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Metabolic control of the membrane fluidity in *Bacillus subtilis* during cold adaptation

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Abstract

Membrane fluidity adaptation to the low growth temperature in *Bacillus subtilis* involves two distinct mechanisms: (1) long-term adaptation accomplished by increasing the ratio of anteiso- to iso-branched fatty acids and (2) rapid desaturation of fatty acid chains in existing phospholipids by induction of fatty acid desaturase after cold shock. In this work we studied the effect of medium composition on cold adaptation of membrane fluidity. *Bacillus subtilis* was cultivated at optimum (40 °C) and low (20 °C) temperatures in complex medium with glucose or in mineral medium with either glucose or glycerol. Cold adaptation was characterized by fatty acid analysis and by measuring the midpoint of phospholipid phase transition T_m (differential scanning calorimetry) and membrane fluidity (DPH fluorescence polarization). Cells cultured and measured at 40 °C displayed the same membrane fluidity in all three media despite a markedly different fatty acid composition. The T_m was surprisingly the highest in the case of a culture grown in complex medium. On the contrary, cultivation at 20 °C in the complex medium gave rise to the highest membrane fluidity with concomitant decrease of T_m by 10.5 °C. In mineral media at 20 °C the corresponding changes of T_m were almost negligible. After a temperature shift from 40 to 20 °C, the cultures from all three media displayed the same adaptive induction of fatty acid desaturase despite their different membrane fluidity values immediately after cold shock.
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Keywords: Bacillus; Fatty acid synthesis regulation; Cold shock; DPH; DSC

1. Introduction

Ambient temperature decrease induces substantial adaptive changes in the prokaryotic cell. First, bacteria change the composition of their membrane fatty acids in order to maintain the membrane in a liquid crystalline phase which is essential for its proper function [1]. Besides, a set of cold inducible proteins responsible for the modification of the protein translation machinery is induced [2]. The fatty acid profile of *Bacillus subtilis*, a model gram-positive organism, is dominated by the iso- and anteiso-branched fatty acids. Branched amino acids are used as the precursors for their synthesis: isoleucine for anteiso- and valine and leucine for iso-branched fatty acids. The melting point of anteiso-branched fatty acids is significantly lower than for their iso-branched isomers [3].

Bacillus subtilis cells use two distinct mechanisms of adaptation to low temperature. The long-term membrane adaptation employs an increase in low melting anteiso-branched fatty acids (mostly a-15:0 and a-17:0) that effectively fluidize the membrane [4,5]. The regulation of the anteiso-/iso-branched fatty acid ratio is, however, far from being understood. After cold

Abbreviations: CM, complex medium with glucose; MMGlu, mineral medium with glucose; MMGlyc, mineral medium with glycerol; DPH, 1,6-diphenyl-1,3,5-hexatriene; FA, fatty acid; i-, iso-branched fatty acid; a-, anteiso-branched fatty acid; n-, non-branched fatty acid; r_{ss}, steady state anisotropy of DPH fluorescence; T_m, gel to liquid-crystalline phase transition temperature; T_c, temperature of cultivation

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shock, a different mechanism for membrane fluidization is employed. Synthesis of the fatty acid desaturase (Des) is induced that catalyses fatty acid desaturation of phospholipids already incorporated in the membrane [6,7]. The induction of fatty acid desaturase was found to be regulated by a two-component system consisting of sensor kinase DesK and the response regulator DesR [7], with the transmembrane domain of the DesK protein serving presumably as a sensor of membrane fluidity [8]. Des synthesis is inhibited by the presence of anteiso-branched fatty acid precursors or by unsaturated fatty acids in medium [9].

It has been hypothesized that bacterial cytoplasmic membranes can compensate for different cultivation temperatures by a process known as homeoviscous adaptation. This concept was introduced by Sinensky [10] as the mechanism for the maintenance of the constant membrane viscosity independent of ambient temperature. Further studies, however, did not validate homeoviscous adaptation as a universal mechanism of membrane cold adaptation [11]. The concept of "homeoviscous efficacy" was introduced later as a measure of the extent to which temperature-induced changes of membrane state are compensated by an adjustment of membrane lipid composition [12].

In *Bacillus subtilis*, the homeoviscous adaptation concept was demonstrated to be invalid [5] despite the fact that this organism substantially increases the level of fluidizing anteiso-branched fatty acids at low cultivation temperature. We also demonstrated later that the biophysical parallel to the changes in the lipid composition in *Bacillus subtilis* is the maintenance of an optimal lipid order of the cytoplasmic membrane rather than membrane microviscosity [13].

Few data are available concerning the adaptation of membrane composition and membrane physical parameters to the growth rate or carbon source. In *Escherichia coli* grown at various growth rates brought about by different carbon sources in the cultivation media, its membrane fluidity (determined on the basis of DPH fluorescence polarization) was shown to be the highest in the culture with the highest growth rate [14]. The authors concluded that the faster growing cells required a faster transport of nutrients which was enabled by higher membrane dynamics. Recently, growth rate dependent changes in *Escherichia coli* membrane were studied in chemostatic culture [15]. The authors analyzed the fatty acid composition, membrane mechanical strength and the leakage of the recombinant periplasmic proteins into mineral medium with glucose within a dilution rate interval of $D=0.6\ h^{-1}$ (maximum growth rate) and $D=0.05\ h^{-1}$. The maximum protein transport and the highest content of fluidizing fatty acids were found at $D=0.3\ h^{-1}$. This dilution rate and the corresponding growth rate were interpreted as the state with maximum membrane fluidity.

