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Cytoplasmic membrane of *Bacillus subtilis* : Regulation of the physical parameters

Regulace fyzikálních vlastností cytoplazmatické membrány u *Bacillus subtilis*

Ph.D. thesis

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Prague, January 27th, 2011

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Dedicated to Eva Beranová

my grandmother, deceased in 2010

Abstract (English)

Bacillus subtilis, a model Gram-positive soil bacterium, employs two distinct mechanisms in its membrane adaptation to low temperature: 1) Long-term adaptation to suboptimal temperature is accomplished by increasing the ratio of anteiso- to iso-branched fatty acids in the membrane lipids. 2) After a sudden temperature decrease, the oxygen-dependent fatty acid desaturase (Des) is induced which desaturates fatty-acyl chains incorporated in membrane lipids. The transcription of the gene encoding desaturase, *des*, is activated by the decrease of the membrane order, via two-component system DesK-DesR.

In this work, I studied the influence of cultivation conditions on the mechanisms of *B. subtilis* membrane adjustments for a low temperature employing fatty acid analysis, fluorescence spectroscopy, differential scanning calorimetry and methods of molecular biology.

In the first part of this work, I examined the impact of the cultivation medium on the composition and biophysical features of the *B. subtilis* cytoplasmic membrane during growth under the optimal (40 °C) and suboptimal (20 °C) cultivation temperature. I compared the nutrient-rich complex medium containing glucose and the mineral medium supplemented with either glucose or glycerol. The results obtained showed the crucial importance of medium composition for the membrane adaptation. The differences in membrane fatty acid profiles recorded in the particular media were probably induced by the different levels of branched fatty acid precursors, which resulted from the different metabolic pathways employed by the cells. At the optimal temperature (40 °C), the cells grown on nutrient-poor medium with glycerol were the ones that exhibited the highest membrane fluidity and the lowest transition temperature (T_t) of the membrane lipids. On the contrary, at the lower temperature (20 °C), the cells cultivated in complex medium had the most fluid membranes. The extent of adaptation, expressed as the difference of T_t obtained for the cells cultivated at the optimal and suboptimal temperature, was the greatest where the rich medium was used. However, the choice of cultivation medium did not have any substantial effect on the induction of fatty acid desaturase after the cold shock.

In the second part of my work, I focused on the adaptation of *B. subtilis* membrane to a low temperature under anaerobic conditions that were predicted to inhibit Des activity. I found that in anaerobiosis, as opposed to aerobic growth, the induction of *des* expression after the temperature downshift (from 37 °C to 25 °C) was not down-regulated. However, the transfer from anaerobic to aerobic conditions rapidly restored the down-regulation. Under both aerobic and anaerobic conditions, the induction of *des* expression was substantially reduced by the addition of external fluidizing oleic acid and was fully dependent on the DesK-DesR two-component regulatory system. Fatty acid analysis proved that there was no desaturation after *des* induction under anaerobic conditions despite the presence of high levels of the Des protein product, which was shown by immunoblot analysis. The cold adaptation of *B. subtilis* in anaerobiosis is, therefore, mediated exclusively by the increased anteiso/iso- ratio of branched-chain fatty acids and not by the temporarily increased level of unsaturated fatty acids that is typical under aerobic conditions. The degree of membrane fluidization, as measured by 1,6-diphenyl-1,3,5-hexatriene fluorescence anisotropy, was found to be similar under both aerobic and anaerobic conditions.

Keywords: *Bacillus subtilis*, cytoplasmic membrane, cold adaptation, anaerobiosis, membrane fluidity

Abstract (Czech)

Bacillus subtilis, modelová Gram-pozitivní bakterie, uplatňuje při adaptaci své membrány k nízkým teplotám dva odlišné mechanismy: 1) Při dlouhodobé adaptaci k nižším teplotám dochází ke zvýšení poměru anteiso/iso-větvených mastných kyselin v membránových lipidech. 2) Po náhlém poklesu teploty je indukována syntéza desaturázy (Des), proteinu, který v aerobních podmínkách desaturuje řetězce mastných kyselin v lipidech cytoplazmatické membrány. Transkripce genu *des,* kódujícího desaturázu mastných kyselin, je indukována zvýšením uspořádanosti membrány. Pro indukci transkripce genu *des* je nezbytná účast dvoukomponentového systému DesK-DesR.

V této práci jsem sledovala, jak kultivační podmínky ovlivňují mechanismus adaptace membrány *B. subtilis* k nízkým teplotám. Použity byly metody fluorescenční spektroskopie, analýza mastných kyselin, diferenciální skenovací kalorimetrie a metody molekulární biologie.

V první části práce jsem se zabývala vlivem složení kultivačního media na chemické složení a biofyzikální parametry cytoplazmatické membrány *B. subtilis* během růstu v optimální (40 °C) a suboptimální (20 °C) teplotě. Srovnávala jsem komplexní medium s glukózou bohaté na živiny a dvě média minerální obsahující buď glukózu nebo glycerol jako zdroj uhlíku. Získaná data jasně ukazují zásadní vliv složení kultivačního media na chladovou adaptaci membrány. Zaznamenané rozdíly ve složení mastných kyselin byly velmi pravděpodobně indukovány různou hladinou prekurzorů pro syntézu mastných kyselin s větveným řetězcem, které jsou důsledkem zapojení rozdílných metabolických drah při růstu buněk v různých mediích. Při růstu blízko teplotního optima (40 °C) byla nejvyšší membránová fluidita a nejvyšší teplota fázového přechodu (T_t) zjištěna u buněk rostoucích na nejchudším substrátu, tj. minerálním mediu s glycerolem. Oproti tomu při v nízké teplotě (20 °C) vykazovaly nejvyšší membránovou fluiditu buňky kultivované na komplexním médiu. Rozsah adaptace, vyjádřený jako rozdíl hodnot T_t naměřených pro optimální a suboptimální teplotu, byl největší v případě komplexního media. Složení kultivačního media ale nemělo vliv na indukci desaturázy po chladovém šoku.

V druhé části práce jsem se zaměřila na chladovou adaptaci membrány *B. subtilis* v anaerobních podmínkách, které by podle předpokladů měly inhibovat aktivitu desaturázy. Zjistila jsem, že při růstu bakterie bez přístupu kyslíku dochází po přenosu z 37 °C do 25 °C k indukci syntézy desaturázy, která ale později není mechanismem zpětné vazby zastavena tak, jak je tomu v aerobních podmínkách. Tato regulace byla však obnovena krátce po dodání kyslíku k původně anaerobní kultuře. Jak při kultivaci aerobní, tak při kultivaci anaerobní byla indukce transkripce genu *des* podstatně snížena přidáním fluidizující kyseliny olejové. Tento jev byl závislý na přítomnosti intaktního dvou komponentového systému DesK-DesR. Analýza mastných kyselin potvrdila, že v anaerobních podmínkách nedošlo k syntéze nových nenasycených mastných kyselin, přestože technikou immunoblotu byla detekována vysoká hladina proteinu Des v buňkách anaerobní kultury. Adaptace membrány *B. subtilis* k nízké teplotě je tedy v anaerobních podmínkách realizována výhradně zvýšením poměru anteiso/iso-větvených mastných kyselin v membránových lipidech a nikoli přechodným zvýšením hladiny nenasycených mastných kyselin typickým pro aerobní podmínky. Rozsah snížení membránové fluidity, měřené jako pokles anizotropie fluorescence 1,6-diphenyl-1,3,5-hexatrienu, byl pro aerobní a anaerobní podmínky srovnatelný.

Keywords: *Bacillus subtilis*, cytoplazmatická membrána, chladová adaptace, anaerobióza, membránová fluidita

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Abbreviations

ABD	ATP-binding domain (of DesK)
ACP	acyl carrier protein
ALM	alkaline lysis mix
BCKA	branched short-chain α-keto acids
BSA	bovine serum albumin
CoA	coenzyme A
CM	complex cultivation medium
DesKC	N-truncated form of membrane sensor DesK
DesR~P	response regulator DesR, phosphorylated
DSC	differential scanning calorimetry
DPH	1,6-diphenyl-1,3,5-hexatriene
DR	direct repeat (in DesR-binding sequence of *des* gene)
EDTA	ethylene diamine tetraacetic acid
FA	fatty acid
FAS	fatty acid synthetase
IR-L	inverted repeat – left (in DesR-binding sequence of *des* gene)
IR-R	inverted repeat – right (in DesR-binding sequence of *des* gene)
KAS	β-keto-acyl-ACP synthetase
MMGlu	mineral cultivation medium with glucose
MMGlyc	mineral cultivation medium with glycerol
MS	minimal sensor
OD_{450}	optical density (at 450 nm)
ONPG	ortho-nitrophenyl-β-galactoside
PAGE	polyacrylamid gel electrophoresis
PEB	plasmid extraction buffer
PDH	pyruvate dehydrogenase
Pdes	the promoter of *des* gene
PL	phospolipid
RA	region A (in DesR-binding sequence of *des* gene)
RB	region B (in DesR-binding sequence of *des* gene)
ResD~P	response regulator ResD, phosphorylated
RNAP	RNA - polymerase
r_{ss}	steady state anisotropy of fluorescence
RT	room temperature
SDS	sodium dodecyl sulphate
T_c	cultivation temperature
T_m	measurement temperature
TM	transmembrane domain
T_t	transition temperature (of lipids)
UFA	unsaturated fatty acid

1 Introduction

Temperature is one of the most important environmental factors to which living organisms need to adapt. Generally, there are two basic strategies for coping with temperature fluctuations. Homoiothemic organisms, such as birds and mammals, have evolved mechanisms for maintaining a stable body temperature. Poikilothermic ones, i.e. reptiles, amphibians, invertebrates and of course microorganisms, do not posses this ability and deal with temperature changes by adjusting their behavior, metabolism and and/or cell composition. For bacteria, due to the microscopic dimensions of their “bodies”, the adaptation of cell composition is essential for their survival in changing environment. Concerning temperature adaptation, we must distinguish between adaptations to low or high temperatures. While the main negative effect of high temperatures is protein denaturation, suboptimal temperatures bring about membrane rigidization and retardation of essential biochemical processes, such as nucleic acid and protein synthesis.

