter reflects the rotation of the DPH probe in the membrane, which is dependent upon the constraints imposed by the neighbouring lipid molecules. ii) The general polarization of the fluorescent probe Laurdan (GP) was used to assess the effect of alcohols on the membrane hydration. iii) Differential scanning calorimetry (DSC) measurements were carried out to determine the effect of alcohols on the midpoint of the phase transition of the membrane phospholipids (T_m). These studies were carried out in vitro using isolated membranes of *B. subtilis* M19 for fluorescence assays and isolated phospholipids for DSC measurements.

The membrane vesicles isolated from *B. subtilis* M19 were labelled with the membrane fluorescent probe DPH. After the fluorescence was stabilized, the alcohols were added to the final concentrations indicated in Fig. 3, and rss was measured over the temperature range 40 °C to 20 °C. The results are presented in Fig. 3.

DPH anisotropy values decreased from approximately 0.25 (20 °C) to 0.18 (40 °C) in control samples without alcohol (Fig. 3). Increasing concentrations of alcohol caused a downshift in anisotropy values in a concentration-dependent manner. Moreover, the resulting dependence of anisotropy on temperature was always nearly parallel with the control without alcohol. The largest downshift was found for benzyl alcohol, which even at 2 mM caused a greater decrease in anisotropy than any other alcohol used in this work. As for the straight-chain alcohols, butanol decreased the anisotropy values most effectively. These results suggest that alcohols that inhibit *des* transcription have a direct effect on membrane lipid fluidity. The decrease in anisotropy produced by butanol, hexanol, octanol and benzyl alcohol is, however, lower than the one produced by a temperature shift from 25 to 37 °C, which has