In this work we studied the cold adaptation of the cytoplasmic membrane in *Bacillus subtilis* during growth in media with different carbon sources (complex medium, mineral medium with either glucose or glycerol). In contrast with complex medium, the branched fatty acid precursors that are important for an effective membrane fluidization were not present in mineral media. First, the membrane cold adaptation in different media was characterized by changes in fatty acid composition that enabled membrane fluidization at cold temperature. These changes were further correlated with the shifts of gel to liquid-crystalline phase transition temperatures of membrane lipids and with the temperature profiles of DPH fluorescence anisotropy used here as a measure of membrane fluidity. With this approach it was possible to show that the extent of cold adaptation was markedly dependent of medium used. At the same time, we suggested the possible mechanisms for metabolic control of fatty acid synthesis in different media.

2. Materials and methods

2.1. Bacterial strains

Bacterial strains used in this study were *Bacillus subtilis* 168 (*trpC2 sfp0*) (wild type, laboratory stock) and *B. subtilis* strain M19 (168 *amyE::Pdes-lacZ*) constructed in this work. The transcriptional fusion between *des* promotor and *lacZ* gene on the strain 168 background was constructed according to [6] using the same primers for the fragment amplification and integrational vector pDG1661 [16], for ectopic integration into *AmyE* locus. The sequence of the construct was confirmed by sequencing.

2.2. Cultivation of bacteria

Bacteria were grown aerobically in three different media containing the same minimal salt basis supplemented with glutamic acid (0,025%) [17] and different carbon sources — either glucose 5 g/l (for minimal medium with glucose, MMGlu) or glycerol 2.5 g/l (for minimal medium with glycerol, MMGlyc). The minimal media were also supplemented with L-tryptophan (50 mg/l). Complex medium (CM) contained the same minimal salt basis as the mineral ones, glucose (5 g/l), beef extract (1.5 g/l, Difco), yeast extract (1.5 g/l, Oxoid) and peptone (5 g/l, Oxoid). Chloramphenicol in final concentration 5 µg/ml was added in the media for strain M19 cultivation.

The temperature of cultivation (T_c) was 40 °C or 20 °C, liquid cultures were cultivated with shaking. The cold shock was performed as a rapid transfer of bacteria from 40 °C to 20 °C during the mid-exponential phase of culture growth, which was estimated turbidimetrically at 450 nm (spectrophotometer Beckman DU530).

2.3. Membrane isolation

The cells in mid-exponential phase of growth were harvested by filtration (filters Pragopor No. 5, ø 0.6 µm, Pragochema, Czech Republic) and membrane isolation was performed as described before [18] with a few modifications. The incubation with lysozyme was performed at the temperature of cultivation of the initial culture, e.g. at 40 °C (for 30 min) or at 20 °C (for 60 min). The membrane pellet was resuspended in 10 mM Tris–HEPES buffer, pH 7.4 and stored at −80 °C.

For anisotropy measurement the samples were thawed only once and diluted in the same buffer to the final protein concentration of approximately 70–100 µg/ml. The Pierce BCA Protein Assay (Pierce Biotechnology) was used for protein determination.

2.4. Lipid isolation

Chloroform–methanol extraction was used for lipid isolation. The harvested cells from 500 to 1000 ml of culture in mid-exponential phase were resuspended in a small amount (approximately 2 ml) of 60 mM phosphate buffer, pH 7.4, and mixed thoroughly with extraction solution (a mixture of chloroform, methanol and phosphate buffer 1:2:0.8 v/v/v) and extracted at room temperature with mild shaking for 2 h. The cells were separated by centrifugation (3200×*g*, 10 min). The supernatant was removed into a clean tube and the phases were separated by adding one volume of chloroform (equal to the volume of chloroform in initial extraction solution) and the same volume of distilled water. After vigorous shaking for 1 min the mixture was left standing for 12 h at 6 °C and then centrifuged (3200×*g*, 10 min). The upper methanol–water phase was discarded

and the lower chloroform phase was removed to a clean tube and evaporated using a flow of nitrogen. The lipid isolates were stored at −20 °C in a desiccator.

For fluorescence anisotropy measurement 0.3–0.5 mg of lipids was dissolved in chloroform and then evaporated by a flow of nitrogen in a thin layer on the walls of a glass tube. Then 3–4 ml of Tris–HEPES buffer (10 mM, pH 7.4) was added and multilamellar liposomes were formed by vigorous shaking of the tube. Large unilamellar vesicles of mean size of 400 nm were prepared by repeated extrusion of multilamellar vesicles using the LiposoFast Basic apparatus (Avestin, Canada) with a polycarbonate membrane of 400 nm pore diameter (Avestin), as previously described [19].

2.5. Fluorescence anisotropy measurement

The fluorescent probe 1,6-diphenylhexatriene (DPH, Sigma) in acetone was added at a final concentration of 10^{-6} M to the *Bacillus subtilis* membrane vesicles or to the unilamellar liposomes from *Bacillus* lipids prepared as described above. The volume of a sample was 2.5 ml. The sample was then incubated in the dark at 37 °C for 30 min.

Steady state fluorescence anisotropy measurements were performed with FluoroMax-3 spectrofluorimeter (Jobin Yvon Horriba, France) equipped with DataMax software and polarization accessory, with excitation and emission wavelengths 360 nm and 430 nm, respectively. The temperature of the sample was checked with an accuracy of 0.1 °C using a thermistor thermometer. The background fluorescence of non-labelled samples did not exceed 2% of the experimental values. The steady-state anisotropy was calculated according to Lakowicz [20]. The steady-state anisotropy values presented below correspond to the average of at least three determinations performed with independent membrane preparations.

2.6. Fatty acid analysis

0.3 mg of isolated whole cell lipids (see part 2.4) was an amount sufficient for identification of the fatty acid (FA) profile of *B. subtilis* under different growth conditions. The concentrated lipids were subjected to a mild alkaline methanolysis by dissolving in 1 ml methanol–toluene mixture (1:1, v/v) and 1 ml of 0.2 M KOH, and heating for 15 min at 37 °C. Then 2 ml H_2O and 0.3 ml 1 M acetic acid were added; the resulting fatty acids methyl esters (FAME) were extracted twice with 2 ml of a hexane–chloroform mixture (4:1, v/v). The FA (in the form of FAME) were separated by gas chromatography (Agilent 6850, Agilent Technologies, USA) with flame ionization detector on a capillary column (Ultra 2, 25 m, 0.20 mm, 0.33 µm, Agilent Technologies, USA). The samples (1 ml) were injected in split mode (1:100); injection temperature was 250 °C; the carrier gas was hydrogen; the temperature regime on the column was 170 °C–5 °C min^{-1}–260 °C–40 °C min^{-1}–310 °C–1.5 min. Individual FA peaks were identified using an automatic identification system (MIDI Inc., USA).