Also, there is a difference between adaptation to abrupt temperature fluctuations and long-term thermal stress. Sudden temperature change represents an immediate stress and necessitates quick reaction by the cell, whereas prolonged influence of non-optimal conditions needs long-lasting adaptation. Hence, each of these temperature-related challenges requires a slightly different adaptation strategy.

Among bacteria, great variability occurs between different groups and species with respect to temperature tolerance. For example, pathogens of homoithermic vertebrates living in the thermally stable environment of their host bodies do not frequently have to face up the thermal stress. On the contrary, bacteria that occur “outside”, such as in water or soil need to be much better at adapting to temperature changes, as these are very common in their environment.

The thermal tolerance and/or resistance of different bacteria have many practical implications for human life. In processes such as food storage, sterilization or various biotechnology productions, the relation of bacteria and temperature plays a key role. Hence, the understanding of principles and mechanisms of bacterial adaptation to temperature is very important, not only for the purposes of basic molecular biology and microbiology research but also for applications in healthcare and industry.

This thesis deals with the adjustment of *Bacillus subtilis*, a Gram-positive soil bacterium, to suboptimal temperatures, both to sudden temperature drop and long-term survival at low temperatures, with a focus on adaptation of its cytoplasmic membrane. In our laboratory, this organism and the adaptation of its membrane to various types of environmental stress have been studied for many years. My work, the results of which I present here, has focused on the mechanisms employed in *B. subtilis* for chemical remodeling of the membrane at low temperatures and their impact on its physical features, such as membrane fluidity and transition temperature of membrane lipids. I have also been exploring the role and importance of fatty acid desaturase (Des) in this adaptation process. The influence of nutrient conditions and anaerobiosis on the mechanisms and the extent of adaptation of this model microorganism to low temperature has been investigated. Both are supposed to be biologically relevant, as in its natural environment, soil, *B. subtilis* cells encounter fluctuations both in nutrient and oxygen availability. My work also includes the construction of two new *Bacillus subtilis* strains, from which one has been employed directly in the experiments presented in this dissertation and the other will be used for further experiments by our research team.

2 Aims

In my work, I have pursued the following aims:

1. **To elucidate the influence of cultivation medium (namely the carbon source) and the resulting type of metabolism on the adaptation of *B. subtilis* membrane to the optimal (40 °C) and suboptimal (20 °C) cultivation temperature during exponential growth.**

    The main focus has been on the changes in the composition of membrane lipids, namely its fatty-acyl chains, under different cultivation conditions, and the extent to which these are reflected in the biophysical characteristics of the membrane. These characteristics include membrane fluidity (assessed using the technique of fluorescence anisotropy measurement) and transition temperature of membrane lipids. This aim also includes the construction of a new strain based on the genetic background *B. subtilis 168,* harboring the fusion of reporter β-galactosidase with the promoter of gene *des*, encoding the fatty acid desaturase. This strain should allow for the monitoring of desaturase expression under different growth conditions and help us to better define the role of this protein in adaptation process.

2. **To compare the cold adaptation of *B. subtilis* membrane during aerobic growth and under anaerobiosis.**

    To achieve this, first the technique of *B. subtilis* anaerobic cultivation (that allows for anaerobic respiration of nitrate) needed to be introduced and attested in our laboratory. Further, I was interested in the features (both chemical and physical) and differences of the membranes of cells cultivated with and without oxygen and their difference. Apart from the analysis of lipid composition and resulting membrane fluidity, the next aim was to focus on the role of fatty acid desaturase, which was proved to need oxygen for its function, under the conditions that should prevent its activity.

3. **To construct a new *B. subtilis* strain bearing an introduced binding motif Cys-Cys-X-X-Cys-Cys in the DesK protein sequence that would allow for employing the fluorescent probe (FLASH) for exploring the dimerization of the membrane fluidity sensor DesK.**

    This strain is planned to be used in further research by our laboratory team. It will be employed in the investigation of the DesK signaling mechanism, namely its dimerization.

3 Literature review

3.1 *Bacillus subtilis*

Bacillus subtilis is a rod shaped bacterium that is able to form very resistant endospores. Its cells stain Gram-positive and are usually motile with peritrichous flagella. Its metabolism is chemoorganotrophic, facultatively anaerobic. *Bacillus subtilis* is not pathogenic to humans or animals. With respect to the growth temperature, it is a mesophilic bacterium. It is able to grow in the temperature range of ca. 11 °C to 53 °C with the optimum temperature of around 40 °C (Weber and Marahiel 2002). The natural habitat of this microorganism is the upper layer of soil where conditions change frequently and transiently. Hence, this bacterium must be capable of rapid adjustment to survive different conditions which makes it a good model for adaptation studies.

Bacillus subtilis is one of the most studied and best characterized organisms used in basic and applied microbiology research. The complete genome sequence of *Bacillus subtilis* has been available since 1997 (Kunst *et al.* 1997).

3.1.1 *Bacillus subtilis* membrane lipids

In *B. subtilis*, the membrane lipid/membrane protein ratio was reported to be around 0.7 for strain BD99 grown at 30 °C on minimal medium with yeast extract (Guffanti *et al.* 1987). It is generally believed that this ratio is quite a stable feature for the cell. However, too few experiments have been conducted in this field to date; therefore, it is not evident whether this general concept is really valid. The mechanism that would control the ratio of lipid/protein ration in membranes has not yet been elucidated.

Lipid synthesis is believed to be a membrane-bound process that occurs on the inner face of the cytoplasmic membrane (de Mendoza *et al.* 1993).

Polar lipids constitute over 75 % of the lipid fraction, with phosphatidylglycerol and phosphatidylethanolamine being the most abundant species, together comprising over 80 % of this fraction. Phosphatidylserine, lysophosphatidylglycerol and diphosphatidylglycerol (cardiolipin) are usually found in minor amounts among *B. subtilis* phospholipids. Glycolipids (diglukosyl-diacylglycerol and monoglukosyl-diacylglycerol) and neutral lipids (1,2-diacylglycerol) contribute each by ca. 10 % to the total lipid fraction (Bishop *et al.* 1967; Guffanti *et al.* 1987; de Mendoza *et al.* 1993; Seydlova and Svobodova 2008). The biosynthesis of lipids and fatty acids seems not to be tightly coupled (de Mendoza *et al.* 1993).

3.1.2 *Bacillus subtilis* membrane fatty acids

For the membrane lipids of the genus *Bacillus*, the predominance is typical of terminally methyl-branched fatty acids (FA) of 12 to 17 carbons. According to the position of the branching methyl group, these branched FA are of three structural types: anteiso-branched and iso-branched with either odd or even carbon number (see Fig. 1).

The most abundant species in *B. subtilis* membrane are anteiso-15:0 and iso-17:0 fatty acids (Kaneda 1977; de Mendoza *et al.* 1993). The straight-chain fatty acids, which are the most common fatty acids found in many other bacteria, are normally minor constituents of the membranes of *Bacilli*.

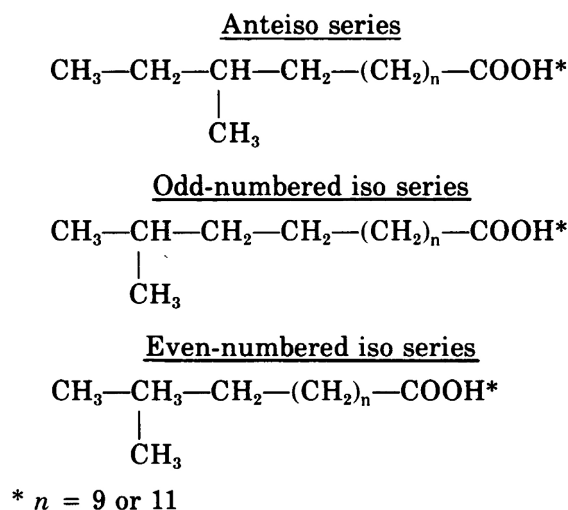


Fig. 1. Three series of branched-chain fatty acids. Reproduced from reference: (Kaneda 1977).