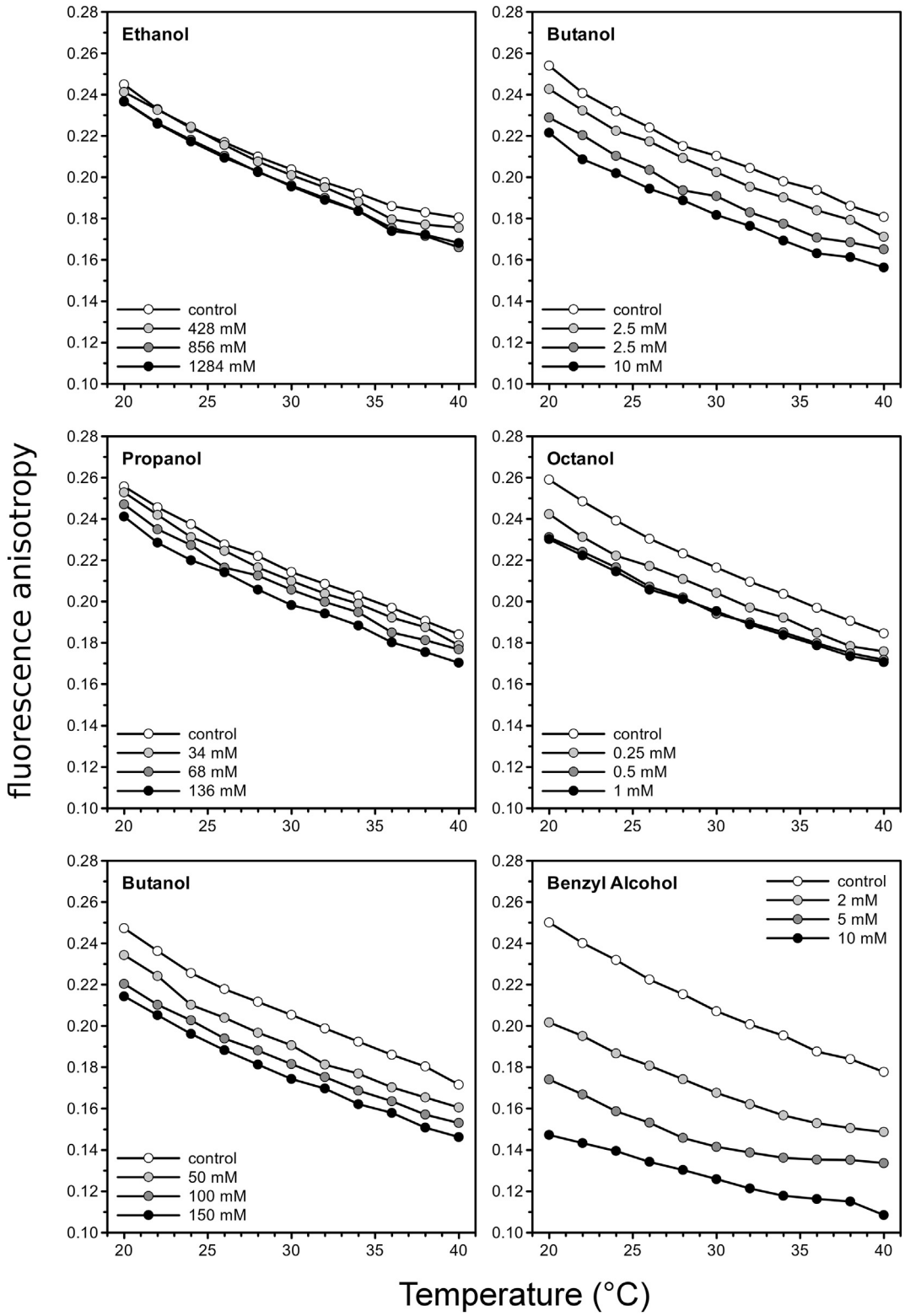

Fig. 3. Effect of alcohols on fluorescence anisotropy of DPH. Cytoplasmic membranes were isolated from *B. subtilis* M19 cells grown at 40 °C in the complex medium and were labelled with DPH. Alcohols were added to the final concentrations indicated. The data shown in the graphs are the representative results (n = 3).

been reported to be the range in which DesK is active (25 °C) or inactive (37 °C) in vivo [4,14,34].

The effect of alcohols on the membranes of *B. subtilis* M19 was further characterized by employing Laurdan. Due to the sensitivity of the Laurdan emission spectrum to solvent relaxation, the general polarization (GP) of Laurdan fluorescence recorded at emission wavelengths of 435 nm and 510 nm reflects the ratio of probe molecules localized in the ordered versus disordered membrane environment. The fluorescent moiety of Laurdan locates itself at the glycerol backbone of the phospholipids [35]. The bacterial membranes from *B. subtilis* M19 were labelled with Laurdan, and GP was measured over the temperature range 40 °C to 15 °C. The decrease in GP values dependent

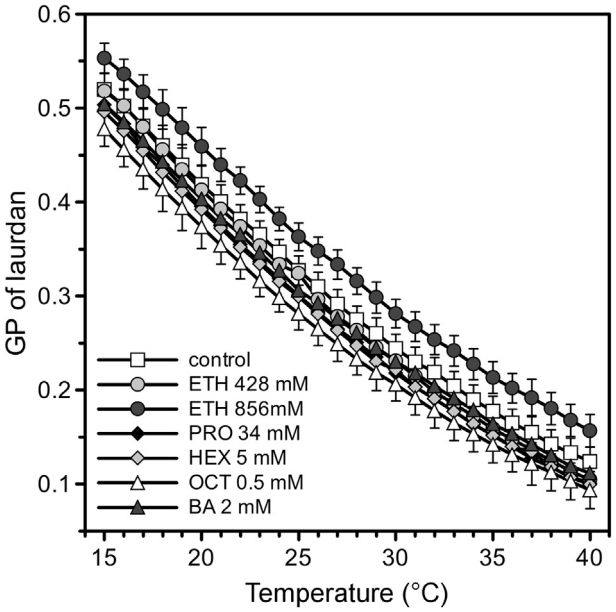

Fig. 4. Effect of alcohol concentration on generalized polarization of Laurdan fluorescence (GP). Cytoplasmic membranes were isolated from *B. subtilis* M19 cells grown at 40 °C in the complex medium and labelled with Laurdan. Alcohols were added to the final concentration indicated. Abbreviations: ETH - ethanol, PRO - propanol, HEX - hexanol, OCT - octanol, BA - benzyl alcohol. The data points correspond to the mean values (n = 3) and their standard errors.

on the alcohol concentration was weak; therefore only concentrations of alcohol which decreased *des* expression by about 50% were used. The effect of alcohols on the GP values is presented in Fig. 4. Control GP values decreased from approximately 0.52 (at 15 °C) to 0.11 (40 °C). With the exception of 856 mM ETH, all other alcohols including 428 mM ETH decreased GP and caused a downshift in the dependence of GP on temperature. The most effective decrease was caused by octanol and hexanol. In contrast with the anisotropy of DPH, benzyl alcohol had only a small effect on GP. However, the downshifts in the temperature dependences of GP caused by alcohols were relatively low compared to those of the anisotropy of DPH. Butanol is not included in Fig. 4 because the GP value could not be stabilized after the application of butanol, for unknown reasons.