2.7. DSC measurement

Thermal properties of isolated lipids were studied using phospholipids model structures. Dry lipid films prepared as described above were suspended in a buffer (1 mM EDTA, 10 mM HEPES, 50 mM KCl, pH 7.4) at a concentration of about 1 mg of lipids per ml. Unilamellar liposomes were prepared as described above. DSC experiments were performed using a CSC Model 6100 Nano II Differential Scanning Calorimeter (Calorimetry Sciences Corporation, USA). The sample cell was filled with 400 µl of a suspension of lipid vesicles in buffer, equal volume of the same buffer was used as a reference. The sample and reference cells were sealed and thermally equilibrated for about 10 min below the starting temperature of the run. The data were collected in the range of 2–70 °C at the scan rate 1 °C min^{-1} both for heating and cooling. The reference scan was subtracted from the sample scan and each data set was analysed for thermodynamic parameters with the CpCalc software package supplied by the producer. The accuracy for the phospholipids phase transition temperature was ± 0.1 °C. The measurements were repeated at least twice for two independently prepared samples. The thermotropic behaviour of extracted lipids was also observed during the successive cooling scans (after 10 min of equilibration at 70 °C, data not shown). The transition pathways in heating and cooling displayed significant hysteresis arising from both the finite response time of the calorimeter and the different lateral mobility of lipid chains in the gel and liquid crystalline phase. The pattern observed for cooling is essentially similar to the corresponding heating one with progressive broadening and shift of phase transition peak to the lower temperatures of about 1.8 °C (not shown).

2.8. β-Galactosidase activity assay

β-Galactosidase activity was analysed according to Miller [21] with some modifications for gram-positive *Bacillus* cells. The samples of the culture were taken during growth, the cultivation medium was removed by centrifugation (1 min, 23,900×*g*) and the samples were frozen in liquid nitrogen and stored at −20 °C until assayed. For the β-galactosidase activity assay cell samples were diluted in Z-buffer and optical density at 525 nm was measured. Aliquots (50–250 µl) were then taken, the volume was adjusted to 730 µl with Z-buffer and 10 µl of lysozyme solution (10 mg/ml in water) was added, samples were thoroughly mixed and incubated at 37 °C for 20 min. Then 10 µl of 10% (v/v) TritonX solution and 100 µl of ONPG solution (4.5 mg/ml in water) were added, samples were again mixed and incubated at 28 °C until yellow colour appeared. The reaction was then stopped by adding 150 µl of 1.2 M Na_2CO_3 solution and the time of the reaction was recorded. Finally the absorbance at 420 nm (A_{420}) was measured for each sample. β-Galactosidase activity in Miller units (MU) was calculated according to the formula $MU = 1000 \times A_{420}/(O.D._{525} \times \text{time in min} \times \text{aliquot volume in ml})$. Each cell suspension sample was analysed in triplicate.

3. Results

3.1. Growth of Bacillus subtilis 168 in different media at cultivation temperatures T_c40°C and T_c20°C and after the cold shock from T_c40°C to T_c20°C

Bacillus subtilis strain 168 was cultivated in different cultivation media at both optimal (T_c40°C) and low (T_c20°C) temperature. Three media types were used containing the same mineral basis and differing in the carbon source: complex medium with glucose and mineral media with either glucose or glycerol, abbreviated here as CM, MMGlu and MMGlyc,

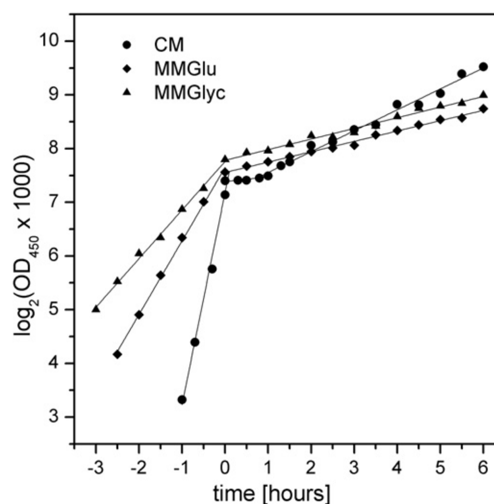

Fig. 1. Growth of *B. subtilis* 168 in different cultivation media at 40 °C and after the cold shock from 40 to 20 °C. *B. subtilis* cultures were grown in complex medium with glucose CM, mineral medium with glucose MMGlu and mineral medium with glycerol MMGlyc. After inoculation (OD_{450} of approximately 0.01–0.03) the cells were grown at 40 °C. In the mid-log phase (OD_{450} of 0.17–0.2) the cultures were subjected to cold shock from 40 to 20 °C. The time of transfer is identified as the zero point at the time scale. Generation times at 40 °C were 15, 44 and 66 min for CM, MMGlu and MMGlyc, respectively. At 20 °C, corresponding generation times were 183, 311 and 312 min, respectively.

respectively. At T_c40°C, the generation times of *Bacillus subtilis* growing in CM, MMGlu and MMGlyc were 15, 44 and 66 min, respectively. At T_c20°C, generation times increased to 186, 311 and 312 min in CM, MMGlu and MMGlyc, respectively.