Unsaturated fatty acids are detected in very low levels when these microorganisms are grown at the optimal temperature (Kaneda 1977) but their synthesis is induced after temperature downshift (Fulco 1969). However, the proportions of particular fatty acid species can vary depending on many factors, such as growth temperature and nutrient conditions (see section 3.3).

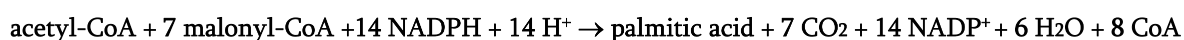
3.2 Fatty acid synthesis

In contrast to *E. coli*, which has been thoroughly studied in respect to fatty acid metabolism, only scarce genetic data had been available for *Bacillus subtilis* until the publication of complete genome sequence (Kunst *et al.* 1997). Afterwards, many genes were identified based on their homology to those of *E. coli* and other bacteria.

3.2.1 Saturated fatty acid synthesis

The fatty acid synthesis is catalyzed by the group of enzymes generally called fatty acid synthetases (FAS). Based on their physical structure, they can be divided in two groups. Type I group includes multifunctional polypeptides that were reported from animals and yeast. Type II group comprise the so-called soluble system of higher plants and bacteria, that is composed of seven individual enzymes (Kaneda 1991). All types of FAS systems, including *B. subtilis* FAS, require acyl carrier protein (ACP) for their function (Kaneda 1977). Acyl carrier proteins are small, highly acidic proteins with a covalently linked phosphopantetheine prosthetic group that are highly conserved among bacteria. *B. subtilis* ACP is coded by gene *acpP*. Its product is of 9000 Da molecular weight and shows 61 % residue identity with *E. coli* ACP (de Mendoza *et al.* 1993; Morbidoni *et al.* 1996).

Regardless of whether complex or soluble FAS are concerned, the general principle of fatty acid synthesis is uniform for most organisms. The best studied of these is the straight-chain FA synthesis with the palmitic acid (n-16:0) being the main product. However, the branched FA synthesis was found to share many common features with the straight-chain synthetic pathway (Kaneda 1991). In bacteria, seven individual enzymes catalyze the reaction:



$$\text{acetyl-CoA} + 7\ \text{malonyl-CoA} + 14\ \text{NADPH} + 14\ H^+ \rightarrow \text{palmitic acid} + 7\ CO_2 + 14\ \text{NADP}^+ + 6\ H_2O + 8\ \text{CoA}$$

that results in palmitic acid formation: acetyl-CoA:ACP transacylase, malonyl-CoA:ACP transacylase, β-keto-acyl-ACP synthetase (KAS), β-keto-acyl-ACP reductase, β-hydroxyl-acyl-ACP dehydrase, enoyl-ACP reductase and palmitoyl thioesterase (Kaneda 1977; 1991; Magnuson *et al.* 1993). Known genes encoding proteins involved in *E. coli* FA synthesis are listed in Table 1.

Table 1. Genes involved in fatty acid synthesis

Gene	Activity
fabD	malonyl-CoA:ACP transacylase
fabB	β-keto-acyl-ACP synthetase I (KASI)
fab F	β-keto-acyl-ACP synthetase II (KASII)
fabH	β-keto-acyl-ACP synthetase III (KASIII)
fabG	β-keto-acyl-ACP reductase
fabA	β-hydroxyl-acyl-ACP dehydrase
fabZ	β-hydroxyl-acyl-ACP dehydrase
fabI	enoyl-ACP reductase

Acetyl-CoA and malonyl-CoA are first converted to their ACP-esters and those are condensed together to give rise to β-ketoacyl-ACP (acetoacetyl-ACP). In *E. coli* this condensation is catalysed by β-keto-acyl-ACP synthetase I. Alternatively, β-ketoacyl-ACP can be formed by condensation of malonyl-ACP with acetyl-CoA, which is performed by β-keto-acyl-ACP synthetase III. Acetoacetyl-ACP then enters the elongation cycle and reduction, dehydratation and final reduction follow. The resulted acyl-ACP then serves as the substrate for another round of elongation. The overall reaction is summarized in Fig. 2.

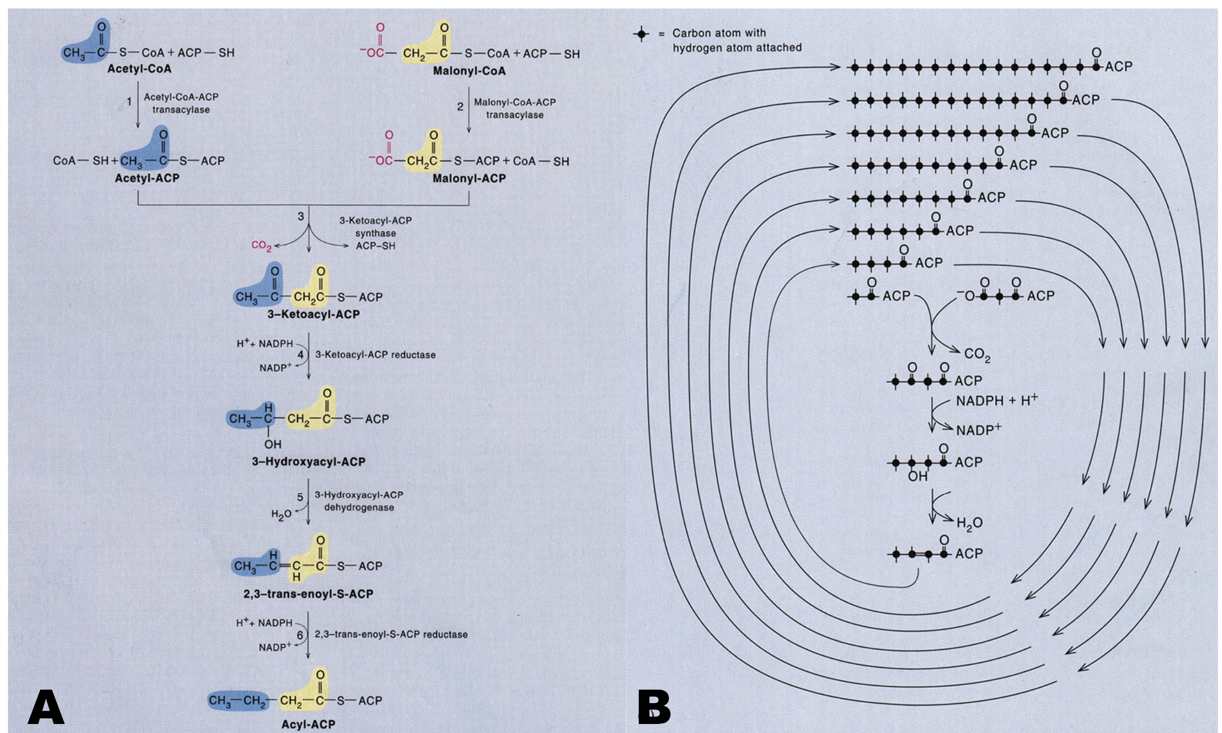


Fig. 2. The synthesis of saturated fatty acids. The initial step of the reaction (**A**) and the consequent elongation cycle (**B**). Reproduced from reference: (Zubay *et al.* 1995)

Each of these chemical reactions can be carried out by multiple, unique enzymes. For example, there are at least three β-ketoacyl-ACP synthases and at least two 3-hydroxyacyl-ACP dehydrases in *E. coli*. Because of their differing substrate specificities, each isozyme makes a unique contribution to the regulation of the distribution of products from the pathway (Magnuson *et al.* 1993).

3.2.1.1 Branched-chain fatty acid synthesis

The branched-chain FA synthesis in *Bacillus subtilis* follows the same principle as described above in the section 3.2.1. The main difference, except of course the final product, was shown to be in the substance that serves as the primer for the synthesis. For straight-chain FAs it is acetyl-CoA, whereas a short-chain acyl-CoA initiates the synthesis of branched FAs (Kaneda 1977).

The biosynthesis of branched fatty acids is closely linked with the biosynthesis of branched aminoacids. Branched-chain α-keto acids, which are used as primer sources (α-ketoisovalerate, α-ketoisocaproate and L-α-keto-β-methylvalerate), are chemically related to valine, leucine and isoleucine, respectively (Kaneda 1966), see Fig. 3.