3.4. Alcohols caused only negligible decreases in the midpoint of the phase transition in membrane phospholipid vesicles of B. subtilis

In our previous work, we demonstrated that *B. subtilis* cultivated at 20 °C decreases the midpoint of the phase transition (T_m) of its membrane phospholipids by approximately 10 °C compared to those of the cells cultivated at 40 °C [30]. Here, we investigated whether the alcohols were able to lower the T_m of the membrane phospholipids. The concentrations of alcohols used for these experiments were the ones that substantially decreased the induction of Des after cold shock (see Fig. 1). The corresponding T_m values were calculated from the DSC thermograms obtained. Table 2 summarizes the results and compares the T_m values of the control with those of the samples with added alcohols. In all samples with alcohols, the T_m value was not decreased by > 2 °C. Therefore, the alcohol concentrations that decreased *des* expression after cold shock were not able to substantially decrease the T_m of their membrane phospholipids.

4. Discussion

Recently, the DesK membrane fluidity sensor of *Bacillus subtilis* became a well-defined model thermosensor [36] that detects the decrease in temperature by a conformational change of its transmembrane

Table 2
Midpoint of phase transition (Tm) of bacterial lipids isolated from *B. subtilis* M19 cells grown at 40 °C in complex medium. The values of T_m were deduced from DSC heating curves obtained for unilamellar liposomes from bacterial lipids after the addition of the different alcohols, see Materials and Methods section. Control: lipids without alcohols. Abbreviations: T_m - midpoint of the phase transition.

Alcohol	Concentration (mM)	T_m (°C)
No alcohol (control)	0	11.35
Ethanol	856	9.61
Propanol	68	11.06
Butanol	150	10.25
Hexanol	5	10.52
Octanol	1	9.78
Benzyl alcohol	2	11.13

segments (TMS). A detailed mechanistic explanation of the molecular mechanism of temperature-dependent DesK sensing was enabled by employing a chimerical single-membrane spanning segment [9], a so-called minimum sensor (MS-DesK), a fully functional variant comprising the N-terminal and C-terminal parts of the first and fifth DesK segments, respectively. This chimerical construction and full length DesK contain two hydrophilic motifs critical for temperature sensing: 1) At the N-terminus, Lys-10 was shown to locate the transmembrane segment at the water-lipid interphase, with lysine 10 pulling towards the hydrated region. 2) At the C-terminus of the helix, serines at positions 23, 30 and 33, work together as a serine zipper that reorients the transmembrane segments according to temperature and this is critical for the signal transduction to the cytoplasmic domain [13]. It has been proposed that the hydrogen-bond zipper would stabilize in thicker membrane when temperature decreases, while it would destabilize in a thinner membrane when temperature increases, due to competition with water molecules [13]. The opposing forces of Lys-10 and the relative position of Ser- 23, Ser-30 and Ser-33 with respect to the lipid-water interface were shown to be dependent on the membrane thickness. The change in the total hydrophobic thickness in membrane lipids of *B. subtilis* cooled from 37 to 25 °C was determined to be approximately 1 Å by employing small-angle X-ray scattering [14]. A hypothesis was proposed that a hydrophobic mismatch between the C-terminus of MS-DesK and membrane lipids created by a slight thickening of the membrane is sufficient to overcome the free energy barrier between the phosphatase-active and kinase-active forms of DesK. Naturally, the change in the membrane thickness is dependent on the magnitude of the temperature downshift, but it could be higher when the membrane lipids approach the fluid-to-gel transition; therefore, other factors that influence the phase transition can also modulate the activity switch of DesK [13].

Our previous results from differential scanning calorimetry [30] showed that lipids from *B. subtilis* cells cultivated at 40 °C exert a broad phase transition with a T_m (transition peak at maximal peak height) at 14.8 °C. Hence, the cells transferred from 40 °C to 20 °C must be more sensitive to the disturbance caused by alcohols than cells transferred from 40 °C to temperatures higher than 20 °C, outside the interval of the phase transition.