After cold shock, two types of growth behaviour were observed (Fig. 1). In the case of cells grown in complex medium (CM) the cold shock induced a 60-min long growth-lag after which the cells started to grow with a generation time corresponding to their steady-state exponential growth at T_c20°C. In contrast, cells grown in both mineral media (MMGlu and MMGlyc) continued to grow after the cold shock without any lag, with the same generation time values corresponding to their steady-state exponential growth at T_c20°C.

3.2. Fatty acid profiles of Bacillus subtilis cells cultivated in different media at T_c40°C and T_c20°C

To identify the profile of fatty acids (FA) synthesized by *B. subtilis* under different growth conditions, we carried out comparative membrane FA analysis of *B. subtilis* strain 168 grown in CM, MMGlu and MMGlyc media at both cultivation temperatures T_c40°C and T_c20°C. We studied how medium composition modified the cold adaptation at T_c20°C by comparing the FA profiles for given medium at T_c40°C and T_c20°C. The cell cultures used for isolation of membrane lipids and cytoplasmic membranes (see below) are abbreviated further as CM-T_c40°C, MMGlu-T_c40°C, MMGlyc-T_c40°C and CM-T_c20°C, MMGlu-T_c20°C, MMGlyc-T_c20°C in case of T_c40°C and T_c20°C cultures, respectively. Fig. 2A shows nine main fatty acid species including unsaturated fatty acids of *Bacillus subtilis*. In addition, some minor fatty acid species were detected showing no significant temperature dependence.

In the complex medium CM at either T_c40°C or T_c20°C the FA profiles showed more than 80% of iso- and anteiso-branched fatty acids (dominated by i-15:0, a-15:0, i-17:0 and a-17:0 FA). The lipids from lower cultivation temperature T_c20°C exhibited an increase in anteiso-branched fatty acids and a concomitant decrease of iso-branched and straight chain FA species (Fig. 2A, B).

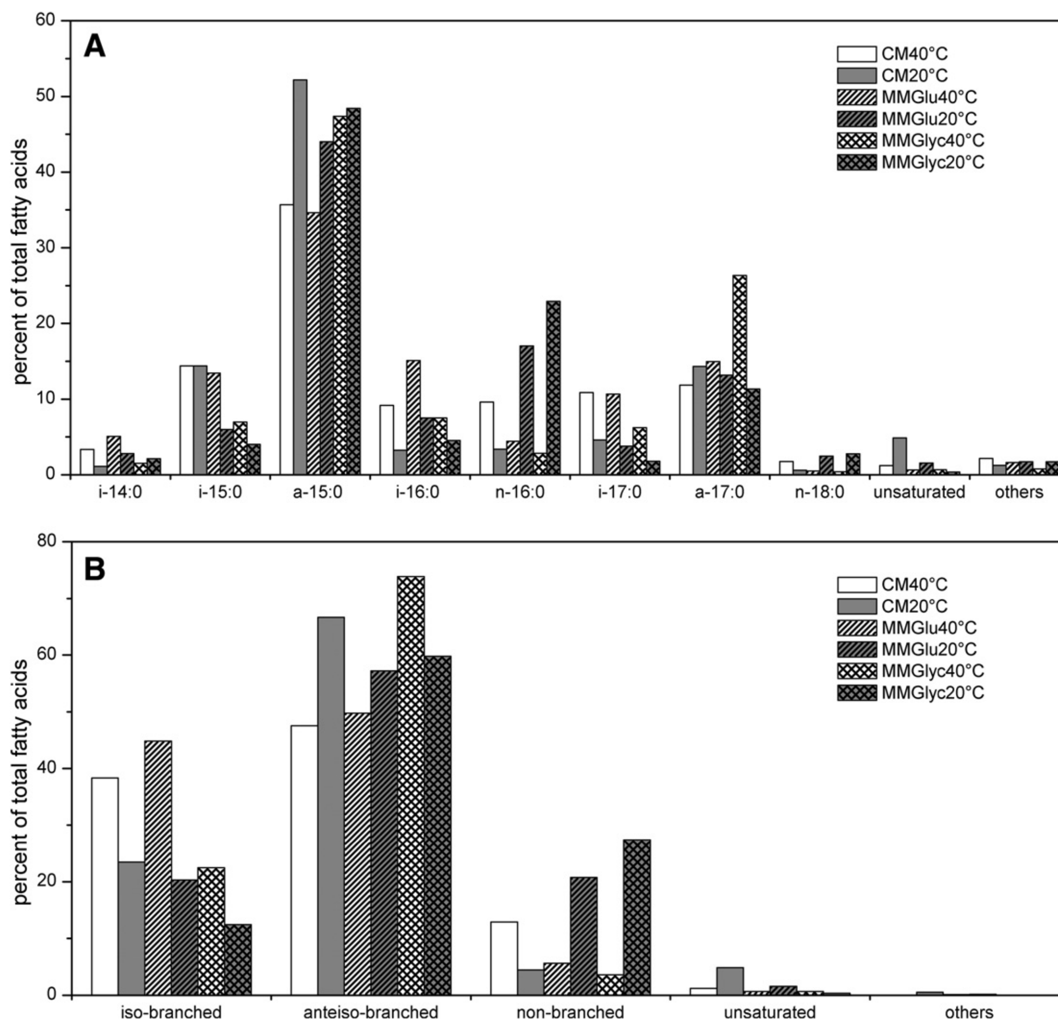

Fig. 2. The effect of growth conditions on the composition of *B. subtilis* membrane fatty acids. *B. subtilis* cells were grown in three different media at a constant temperature of 40 °C or 20 °C and harvested in the mid-log phase (at OD_{450} of 0.5–0.6) for lipid isolation and fatty acid analysis. From a complete fatty acid profile recorded for each FA sample we show the frequency of the most prominent species (A) and main structural fatty acid types (B). *i-*, *a-*, *n-* denotes the respective branching pattern for *iso-*, *anteiso-* and *non-*branched fatty acids, respectively. CM, complex medium with glucose; MMGlu, mineral medium with glucose; MMGlyc, mineral medium with glycerol; 40 °C or 20 °C denotes the temperature of cultivation.