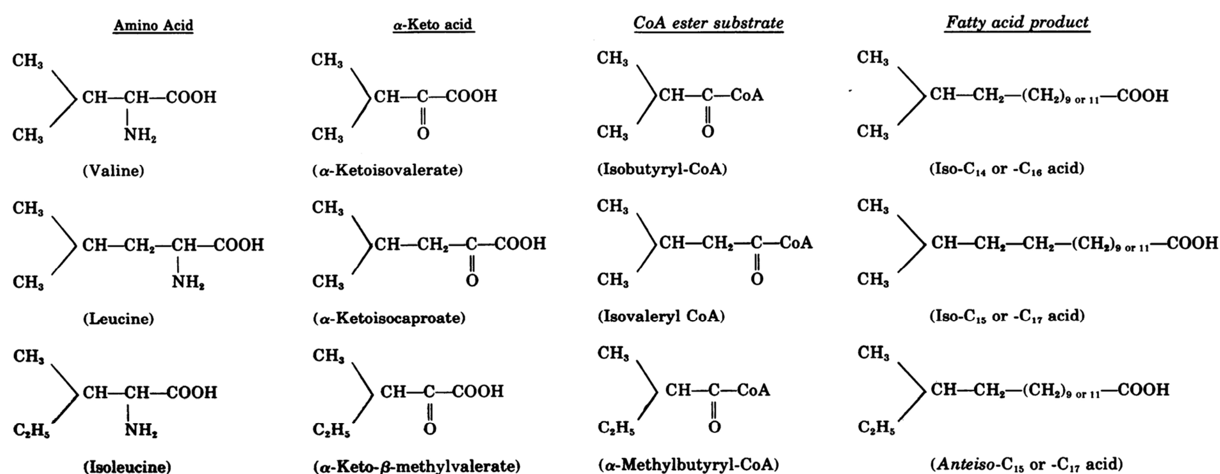


Fig. 3. Structural correlation of branched -chain substrates and fatty acids. Reproduced from reference: (Kaneda 1977).

These branched short-chain α-keto acids (BCKAs) are transformed by oxidative decarboxylation to the corresponding acyl-coenzyme A esters (isobutyryl-CoA, isovaleryl-CoA and 2-methylbutyryl-CoA). The BCKA-dehydrogenase (α-ketoisovalerate dehydrogenase) complex catalyzes this reaction that requires NAD and CoA as cofactors (Namba *et al.* 1969; Wang *et al.* 1993). BCKA-dehydrogenase complex is composed of four components E3, E1α, E1β and E2 that are encoded by genes *lpd*, *bkdA1*, *bkdA2* and *bkdB*, respectively (Wang *et al.* 1993; Debarbouille *et al.* 1999). A *bfmB* mutant of *B. subtilis* with defect in BCKA-dehydrogenase activity, requires short branched-chain fatty acids for growth (Willecke and Pardee 1971; de Mendoza *et al.* 1993). Genes encoding the BCKA-dehydrogenase components and three genes encoding proteins involved in utilization of isoleucine and valine are organized in a seven gene operon, called *bkd,* in the following order: *ptb*, *bcd*, *buk*, *lpd*, *bkdA1*, *bkdA2*, *bkdB*. Gene *ptb* encodes phosphate butyryl-CoA transferase, *bcd* leucine dehydrogenase and *buk* butyrate kinase, see Fig. 4. The expression of *bkd* operon is controlled by the regulatory gene *bkdR*, located upstream, strictly depends on alternative sigma factor σ^L (SigL) and is negatively controlled by a global regulator CodY. The operon was induced by the presence of isoleucine and valine in the growth medium (Debarbouille *et al.* 1999). The target genes of the CodY repressor also includes the operon *ilvBHCleuABCD* and genes *ilvD* and *ybgE* which are involved in the biosynthesis of branched-chain amino acids (Shivers and Sonenshein 2004).

Alternatively, BCKAs can enter the branched fatty acid synthesis pathway without being esterified to CoA. BCKA-decarboxylase is involved in the synthesis of this kind of primer converting BCKAs to an aldehyde derivative. This primer is then condensed with malonyl-ACP and then elongated as described below (Oku and Kaneda 1988).

The priming CoA-esters are first converted to ACP-esters by the activity of acyl-CoA:ACP transacylase. Hence, the substrate specificity of this enzyme is the factor that determinates the character of the resulting product (Butterworth and Bloch 1970) . The transacylases involved in the branched FA synthesis prefer branched primers with 3 to 6

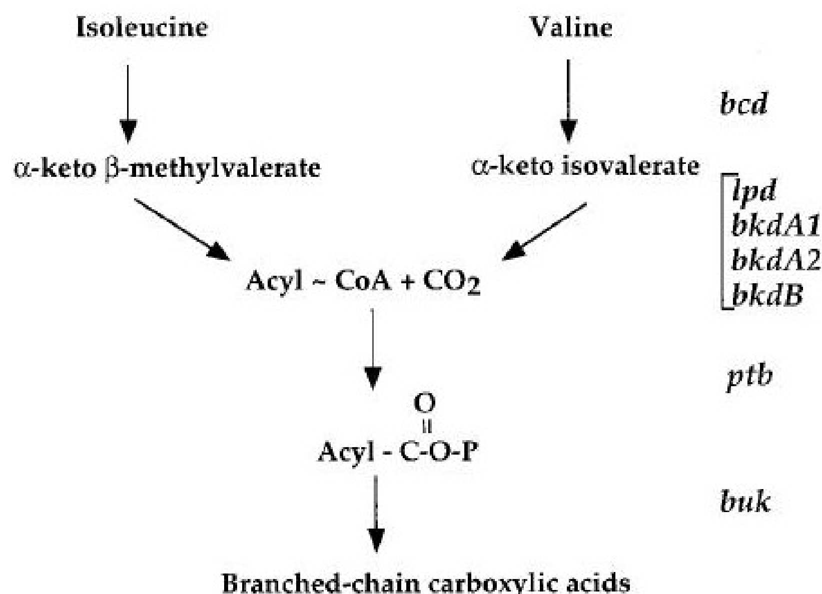


Fig. 4. Proposed pathway for the degradation of branched-chain amino acids in *B. subtilis*. Reproduced from reference: (Debarbouille *et al.* 1999).

carbons to acetyl-CoA for their substrates. *B. subtilis bfmA* mutant strain with defect in this enzymatic activity needs branched acyl-CoA to support growth (Boudreaux *et al.* 1981). Both acetyl-CoA:ACP transacylase and acetoacetyl-ACP synthase activities were detected for the product of *fabH* gene in *E. coli*, that codes for KAS III (Tsay *et al.* 1992). In *B. subtilis*, two homologues of this gene (*fabH1*, *fabH2*) were reported (Choi *et al.* 2000). Products of these genes carry out the initial condensation reaction in *B. subtilis* FA biosynthesis and (in contrast to *E. coli* FabH) exhibits high specificity to branched-chain acyl-CoA and the ability to use a wide range of branched precursors. No major difference was found in substrate specificity (straight vs. branched-chain or anteiso- vs. iso-branched primers) between FabH1 and FabH2. However, *fabH1* gene was detected in an operon together with gene originally named *yajY,* which is the *B. subtilis* homologue of *E. coli fabF.* Gene *fabF* encodes KASII, the condensing enzyme that carries out the subsequent elongation reaction. It has been hypothesized that *fabH1* is constitutively expressed initiator of FA synthesis, whereas *fabH2* expression might be regulated by any environmental or developmental signal to adjust the fatty acid composition according to the momentary need (Choi *et al.* 2000).

The initial carbon chain esterified to ACP is then further elongated by addition of two-carbon segments by the condensation of malonyl-CoA to the primer. This process is repeated four or five times to generate the ACP derivative with 14 to 17 carbons. The concentration of malony-CoA was reported to affect the chain length of the final product (Naik and Kaneda 1974). Malonyl-CoA is produced from acetyl-CoA by carboxylation which is catalyzed by acetyl-CoA carboxylase. This enzyme consists of four subunits. The

operon *accB-accC*, coding for two of them, has been shown to be growth-rate regulated in *B. subtilis* (Marini *et al.* 1995; Marini *et al.* 2001). The final reaction products of *B. subtilis* fatty acid synthetase were identified as free acids that are formed from FA acyl-ACPs by their enzymatic hydrolysis (Kaneda 1973).

3.2.1.2 Straight-chain fatty acid synthesis

Branched-chain fatty acid synthetase from *B. subtilis* is not able to convert effectively acyl-CoA to acyl-ACP because the branched-chain CoA esters are the preferred substrates for acyl-CoA:ACP transacylase. However, if acetyl-ACP is provided to this organism, the straight-chain FA synthesis occurs (Butterworth and Bloch 1970). The alternative pathway of acetyl-ACP formation consists of the decarboxylation of malonyl-ACP that is catalyzed by 3-ketoacyl-ACP synthetase (Kaneda and Smith 1980). This enzyme is also involved in the condensation of malonyl-ACP with acetyl-ACP that yields acetoacyl-ACP. Therefore, a malonyl-ACP serves two fuctions in *B. subtilis*, as the primer and the chain extender. Malonyl-ACP is synthesized from malonyl-CoA by malonyl-CoA:ACP transacylase (Kaneda 1991). This protein is encoded by the gene *fabD*, that clusters with *fabG* and the gene for ACP (*acpP*) in *B. subtilis* genome (Morbidoni *et al.* 1996).

Propionic, butyric and valeric acids also serve as primers in the synthesis of straight-chain FA synthesis in *B. subtilis* and yield odd numbered (n-15:0, n-17:0) or even numbered (n-14:0 and n-16:0) non-branched FAs (Kaneda 1977).