4.1. Role of membrane thickness in membrane-alcohol interaction

Alcohols used in this work were able to suppress *des* expression at low temperature, suggesting that alcohol incorporation into lipid membranes has a fluidizing effect, mimicking the conditions of the increase in temperature. When PRO, HEX, OCT and BA are considered, all these alcohols induced fluidization in the membrane hydrophobic region sensed by DPH. ETH, on the other hand, caused the dehydration in the phospholipid headgroup region sensed by Laurdan. Both effects had to be appropriately sensed by DesK, because the measured biophysical changes were reflected accordingly in *des* expression. The hydrophilic motifs that localize at both ends of the transmembrane

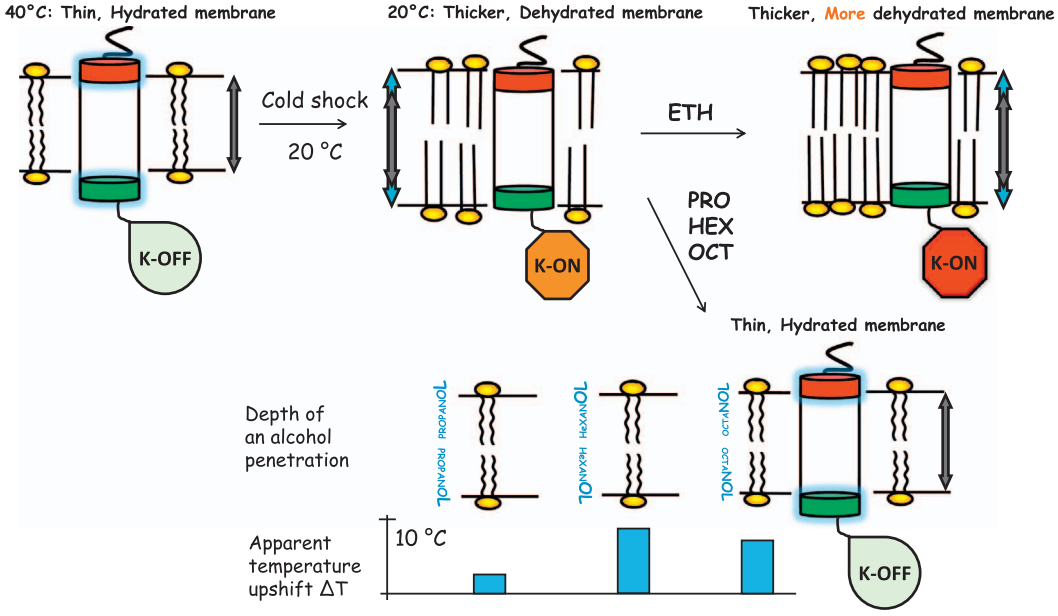

Fig. 5. A model for the interaction between DesK and aliphatic alcohols. Model schematically shows the membrane-spanning part of DesK with hydrophilic motifs (red and green cylinders) connected to its cytoplasmic part in kinase-on or kinase-off states. Penetration of propanol, hexanol and octanol into the membrane hydrophobic region is roughly proportional to the the relative height of their letters. At 20 °C, these three alcohols mimic the different temperature upshifts. For explanation see text. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

segment of DesK, which are responsible for sensing membrane fluidity, would be accessible to water in a more fluid and thinner lipid membrane [14]. Unmasking the hydrophilic motifs is associated with the kinase-off conformation of the kinase, and consequently null *des* expression. The question arises of whether the effect of alcohols shown in this work can also be universally explained by the influence of these alcohols on membrane thickness.

Reduction of the membrane thickness by alcohols was reported recently for synthetic phospholipids with propanol, butanol, methanol and ethanol [21]. Similar results were obtained using molecular dynamics simulations for ethanol and methanol [26]. Recently published data indicate that 1.28 M ethanol shortens the transmembrane interphosphate distance by 10% in POPC membranes [25]. In SOPC vesicles, a 5% decrease in membrane thickness was calculated for 1 M ethanol and for 150 mM propanol. Such effects are comparable with the membrane thickness decrease of 4% after the temperature downshift from 37 °C to 25 °C observed in *Bacillus* lipids [14]. Therefore, we can conclude that the decrease in membrane thickness by alcohols must also play a role here.

Another factor that can be also responsible for the decreases in membrane thickness is the interdigitated state of the lipids that can be induced by alcohols. Such an interdigitated phase, $L_{\beta I}$, where the terminal methyl groups of one monolayer are located near the polar interface of the other membrane leaflet, is more tightly packed and consequently increases the transition temperature [37]. Based on our DSC results (see Table 2), we can conclude that none of the investigated alcohols at the concentrations used in this work were able to induce interdigitation in the *B. subtilis* lipid bilayer.

4.2. Ethanol and butanol toxicity

Ethanol is one of the growth-limiting environmental stresses that induce the general stress response, both dependent on and independent of the sigma factor sigB of *Bacillus subtilis* when applied at 3–5% (v/v, i.e. 513–856 mM, respectively) [38,39]. At these concentrations ethanol slightly decreased growth rate, similar to our results.