These FA profiles were in agreement with previously published data [4,5,22].

When the FA profiles of the CM-T_c40°C cells and MMGlu-T_c40°C cells were compared, the latter showed an increased level of i-16:0 and a-17:0 and a decreased level of the straight chain n-16:0 (Fig. 2A). Such result can be interpreted as the fluidization of MMGlu-T_c40°C membranes. In case of MMGlyc-T_c40°C cells, their FA profile displayed obvious differences in almost all major FA types, when compared with that of CM-T_c40°C cells. The most striking feature in MMGlyc-T_c40°C FA profile was the very high level of low-melting a-C15:0 and a-C17:0 FA, together with the low proportion of iso-branched and straight-chain FA. Such high levels of fluidizing anteiso-branched fatty acids have so far been considered to be typical for cold-adapted cells [4,5] and not for cells cultivated near their growth temperature optimum.

Further, we studied the extent of cold adaptation in CM, MMGlu and MMGlyc media by comparing T_c20°C and T_c40°C lipids for given medium (Fig. 2A, B). The low-temperature-induced changes in FA compositions in mineral media were compared with the ones for the complex medium CM that represented the cold-induced FA adaptation already published by several authors [4,5]. Our comparison showed that low-temperature-induced FA profile changes for MMGlu medium were rather close to that of CM lipids while the respective adaptations of MMGlyc lipids were different. MMGlu-T_c20°C cells (in comparison with CM-T_c20°C cells) displayed marked decrease of i-15:0, a much lower increase of a-15:0, and a striking increase of straight-chain n-16:0 fatty acid (Fig. 2A). When the same comparison was made between MMGlyc and CM cells, the differences between T_c40°C and T_c20°C lipids were much more obvious. In MMGlyc-T_c20°C the proportion of a-17:0 drastically decreased while the level of straight chain n-16:0 FA increased. In case of the major FA a-15:0, almost the same level was maintained at both T_c40°C and T_c20°C in MMGlyc derived lipids.

In further experiments we addressed the question how the alterations in chemical composition of membrane fatty acids induced by growth in different media changed the respective biophysical characteristics of membranes.

3.3. The same membrane physical state was maintained in the membranes of cells cultivated at T_c40°C in different media. Their membrane lipids, however, exhibited different phase transition temperatures

Fig. 3A shows the DPH fluorescence anisotropy r_{ss} measured over the temperature interval from 10 °C to 45 °C. These measurements were made with both DPH-labelled phospholipids and cytoplasmic membrane vesicles, derived from CM-T_c40°C, MMGlu-T_c40°C and MMGly-T_c40°C cells. Anisotropy measurements of cytoplasmic membrane samples at 40 °C (where temperatures of cultivation, T_c, and temperatures of measurement were the same) provided the same r_{ss} values for all three types of membranes. *B. subtilis* at T_c40°C, therefore, maintained the same membrane fluidity independent of the growth rate, carbon source or the presence of branched fatty acid precursors. With phospholipid samples the DPH anisotropy measurements provided very similar results (Fig. 3A).

At lower temperatures of measurement (10–25 °C), different r_{ss} values were observed for the three types of membranes showing the highest, medium and lowest r_{ss} values for CM-T_c40°C, MMGlu-T_c40°C and MMGly-T_c40°C cytoplasmic membranes, respectively (Fig. 3A). In this temperature interval, therefore, the CM-T_c40°C membranes were, surprisingly, the most rigid ones. Measurements of phospholipid vesicles at lower temperatures showed qualitatively similar but lesser r_{ss} differences between lipid samples.

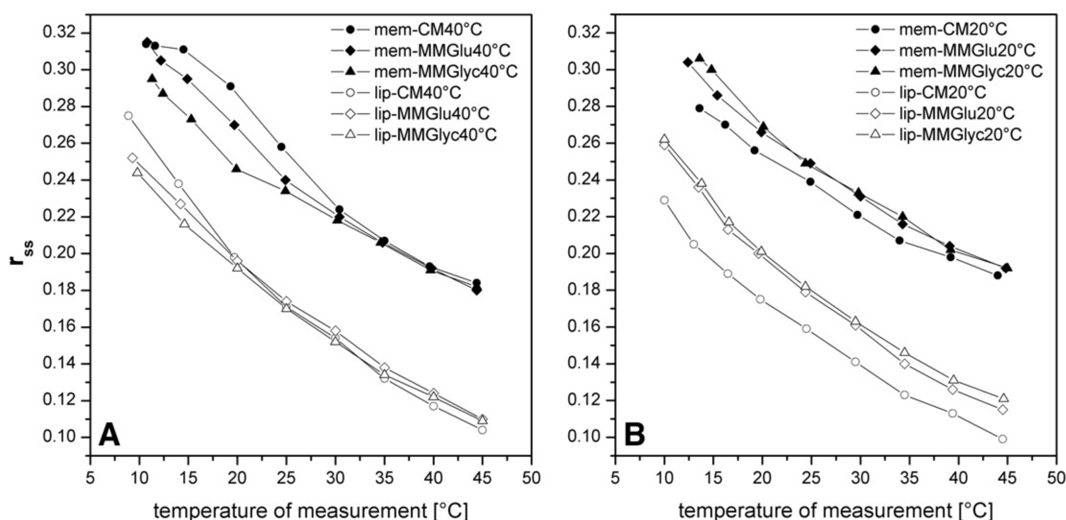

Fig. 3. Temperature dependence of DPH fluorescence anisotropy in cytoplasmic membranes and membrane lipids isolated from *B. subtilis* cultivated in different media at 40 °C (A) and 20 °C (B). Steady state fluorescence anisotropy (r_{ss}) of DPH embedded in cytoplasmic membrane vesicles (full symbols) or liposomes from membrane lipids (open symbols) was measured along the temperature scale as indicated on *x* axis. Cytoplasmic membranes or membrane lipids were isolated from *B. subtilis* 168 cells grown in different media at a constant temperature of 40 °C or 20 °C and labeled with DPH as described in Materials and methods. Abbreviations: "mem" and "lip" denotes membranes and membrane lipids, respectively. CM, complex medium with glucose; MMGlu, mineral medium with glucose; MMGlyc, mineral medium with glycerol; 40 °C or 20 °C denotes the temperature of cultivation.