3.2.2 Unsaturated fatty acid synthesis

Mostly monounsaturated fatty acids (UFAs) can be detected in *B. subtilis* membrane (Kaneda 1977; de Mendoza *et al.* 1993). Two mechanisms of unsaturated fatty acids synthesis in bacteria have been described: anaerobic and aerobic (Bloch 1969). An example of the former (anaerobic) is the *E. coli* system, *Bacillus* species serves as the model of the latter (aerobic).

E. coli membrane has a very simple composition of cytoplasmic membrane. Only three major lipid head-groups and three different fatty-acyl-chains (n-16:0, n-16:1 and n-18:1) form its membrane lipids (Cronan and Rock 1987; Neidhart 1987). The proportion of unsaturated fatty-acyl-chains in membrane lipids increases with the decreasing temperature (Marr and Ingraham 1962; Cronan 1975; Grau and de Mendoza 1993). The synthesis of UFAs is catalyzed by three enzymes, encoded by the genes *fabA*, *fabB*, and *fabF*. β-Hydroxydecanoyl dehydrase (FabA) introduces the double bond into 10-carbon intermediate. This intermediate is then elongated by 3-ketoacyl-ACP synthetase I (FabB) to give rise to the palmitoleoyl-ACP (n-16:1). Finally, FabF (3-ketoacyl-ACP synthasese II, KAS II) converts the n-16:1 fatty-acyl chain to n-18:1 (Cronan and Rock 1987). The activity of FabF, rather than its level in the cell, is regulated by temperature; the enzyme is more active in low temperatures (Fulco 1969; Garwin and Cronan 1980; Garwin *et al.* 1980; de Mendoza and Cronan Jr 1983). *E. coli* strains lacking *fabF* gene are not able to regulate their UFA content in the membrane in response to temperature (de Mendoza *et al.* 1983), which indicates that this is the sole protein responsible for thermal modulation

of inner membrane lipid composition and thus membrane fluidity. This pathway of UFA synthesis (see Fig. 5A), that was originally considered to be universal among most of the bacteria, seems to be possessed only by alpha and gamma proteobacteria (Mansilla *et al.* 2004; Albanesi *et al.* 2009).

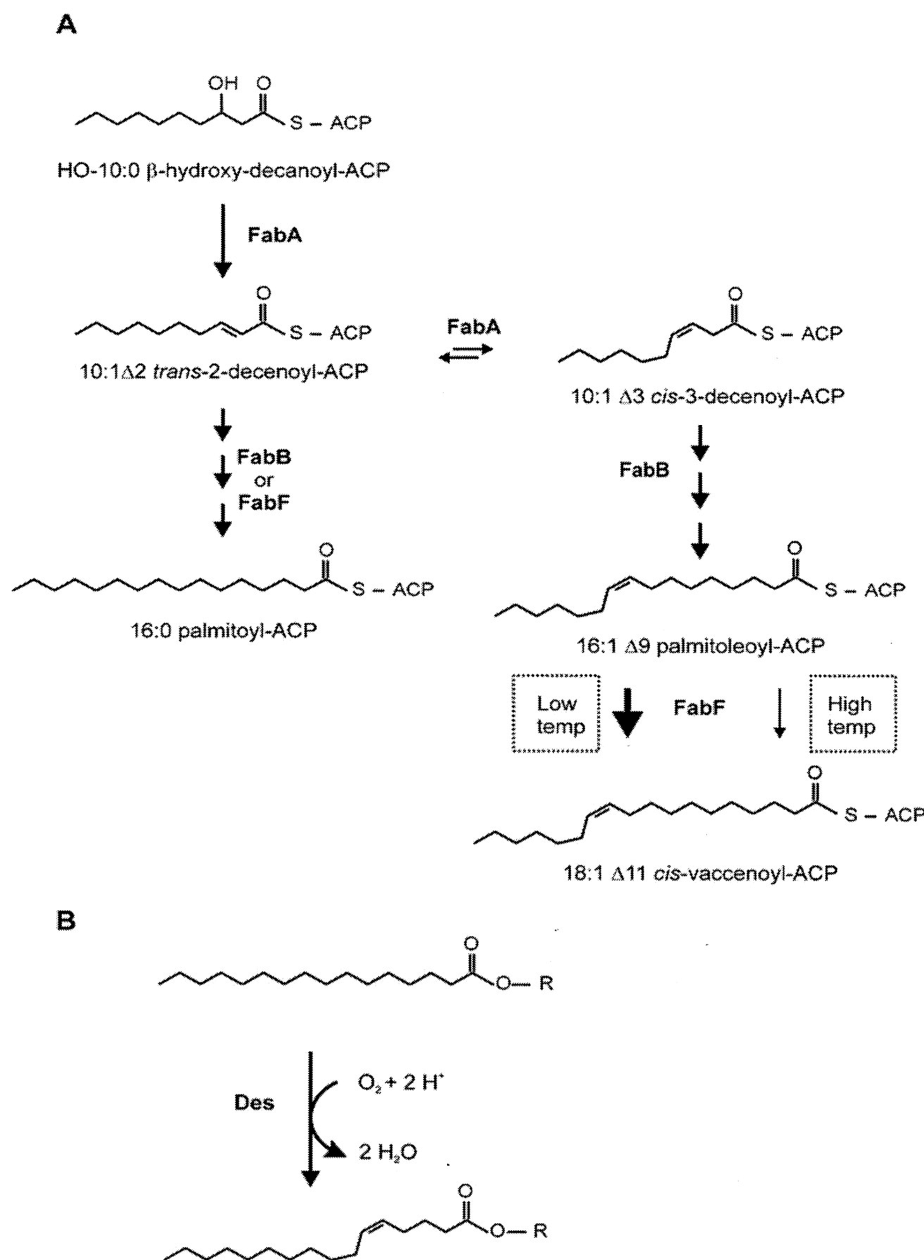


Fig. 5. **Unsaturated fatty acid biosynthesis in (A) *E. coli* (anaerobic pathway) and (B) *B. subtilis* (aerobic pathway).** R indicates a phospholipid. Reproduced from reference: (Mansilla *et al.* 2004).

Bacillus subtilis synthesize only traces of unsaturated fatty acids when grown close to the optimum temperature (37–40 °C). However, the synthesis of unsaturated fatty acids is induced after downshift to cold temperature (20–25 °C) (Fulco 1969; Fujii and Fulco 1977; Grau and de Mendoza 1993) and significant amounts of UFAs have also been reported for the *long-term* growth at low temperatures (Suutari and Laakso 1992).

The gene encoding the acyl-lipid desaturase, *des*, was identified and characterized by Aguilar *et al.* (1998). The gene is present in a single copy in the genome of *Bacillus subtilis* and encodes a polypeptide of 352 amino acid residues. Its product catalyzes O_2-dependent dehydrogenation at an unactivated position of the fatty-acyl chain which results in *cis* double bond formation (Fig. 5B). It is the sole desaturase that this organism possesses. The details are given in the next sections (3.2.2.1 and 3.2.2.2).

3.2.2.1 *Bacillus subtilis* desaturase

Desaturase is an iron-dependent integral membrane protein with six transmembrane and one membrane associated domain. Both N- and C-terminus of the protein are located in cytoplasm (Diaz *et al.* 2002). It contains four histidine (His) clusters, three of which are conserved among related desaturases of plants, mammals and cyanobacteria. The fourth non-conserved His-patch is located close to the C-terminus (Diaz *et al.* 2002). Histidin-rich regions are supposed to be involved in binding iron which is necessary for enzyme activity (Shanklin *et al.* 1994). Single mutations of His residues (His→Ala) in three conserved clusters totally abolished the in vivo activity of desaturase, therefore, they are believed to participate in the building of the desaturase active site. In contrast, the His residues of the fourth cluster have been shown to be non-essential for enzyme activity. However, the carboxy-terminal truncation (residues 294 to 352) of the protein gave rise to the non-functional enzyme that indicates its important role of C-terminal portion either in enzyme activity or its folding (Diaz *et al.* 2002). The study of the desaturase topology revealed that all the histidine-rich regions – and hence the active site – are located in the cytoplasm. In addition, a hydrophobic segment (residues 89 to 109) was found between the first two His clusters and is hypothesized to be involved in substrate recognition (Diaz *et al.* 2002). See the membrane topology of Des in Fig. 6.

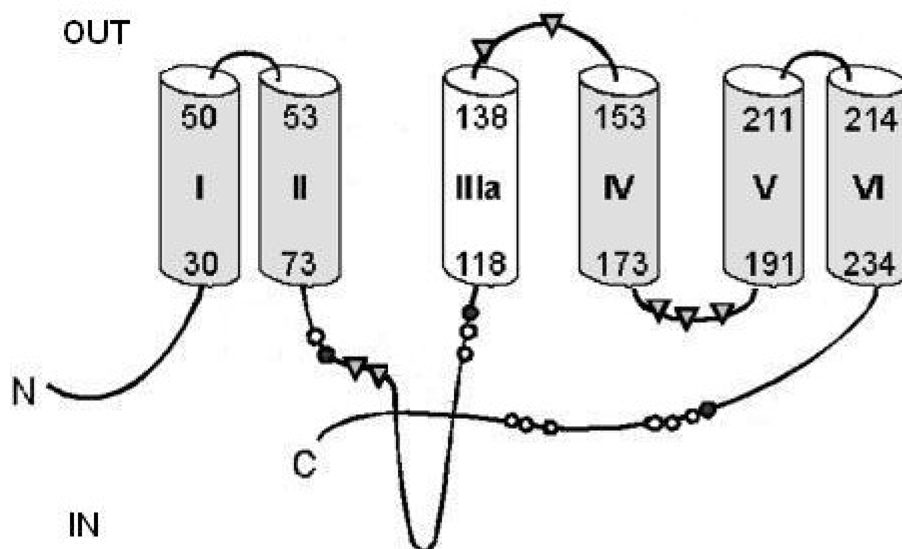


Fig. 6. Membrane topology of *B. subtilis* fatty acid desaturase. Cylinders represent the membrane-spanning segments, circles represent His residues (dark circles indicate the mutated His residues that caused activity loss), triangles represent Lys residues. Figure adapted from reference: (Diaz *et al.* 2002).