Ethanol-induced increase in *des* expression is associated to lowered membrane hydration measured by Laurdan GP, but the effect was only confirmed for the higher ethanol concentration of 856 mM. However, the ordering of the membrane periphery by ethanol was also supported by a slight increase in TMA-DPH anisotropy (for both 428 and 856 mM concentrations of ethanol, results not shown). TMA-DPH resides in a similar region to Laurdan.

1-butanol (BUT) decreased the growth rate at all concentrations that also decreased *des* expression (Table 1) and, at the same time, abolished constitutive Des synthesis (Fig. 2) in LCK20. BUT also delayed the onset of *des* expression after cold shock (Fig. 1). The inhibiting effect of BUT on the cellular metabolism was studied in eukaryotic organisms and, to a lesser extent, in bacteria. It was recently shown that BUT, but not its secondary and tertiary isomers, inhibits intracellular signalling by phosphatidic acid because only BUT could serve as a substrate for phospholipase D [40]. Also, only BUT was shown to inhibit voltage-gated channels in eukaryotes [41]. In order to compare the effects of BUT and isobutanol (ISB) on *des* expression and membrane fluidization, we performed control experiments with ISB at concentrations of 100, 125, 150 mM (results not shown). We concluded that the effects of BUT and ISB on the physiology of *Bacillus subtilis* were comparable, and that the mechanism of inhibition is probably different from the one observed in eukaryotes. Interestingly, both BUT and ISB exhibited an inhibitory effect on *E. coli* respiration, as was shown by transcriptome analysis [42].

4.3. Effect of alcohols on DesK sensing

At 20 °C, the aliphatic alcohols propanol, hexanol and octanol were able to mimic the state of the membrane at 40 °C. To make a quantitative comparison between the temperature effect and the one caused by a given alcohol, it is possible to compare the drop in fluorescence anisotropy caused by increase of temperature with the one caused by the isothermal addition of alcohol. Data from Fig. 3 show that downshifts in anisotropy dependences caused by alcohols in concentrations that suppressed *des* expression mimic a lower than 20 °C increase in temperature. For PRO, HEX and OCT (68, 10 and 1 mM concentrations, respectively), the corresponding temperature increases were 2.8, 9.5 and 7.8 °C. The value for HEX is slightly underestimated, as 10 mM HEX does not abolish *des* expression completely. The discrepancy between

the effect of alcohols sensed by DesK and the one sensed by DPH anisotropy can be explained in terms of the effect of aliphatic alcohols on the membrane order depending on their length. Generally, the potency of shorter alcohols to fluidize the membrane increases with their chain length and decreases beyond a certain chain length limit [43]. The limit is approximately equal to the half of the acyl chain length of the bilayer-forming lipid [44], probably due to compensation for the disordering in the polar headgroup region by the longer chain of the alcohol [43]. At the same time, alcohols with longer chains decrease the lipid order deeper in the bilayer [45]. We concluded that the disordering effect of propanol does not reach the region sensed by DPH while with hexanol, regions of disorder and those sensed by DPH overlap better. With octanol, however, the hydrophobic region sensed by DPH becomes more ordered, because the length of OCT exceeds the half-length of the average fatty acid length of *Bacillus* phospholipids (approximately 15.5 carbon atoms) [46].

The effect of alcohols is summarized in Fig. 5, a model for the interaction between DesK and aliphatic alcohols. At 40 °C, the hydrophilic motifs (red and green cylinders) are exposed to water (highlighted with blue). This conformation is associated to a kinase-off state of DesK. At low temperature, the increase of membrane thickness results in dehydration of the sensing motifs and activation of the kinase state. Incorporation of propanol, hexanol and octanol to the membrane cause fluidization and increase of hydration of the membrane, leading to a kinase-off conformation even at low temperature. In contrast, ethanol decreases the hydration of the membrane and further enhances kinase state of DesK.

5. Conclusions

In this work we proved that the fluidization of the membrane by alcohols resulted in a decrease in P*des* expression. Such a change in DesK-mediated signalling was probably due to enhanced hydration of the membrane periphery, which was able to unmask the sensible part of DesK, inducing its conformation change and decreasing its phosphorylation activity. We concluded that the membrane thickness is the most likely factor to mediate the effect of alcohols after cold shock in *Bacillus subtilis*. We can therefore expect that other exogenous fluidizing agents will decrease the kinase activity and rigidifying agents will support the kinase activity of DesK.

Transparency document

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