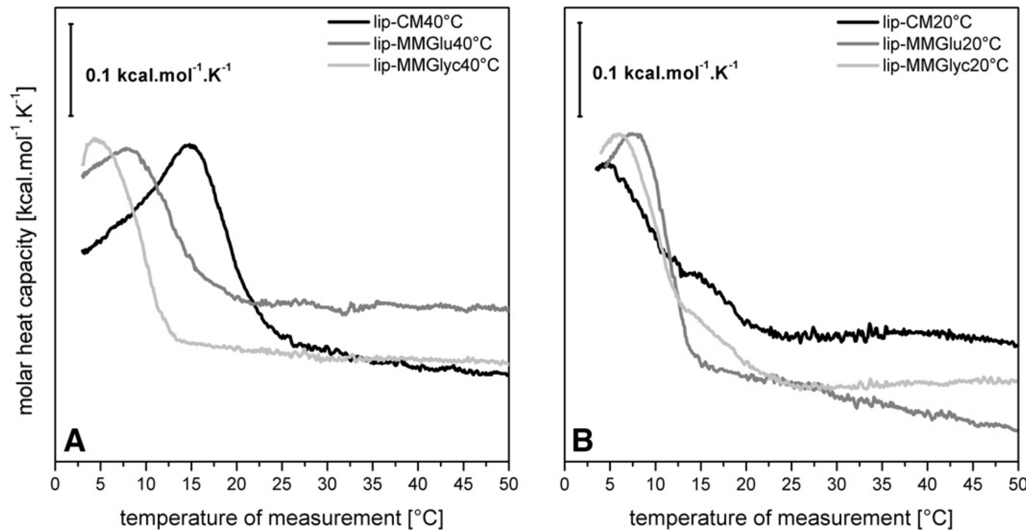

Fig. 4. DSC thermograms of membrane lipids isolated from *B. subtilis* cells cultivated in different media at 40 °C (A) and 20 °C (B). Shown here are the representative DSC heating curves obtained for unilamellar liposomes from bacterial lipids. Scans were collected at a heating rate of 1 °C min^{-1} from 2 to 70 °C, only relevant parts of the curves from 4 to 50 °C are shown. Calculated phase transition temperatures T_m that correspond to the transition peak at the maximal peak height were (A): 14.8, 7.8 and 4.3 °C for CM, MMGlu and MMGlyc, respectively, (B): 4.6, 7.3 and 6.2 °C for CM, MMGlu and MMGlyc, respectively. CM, complex medium with glucose; MMGlu, mineral medium with glucose; MMGlyc, mineral medium with glycerol; 40 °C or 20 °C denotes the temperature of cultivation.

DSC measurements of membrane lipids were employed to assess their respective melting temperatures T_m, the transition peaks at the maximal peak height (Fig. 4A). For CM-T_c40°C, MMGlu-T_c40°C and MMGly-T_c40°C lipid samples, their respective T_m were located at 14.4, 7.6 and 4.5 °C, respectively.

The same measurements employing DPH anisotropy and DSC were further performed with membranes and phospholipids derived from cells grown at T_c20°C to elucidate the extent of membrane fluidity adaptation in different media.

3.4. At cultivation temperature T_c20°C, *Bacillus subtilis* maintained the highest membrane fluidity in the complex medium

Fig. 3B shows the DPH anisotropy values r_{ss} for the membrane and phospholipid vesicles derived from cells grown at T_c20°C in CM, MMGlu and MMGlyc media. In contrast with T_c40°C cultivation temperature, the r_{ss} values for cytoplasmic membrane samples derived from CM-T_c20°C were always lower than those of MMGlu-T_c20°C or MMGlyc-T_c20°C ones, at any temperature of measurement. At T_c20°C, therefore, the cells grown in CM medium maintained the most fluid membranes. The differences between r_{ss} values for mineral media samples were not substantial, showing slightly higher values (less fluid membranes) for MMGlyc-T_c20°C membranes at the lower temperatures of measurement. In case of DPH-labelled phospholipid vesicles, higher differences were observed in DPH anisotropy between samples derived from CM and mineral media (Fig. 3B).

The melting temperatures (T_m) of the lipids isolated from CM-T_c20°C, MMGlu-T_c20°C and MMGlyc-T_c20°C cells were found at 3.9, 6.6. and 5.2 °C, respectively (Fig. 4B). For all three lipid samples their respective T_m values were located well below the temperature of cultivation (T_c20°C). In comparison with T_c40°C lipid samples, the T_m of CM-T_c20°C lipids decreased by 10.5 °C while T_m temperatures of lipids from MMGlu-T_c20°C and MMGlyc-T_c20°C cells decreased only by 1 °C or even increased by 0.7 °C, respectively.