B. subtilis desaturase introduces the double bond into the fatty-acyl chains incorporated in membrane phospoholipids. It is not able to desaturate either free fatty acids or those acetylated to coenzyme such as CoA or ACP (Grau and de Mendoza 1993). As for the position of the double bond introduced, some authors have reported UFAs with a double bond at position Δ5, Δ7 and Δ9 (Weber *et al.* 2001), whereas others have posited that the double bond of UFAs synthesized by *B. subtilis* is located exclusively at the Δ5 position, regardless of the growth temperature and the length chain of the fatty acids (Altabe *et al.* 2003).

Desaturase is not essential for *B. subtilis* growth. Disruption of the *des* gene resulted in a survival defect of *B. subtilis* during stationary phase at 20 and 37 °C but did not affect the growth rate of this organism at these temperatures (Aguilar *et al.* 1998). However, *des* null mutant exhibited severe cold sensitive phenotype in the absence of isoleucine (Weber *et al.* 2001). No UFAs were detected in the membranes of *B. subtilis* cells with mutated *des* (Aguilar *et al.* 1998; Weber *et al.* 2001) which proves that fatty-acyl desaturation in *B. subtilis* is strictly dependent on Des protein.

The transcription of the *des* gene is induced by the decrease in temperature. The 1.1 kb long *des* transcript was detectable as soon as 15 minutes after the downshift from 37 °C to 20 °C and reached the maximum level 30 minutes after the cold shock (Aguilar *et al.* 1999). Using the proteosyntesis blocking agent chloramphenicol, it was demonstrated that the induction of *des* does not require the synthesis of any new proteins (Aguilar *et al.* 1999). *des* gene does not contain long 5' untranslated leader region (see section 3.4.1) typical of other cold shock induced genes, both from *E. coli* and *B. subtilis* (Zubay *et al.* 1995; Mitta *et al.* 1997). Derepression of *des* takes place at the level of transcription but is not caused by the stabilization of *des* induction mRNA at low temperatures (Aguilar *et al.* 1998). Transcriptional activity of the *des* promoter was shown to be inhibited by unsaturated fatty acids – either endogenously synthesized or exogenously added (Aguilar *et al.* 2001).

3.2.2.2 Regulation of *des* expression by DesK-DesR two-component system

A two-gene operon formed by *desK-desR* genes, originally named *yocF-yocG* in *Bacillus subtilis* sequencing project (Kunst *et al.* 1997), located immediately downstream of *des* gene was shown to be essential for *des* induction (Aguilar *et al.* 2001). This operon encodes a two-component system that consists of a membrane bound histidine kinase (DesK) and its cognate response regulator (DesR) and is constitutively expressed both at the optimal (37 °C) and suboptimal temperature (25 °C). Both DesK and DesR are essential for *des* gene expression. This two-component system was shown to stringently control the expression of *des* gene (Aguilar *et al.* 2001) but not any other cold inducible gene (Beckering *et al.* 2002). However, the microarray analysis of *B. subtilis* two-component systems revealed some other genes with an unknown function (such as putative two-component system *yvfT-yvfU*) as possible target genes for DesK-DesR two-component system (Kobayashi *et al.* 2001), but direct evidence confirming this heuristic observation has yet to be provided.

Activation of the Des pathway occurs either when the *B. subtilis* cell is shifted to a low temperature (Aguilar *et al.* 2001) or when grown at an optimal temperature in

minimal medium without low-melting anteiso-branched fatty acid precursors (Cybulski *et al.* 2002). Addition of isoleucine, 2-methyl butyrate (isoleucine keto-derivative) or threonine to minimal medium for *B. subtilis* JH642 growth at 37 °C resulted in a substantial decrease in *des* promotor activity. Leucine, valine or their keto-derivatives (isobutyrate or isovalerate) did not have this effect (Cybulski *et al.* 2002). Also, in the presence of isoleucine, neither the *des* transcript nor unsaturated fatty acids were detectable in *B. subtilis* (strain JH642) grown at 37 °C on minimal medium. Cells grown at 37 °C on minimal medium with isoleucine exhibited the markedly increased levels of fluidizing anteiso-branched fatty acid in their membranes (Cybulski *et al.* 2002).

Thus, it was concluded that it is the membrane fluidity rather than temperature that DesK is sensing. The hypothesis was put forward that this bifunctional enzyme with both kinase and phosphatase activity assumes different signalling states according to the membrane physical state. When the membrane is satisfactorily fluid, a phosphatase-dominant state is favored. When the membrane becomes too rigid, kinase-dominant state of DesK prevails, see Fig. 7. It has been hypothesized that the transition of membrane lipids from the disordered (fluid) to the ordered (non-fluid) phase could be sensed by some of the transmembrane domains of DesK protein (Aguilar *et al.* 2001; Cybulski *et al.* 2002).

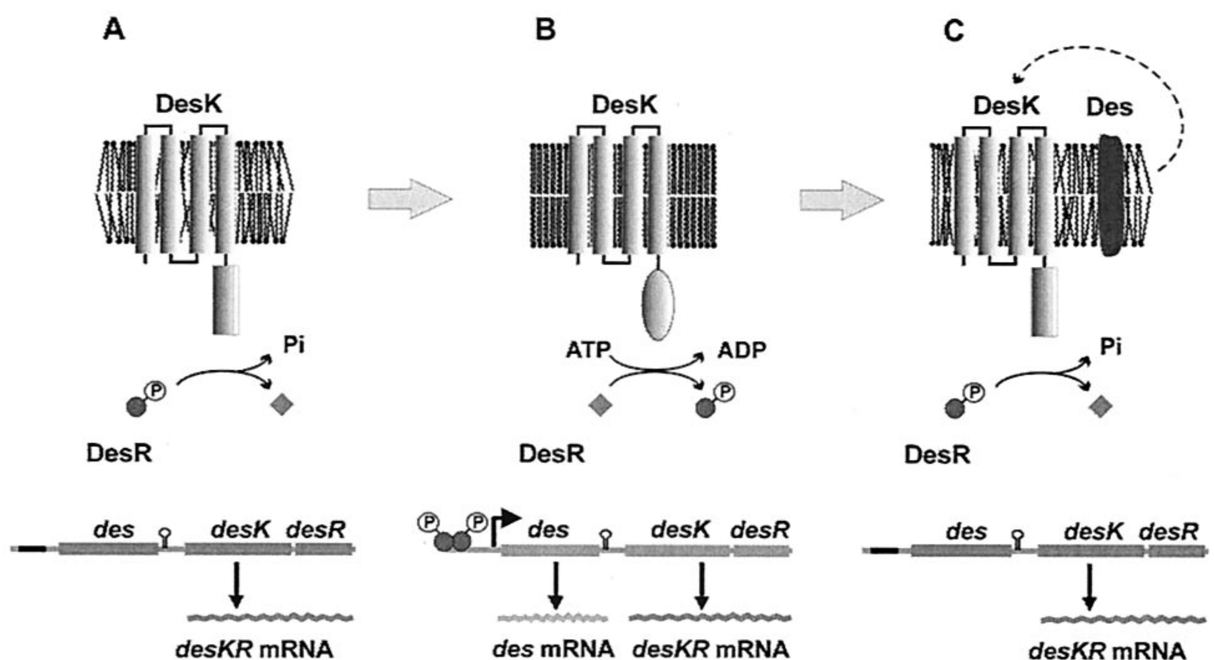


Fig. 7. **Signal transduction pathway leading to membrane fluidity optimization in *B. subtilis.*** (A) A phosphatase-dominant state is present when membrane lipids are disordered. (B) A kinase-dominant state of DesK predominates upon an increase in the proportion of ordered membrane lipids. DesK-mediated phosphorylation of DesR results in transcriptional activation of *des*. (C) Activation of *des* results in synthesis of Des, which desaturates the acyl chains of membrane phospholipids. These newly synthesized UFAs inhibit *des* transcription by favoring the dephosphorylation of DesR. Reproduced from the reference: (Mansilla *et al.* 2004).