3.5. After cold shock, cells grown in different media exhibited the same level of fatty acid desaturase induction despite their different membrane fluidity

In *Bacillus subtilis*, the induction of synthesis of fatty acid desaturase (Des) was shown to be an important short-term

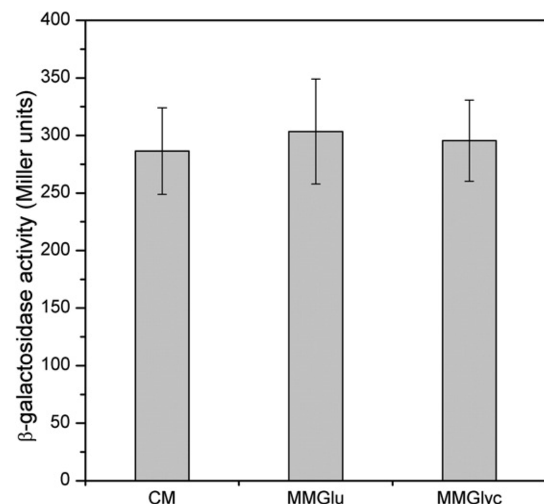

Fig. 5. Effect of the growth conditions on *Pdes-lacZ* expression in *B. subtilis* after cold shock. Fatty acid desaturase induction after the cold shock in different cultivation media was expressed in terms of β-galactosidase activity in *B. subtilis* M19 strain harboring *Pdes-lacZ* fusion. Cells were cultivated at 40 °C either in complex medium with glucose CM, mineral medium with glucose MMGlu or mineral medium with glycerol MMGlyc until the culture reached OD_{450} of 0.2 and then were shifted to 20 °C. β-Galactosidase activity expressed in Miller units (MU) was assayed at maximum induction (3 h after cold shock) as described in Materials and methods. The results shown are means of three independent experiments.

adaptation mechanism for membrane fluidization after cold shock [7,22]. We decided to determine whether the induction of fatty acid desaturase after cold shock from T_c40°C to T_c20°C was dependent on the medium used for *Bacillus subtilis* cultivation (CM, MMGlu, MMGlyc). *B. subtilis* M19 strain with the fusion of the *lacZ* gene to the *des* promoter integrated in the non-essential *amyE* locus was used for this purpose.

M19 cells were cultivated at T_c40°C in CM, MMGlu or MMGlyc medium, transferred to T_c20°C and their β-galactosidase activity was assayed for 6 h after cold shock. The initial β-galactosidase activity in all samples was close to zero (not shown). Maximum level of β-galactosidase activity was reached 3 h after the cold shock. No significant differences in Des induction between CM, MMGlu and MMGlyc cells were found (Fig. 5). We could therefore conclude that the membrane FA composition of cells growing in mineral media that resulted in higher membrane fluidity after cold shock did not lower or prevent the maximum fatty acid desaturase induction.

4. Discussion

The aim of our current work was to elucidate the effect of medium composition on the extent of membrane adaptation for different cultivation temperatures in *Bacillus subtilis*.

The dependence of fatty acid profile in the medium composition in *Bacillus subtilis* has already been demonstrated by Klein et al. [22]. These authors showed that if isoleucine or threonine were present in mineral medium as precursors for anteiso-branched FA biosynthesis, the level of these fatty acids (FA) in membrane phospholipids was increased and the cells were able to survive the cold shock from 37 to 15 °C. However, the authors found substantial differences in the requirements for isoleucine when they compared their *Bacillus subtilis* JH642 strain with the original Marburg strain 168 that was used in our work. Strain JH 642 (*pheA1 trpC2 sfp0*) exhibited strong isoleucine dependence while strain 168 (*trpC2 sfp0)* did not and, therefore, the authors supposed that strain 168 had a higher biosynthetic capacity for threonine than JH642.

As shown in our present work, *Bacillus subtilis* 168 cells grown at T_c40°C in mineral media were able to synthesize sufficient levels of precursors for branched fatty acids (both iso- and anteiso-branched) from glucose or glycerol. The levels of anteiso-branched fatty acids were even higher in mineral media than in the complex medium (Fig. 2A). In glycerol medium the proportion of anteiso-branched fatty acids probably reached the possible limit (nearly 75% of total, see Fig. 2A). Such high level was reported by Suutari et al. for *Bacillus subtilis* growing in a complex medium at 15 °C, the lowest temperature used in their work [4]. The authors demonstrated that the proportion of anteiso-branched fatty acids increased almost proportionally with decreasing cultivation temperature. We further showed that in mineral medium with glucose, MMGlu, the cells also produced a higher proportion of branched fatty acids (in comparison with complex medium, CM), but to a lesser extent than in medium with glycerol, MMGlyc, (Fig. 2A). Nevertheless, even these relatively small changes in overall FA composition of MMGlu cells markedly influenced biophysical parameters of membrane phospholipids, shifting down their T_m by almost 7 °C (Fig. 4A) in comparison with lipids of cells grown in complex medium. Our results, however, did not allow us to decide whether such surprising regulation of fatty acid synthesis was induced by available carbon source only or whether it was also affected by the growth rate. Further experiments should be performed using cultivation in a chemostat to see whether a decreased growth rate (without changing the carbon source) should induce similar changes in FA composition as those observed in glycerol medium.

Relatively few data are available in the literature on the growth rate regulation of fatty acid synthesis in *Bacillus subtilis*. The effect of the growth rate on expression of the *accBC* operon coding for the subunit of Acetyl-CoA carboxylase was reported by Marini et al. [23]. The authors found a direct correlation between the level of transcription of this operon and the growth rate.

The question arises why *Bacillus subtilis* cells grown at T_c40°C preferred to synthesize the highest proportion of anteiso-branched FA in MMGlyc medium, where we expected the least favourable conditions for the branched anteiso-FA synthesis. The increased proportion of anteiso-branched fatty acids should result either from the mechanism of the primer selection by the FabH1 and FabH2 isoenzymes, the condensing enzymes involved in the first step of fatty acid elongation, or from the levels of the primer precursors [24]. It was hypothesized that the increased preference of the FabH for the precursors of the anteiso-branched fatty acids at low temperature was responsible for fluidization of the membrane in cold [25]. This temperature-dependent mechanism, however, could not be the reason for the fluidization in our case because such fluidization was induced by medium composition and not by temperature. We concluded, therefore, that it had to be the increased level of the precursors for anteiso-branched fatty acids leading to the high level of a-15:0 and a-17:0 FA observed in the membranes of cells grown in glycerol medium. Such explanation should be confirmed by further experiments as there are no data published as yet confirming that the growth in minimal media with glycerol (or glucose) enhances threonine and/or isoleucine synthesis in *Bacillus subtilis*. Twofold induction of isoleucine biosynthesis proteins after cold shock has been described recently [26].

Having found that at optimal temperature the choice of cultivation medium has apparently substantial influence on membrane chemical and biophysical characteristics, we studied further how the growth in different media affected the adaptation for low cultivation temperature. In complex medium, the low cultivation temperature was shown to induce an increase in the proportion of anteiso-branched FA [4,5]. This tendency was confirmed in our work. However, when we cultivated *Bacillus subtilis* cells at T_c20°C in mineral media MMGlu and MMGlyc, the levels of fluidizing a-15:0 fatty acids were lower than for cold-adapted cells grown in complex medium CM. Consequently, the proportion of n-16:0 FA increased dramatically in both mineral media (Fig. 2B). Surprisingly enough, the cells grown in MMGlyc at T_c20°C even lowered the level of their anteiso-branched fatty acids in comparison with those cultured at T_c40°C. If we consider the possible higher affinity of FabH for branched fatty acid primers at low temperature [25] together

with the increased level of n-16:0 in our fatty acid profiles for MMGlu and MMGlyc media at T_c20°C (Fig. 2A), then the most probable reason for lower levels of anteiso-branched FA at T_c20°C was the insufficient synthesis of branched-fatty acid primers.

In our work we tried to correlate the changes in fatty acid composition with membrane biophysical parameters. Despite the fact that DPH fluorescence anisotropy as a measure of membrane fluidity was shown to have some limitations [27] the r_{ss} measurements employed in our work provided results consistent with those of our DSC measurements. The only exception was observed in case of slightly higher DPH anisotropy for MMGlyc in comparison with MMGlu samples (T_c20°C, both membranes and lipids) where T_m for MMGlyc lipids was slightly lower (Figs. 3B and 4B). In all other cases lipid samples with higher melting temperatures also exhibited higher DPH anisotropy values at lower temperatures of measurement where the anisotropy differences between samples (both membranes and lipids) were significant.

The membranes of cells grown at T_c40°C in mineral media with either glucose or glycerol were more fluid at lower temperatures of measurement than the ones isolated from complex medium cells (Fig. 3A). This finding was validated by DSC measurements showing that the melting temperature T_m of membrane phospholipids from complex medium was much higher than those from both mineral media (Fig. 4A). Such data are helpful for the analysis of the membrane state after the cold shock. From Figs. 3A and 4A one can conclude that cells grown in mineral media (T_c40°C) shifted to the low cultivation temperature (T_c20°C) have more fluid membranes than cells from complex medium and that CM-T_c40°C lipids are much closer to the phospholipid phase transition. Cells grown in mineral media might be therefore better prepared for performing their membrane-bound activities after cold shock. This conclusion is supported by the data shown in Fig. 1. After the cold shock (cooling from 40 °C to 20 °C) only cells growing in mineral media started their growth immediately. Cells cultivated in complex medium resumed their growth after a 60 min delay. Such growth lag had to be caused by membrane rigidization because the length of the lag could be shortened by addition of the membrane fluidizing agent benzylalcohol [27,28].

We compared our DPH anisotropy data with those already published for *E. coli* [29]. In both cases, the bacteria were cultivated at their temperature optimum and the growth rate was modified by the carbon source. For *Bacillus subtilis* cultivated and measured at 40 °C, the observed DPH anisotropy values were the same for all three types of media (Fig. 3A) despite the substantial differences in fatty acid composition of membrane phospholipids. In *E. coli* cultivated and measured at 37 °C, however, different adaptation was observed — a higher growth rate induced a decrease of DPH fluorescence polarization which was interpreted as an increase in membrane fluidity and decreasing order parameter [14,29].

The extent of membrane cold adaptation can be quantified in terms of homeoviscous efficacy, a measure of adaptation of the membrane state for the lower cultivation temperature [12]. The calculation is based on comparison between membrane biophysical characteristics (e.g. DPH anisotropy) of given organism adapted to optimum and low cultivation temperature. Homeoviscous efficacy for membranes of *Bacillus subtilis* grown in complex medium calculated from r_{ss} data for T_c40°C and T_c20°C membranes comprised 20%. At the same time, the appropriate T_m values of membrane lipids decreased by 10.5 °C for the lipids from lower cultivation temperature (from 14.4 °C to 3.9 °C for CM-T_c40°C and CM-T_c20°C lipids, respectively). However, it is not easy to find an appropriate measure of cold adaptation for the *Bacillus subtilis* cells grown in MMGlyc medium. In terms of homeoviscous efficacy, there is no cold adaptation — the T_c20°C membranes were even more rigid than the T_c40°C ones (compare Fig. 3A with B). However, the midpoint of the phase transition T_m for the cells grown at 40 °C in MMGlyc was already very low (4.5 °C), nearly the same as T_m for the cold-adapted cells in complex medium (3.9 °C). At a cultivation temperature of 40 °C, therefore, the MMGlyc cells surprisingly showed the features characteristic for cold adaptation of their membrane state and fatty acid composition. At lower cultivation temperature, glycerol medium clearly imposed the limitations for the synthetic capacity of the fluidizing anteiso-branched fatty acid precursors and the cells were not able to further fluidize their membranes.

Finally, we examined whether the membrane properties of cells grown in glycerol medium provided some advantage for the short-term adaptation after cold shock, namely for fatty acid desaturase induction. We showed that the “cold temperature phenotype” of MMGlyc cells cultivated at T_c40°C did not prevent the induction of fatty acid desaturase after cold shock which was the same as in the case of the other media MMGlu and CM. For full desaturase induction in the cells from glycerol medium, therefore, a sufficient abrupt change of the membrane fluidity induced by cold shock was more critical than the fact that the membrane after cold shock already exhibited the characteristics typical for the cold adaptation with respect to FA composition and biophysical characteristics necessary for growth at low temperature.

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