Because of the difficulties in expressing the complete *desK* gene in *E. coli,* the N-truncated form of DesK (DesKC), retaining a major part of the cytoplasmic domain that includes the conserved kinase domain was employed in the detailed study. In vitro, DesKC was able to undergo the autophosphorylation in the presence of ATP and Mg^{2+} and transfer the phosphoryl group to Asp-54 residue of response regulator DesR (Albanesi *et al.* 2004; Cybulski *et al.* 2004). At the same time, it was able to desphophorylate the phosphorylated form of DesR in vitro (Albanesi *et al.* 2004). Histidine 188 residue of DesK was shown to be the phosphoacceptor amino acid, essential for the kinase activity but not for the phosphatase one (Albanesi *et al.* 2004). In vivo, the truncated form of DesK retained the kinase activity but totally lacked the phosphatase one. Thus, the authors concluded that the transmembrane domains play a key role in the regulation of the ratio of kinase to phosphatase activity of DesK and transferring the membrane conformational changes to the cytoplasmic portion of the sensor (Albanesi *et al.* 2004). The work of another research group also confirmed that the membrane domain is the temperature-sensing element of this two-component system (Hunger *et al.* 2004).

Just recently, the cytoplasmic portion of DesK, which consisted of a catalytic core and ATP-binding domain, has been crystallized (Albanesi *et al.* 2009). This has revealed detailed information about the structure and function of this sensor. The catalytic core of DesK was found to have a homodimeric structure. Each monomer consists of an N-terminal antiparallel 2-helix hairpin (containing helices α1, α2 and the phosphorylatable residue, His188) connected by a short linker to a C-terminal ATP-binding domain. The helical hairpins of two monomers interact and together form a central four-helix bundle domain, which is responsible for dimerization and phosphotransfer (DHp domain). The ATP-binding domain (ABD) is constituted of a five-stranded β-sheet opposed by a layer of three α-helices. In the proposed model, the sensor domain of the protein induces the structural changes in the central helical domain. This conformational signal is transmitted by a two-helical coiled coil that connects the sensor domain with the catalytic core. DesKC homodimer was found to adopt three distinct conformational states that correspond to the different enzyme activities (Fig. 8). In the phosphatase-competent conformation, the structure is compact, with ABD's attached to DHp domain, which is available for interaction with phosphorylated DesR. When the "cold" signal from the membrane is transmitted the helical rearrangement results in releasing the ABD for histidine 188 phosphorylation (kinase-competent conformation). Autophosphorylation of DesK induces yet different, assymetrical conformation (with a characteristic bend in helix α1) which is able to interact with DesR (Albanesi *et al.* 2009). The most recent experiments, performed on chimerical single-membrane spanning minimal sensor (MS), derived from DesK multimembrane-spanning domain, have revealed that it is the membrane thickness that controls the signaling state of sensor (Cybulski *et al.* 2010). DesK possesses five transmembrane domains (TM) of which the first (TM1) was shown to play a central role in modulating DesK kinase activity and the last (TM5) contains the important coiled coil that propagates the signal to cytoplasmic domain (DesKC). These authors were able to construct the minimal sensor (MS), a fusion construct linking the N-terminal half of TM1 (aminoacids 1-17), the C-terminal half of TM5 (aminoacids 138-151) and DesKC. MS can be, unlike the whole DesK, expressed in *E. coli*. It was shown to fully retain the activity of parental system (both *in vitro* and *in*

vivo) and thus became a very useful tool for the research on DesK sensing (Cybulski *et al.* 2010). The cluster of hydrophilic aminoacids, located at the N-terminus of MS, is crucial for membrane fluidity sensing because it constitutes a built-in instability when fully buried in the membrane lipid phase. Under conditions that invoke increased lipid

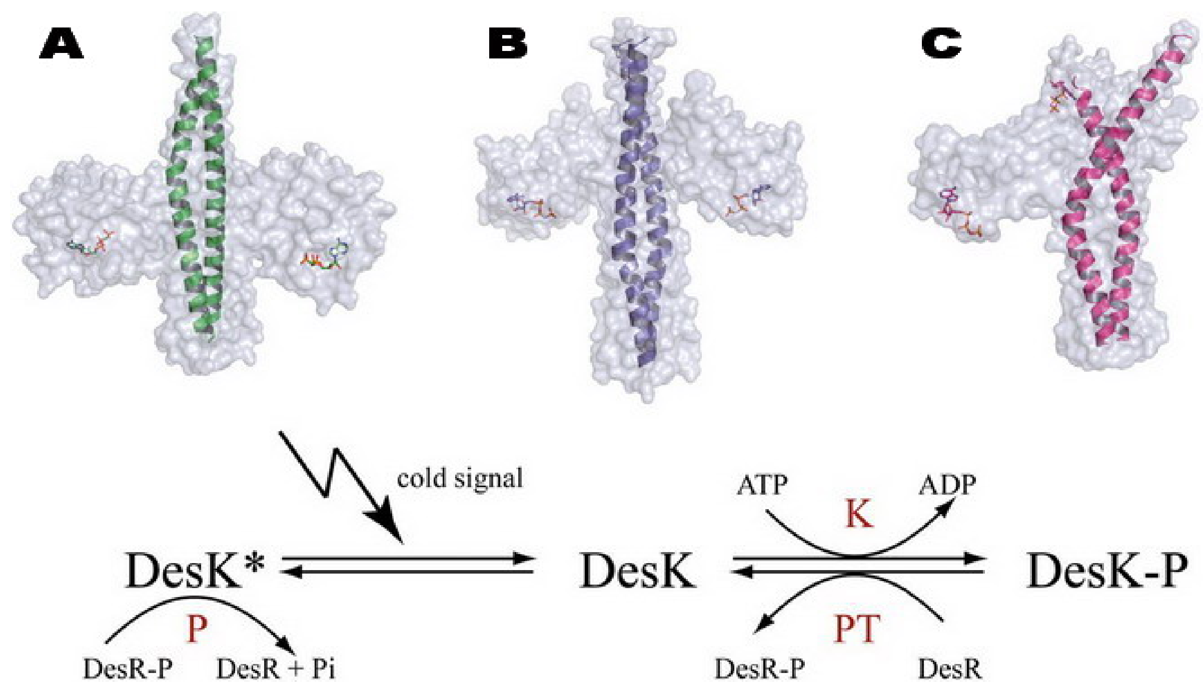


Fig. 8. The conformation of DesKC homodimer in the three functional states: A - phosphatase-competent (DesK*), B- kinase-competent (DesK), and C- phosphotransferase-competent (DesK-P). In the surface models helix α1 is highlighted. The corresponding reactions (P – phosphatase, K – kinase, PT - phosphotransferase) are indicated in the lower panel. Reproduced from reference: (Albanesi *et al.* 2009).

ordering (such as a low temperature) the membrane becomes thicker because of the close packing of lipids. The authors suggested a following “sunken-buoy” (SB) model for temperature sensing. When the membrane becomes thicker, the hydrophilic motif is trapped in the anhydrous environment and the consequent destabilization promotes kinase activity. Conversely, when lipids are more disordered and the membrane narrower, the SB-motif reach the aqueous environment which stabilizes the phoshatase state of DesK (Cybulski *et al.* 2010).

Response regulator DesR is a 22.18-kDa cytoplasmic protein, belonging to the NarL group of response regulators, that contain a helix-turn- helix motif within their C-terminal domain (Fabret *et al.* 1999). This response regulator binds specifically to the same promoter regulatory region of the *des* gene both after the temperature downshift (Aguilar *et al.* 2001) and at 37 °C on minimal medium (Cybulski *et al.* 2002). The phosphorylation of DesR is crucial for its binding activity, since a non-phosphorylated regulator is not able to bind the *des* promoter (Cybulski *et al.* 2004). In unphosphorylated protein, the N-terminal regulatory domain was shown to obstruct the access of the of C-terminal binding domain to its DNA target. However, the phosphorylation of Asp-54, located in the receiver domain, causes the repositioning that releases this sterical inhibition and activate

the binding capacity of DesR to DNA (Najle *et al.* 2009). Unphosphorylated DesR exists in the form of a dimer in solution. The phosphorylation induces new interactions between DesR dimers thus promoting the formation of tetramers.

Phosphorylated DesR (DesR~P) binds to the DNA sequence between positions -77 and -28, relative to the des transcription start site of *des*. Two inverted repeats (5'-TCAT-3') separated by 9 nucleotides were identified in the center of this sequence (Aguilar *et al.* 2001). DesR~P dimers bind in cooperative fashion to the two adjacent binding regions of *des* promoter. One DesR~P dimer binds primarily in the region designated region A (RA) and the occupancy of this region allows another dimer to bind region B (RB). The sequence of RA and RB is not identical. Region A harbors two inverted repeats 5'-ATGACA-3' (IR-L, left), and 5'-TGTCAT-3' (IR-R, right) separated by 2 base pairs. The palindromic motif is absent in region B, except for the short sequence 5'-ATGA-3' which is identical to the first four nucleotides of IR-L that was designated DR (direct repeat). IR-L, IR-R and DR were shown to be essential because mutation in any of them abolished the *des* expression (Cybulski *et al.* 2004).

The response regulator bound to both RA and RB regions efficiently stabilizes the interaction between RNA polymerase (RNAP) and *Pdes*, leading to the formation of a ternary open complex thus leading to *des* gene expression (Cybulski *et al.* 2004). For the contact of RNAP, DesR and DNA the tridimensional orientation is crucial.

3.3 Fatty acid composition of *Bacillus subtilis* membrane

The fatty-acyl chain composition of membrane lipids is not a constant feature of a certain bacterial species (or strain). The pattern and levels of different fatty-acyl-species depend on many factors. The endogenous (genetic) factors, inherent to particular bacterial species and/or strains, comprise the activity and specificity of enzymes involved in the FA synthesis and also the endogenous supply of priming substances. The relative activity of α-keto substrates as primers for FA synthesis in *B. subtilis* was reported to be in the order α-keto-β-methylvalerate ≥ α-ketoisocaproate > α-ketoisovalerate (Kaneda 1973; Naik and Kaneda 1974). Moreover, the cellular pools of chain initiators are basically influenced by the nutrient conditions. Many environmental factors (such as temperature, various types of chemical compounds and different environmental stresses) and physiological factors (such as growth phase) also affect this characteristic of a particular bacterial cell.

The physical properties of phospholipids (PLs) depend on the distribution of fatty acids between the 1- and 2- positions, the nature of the phosphate derivative in the 3-position and the physical properties of fatty acid components (Kaneda 1977).

The PLs of higher plants, animals and also *E. coli* exhibit the positional preference with regard to saturated and unsaturated FA. The 1-position is usually occupied by the saturated FA, whereas the unsaturated species are often found in the 2-position (Kaneda 1977). For *B. subtilis*, it was observed that in the 2-position the anteiso-15:0 FA is the most abundant. The preference towards this position was reported to be in the order of anteiso- > iso- > straight-chain among the three acids with the same carbon number. Within a chosen series, the shorter the chain length, the higher is the preference to the 2-position (Kaneda 1972). Straight-chain FA show the preference mainly to 1-position (Kaneda 1977). However, the mechanism by which the fatty acids are preferentially incorporated into one of the two positions of the phospholipids in *Bacillus* species has yet not been elucidated.

3.4 Cold shock response and low temperature adaptation in bacteria

Many organisms, including bacteria, have to face the fluctuations in the environmental temperature in both directions during certain periods of their life cycles. While homoiothermic organisms employ the strategy of maintaining a stable body temperature, the poikilothermic organisms, including microorganisms, have to fully adjust their bodies and/or cells to the ambient temperature. It is worth noting that, in respect to low temperature adaptation, it has to be distinguished between the response to sudden temperature downshift and the adaptation for *long-term* growth and survival in cold temperatures (Panoff *et al.* 1998).

In bacteria, it was the response to increased temperature, the heat shock response, which was extensively studied first (Neidhardt *et al.* 1984). More recently, however, the adaptation of the bacterial cell to the temperature downshift has also become an attractive research topic. Apart from making the important contribution of the revealing of the basic principles of bacterial cell functions, research in this field is also of great industrial and commercial importance. Knowledge about bacterial adaptation to low temperatures and its regulation can have many useful implications for the food industry, agriculture or the commercial production of temperature sensitive proteins.

The decrease of ambient temperature impacts a bacterial cell on several levels. The target cellular structures that, due to their thermo-dynamical properties, respond physically to a rapid change in temperature are cellular membranes, proteins and nucleic acids. Hence, all these structures can play an important role in the "temperature detecting" systems of a bacterial cell.

Due to low temperature, the membrane lipids undergo a reversible change from a fluid (disordered) to a non-fluid (ordered) phase. Such a rigidified membrane cannot support properly the crucial physiological processes such as transport, replication and energy production. The negative supercoiling of DNA increases and this may adverse the helix opening during transcription (Grau *et al.* 1994; Woldringh *et al.* 1995; Aguilar *et al.* 1998; Graumann and Marahiel 1999b). The secondary structures of RNA are stabilized by the effect of low temperature, which hampers both transcription and translation and requires remodeling of ribosomes. The protein folding is too slow or inefficient, and primary metabolism is also affected due to the decreased activity of numerous enzymes (Jones and Inouye 1994; Panoff *et al.* 1998; Graumann and Marahiel 1999b; Weber and Marahiel 2002).

To overcome the above mentioned problems, bacteria have evolved effective adaptation mechanisms. They adjust their membranes in order to attain the necessary membrane fluidity and induce the synthesis of proteins that help to modify the transcription, translation and metabolic machinery in order to work efficiently under new conditions.

The cold shock response and cold shock proteins have been studied in detail mostly in *E. coli* and *B. subtilis*, which serve as the models of Gram-negative and Gram-positive cells, respectively. However, interesting research has also been performed on other bacterial species recently.

During heat shock response of *B. subtilis*, a specialized alternative sigma factor

σ^B (SigB) has been shown to play a key role in controlling genes involved in the response to high temperatures, together with other regulatory genes (Schumann 2003). Alternative sigma factor σ^B (Haldenwang and Losick 1979) governs the regulon which is believed to provide the non-growing cell with a non-specific, multiple and preventive stress resistance. σ^B regulon comprises more than 120 genes encoding general stress proteins, that are induced by heat, ethanol, salt or acid stress, or during energy depletion. These proteins are involved in non-specific protection against oxidative stress and also protect cells against heat, acid, alkaline or osmotic stress (Hecker and Volker 2001; Petersohn *et al.* 2001). σ^B regulon has also been reported to be induced during the *long-term* growth of *B. subtilis* in a low temperature and seems to be essential for its survival at temperatures around 15 °C (Brigulla *et al.* 2003)

For cold shock, the regulation of the response appears to be more complex, as it is organized more like a stimulon rather than a regulon (Weber *et al.* 2001). However, an alternative *B. subtilis* sigma factor, σ^L (σ^{54}) (Debarbouille *et al.* 1991) was quite recently reported to be involved in the regulation of at least two cold shock adaptation pathways (Wiegeshoff *et al.* 2006).

The DesK-DesR two-component system was proved to act as a thermosensor that directly regulates the synthesis of desaturase in *B. subtilis* (Aguilar *et al.* 2001). However, this regulation pathway seems to be unique only for this protein and does not represent the general temperature perception system for induction of the cold shock response in *B. subtilis* (Beckering *et al.* 2002).

3.4.1 Cold shock and cold inducible proteins

The proteins involved in cold adaptation bring about many different physiological functions. It was shown that they serve as RNA chaperones, bind on ribosomes, are involved in transcription initiation, termination and antitermination, recombination, protein folding, trehalose synthesis, lipid desaturation, chemotaxis and other processes (Phadtare 2004). Those proteins are termed cold shock proteins, CSPs (proteins with the typical cold shock domain, see below), cold induced proteins, CIPs (other proteins induced after a cold shock) and cold acclimation proteins, CAPs (proteins that are detected in higher levels at low temperatures) (Graumann and Marahiel 1999b).

The first cold shock protein (CSP) identified was CspA from *E. coli* which functions as a transcriptional activator and RNA chaperon. *E. coli* possesses five other homologues of this protein: CspB to CspF (Lee *et al.* 1994) and others have been reported for several species of both Gram-negative and Gram-positive bacteria, including *B. subtilis* (Av-Gay *et al.* 1992; Schroder *et al.* 1993; Ray *et al.* 1994; Gumley and Inniss 1996; Mayr *et al.* 1996; Berger *et al.* 1997; Graumann and Marahiel 1998) as well as some archaea (Cronan 1975). Cyanobacteria lack the CspA homologues but posses several cold-inducible RNA-binding proteins from RBD (RNA-binding domain) family (Suzuki *et al.* 2000).

A typical feature for CSPs is the presence of the conserved cold shock domain harboring the nucleic acid binding motifs (Graumann and Marahiel 1998; Weber *et al.* 2002) and characteristic folding that comprises a typical β-barrel structure (Landsman 1992; Schnuchel *et al.* 1993; Weber and Marahiel 2002).

The genes encoding the proteins of the CSP family are often found in multiple copies within the genome of a given species. This duplication and later adaptation resulted in specific groups of genes that are involved in several physiological aspects and respond to different environmental stresses, not only to cold shock (Graumann and Marahiel 1998; Yamanaka *et al.* 1998). Their presence in the earliest diverging bacterial branches indicates the ancient origin of this protein family (Deckert *et al.* 1998; Perl *et al.* 1998).

Regulation of expression of genes encoding this group of cold inducible proteins happens at the post-transcriptional level. The unusually long 5'untranslated region (5' – UTR), containing highly conserved sequence called "cold box", is presumed to be responsible for extreme stabilization of their mRNA at low temperature that otherwise has very short half-time at 37 °C. Besides, another unique sequence located 14-bases downstream of the initiation codon, denoted as the "downstream box", was reported to enhance the initiation of translation of cold shock mRNAs (Mitta *et al.* 1997). The proposed mode of action presumes that cold-inactivated ribosomes first exclusively synthesize CSPs, which then alter the ribosome conformation and hence enable the synthesis of other, so called cold induced proteins (CIPs) which finally act to restore the bulk protein synthesis (Graumann *et al.* 1997).

3.4.1.1 Cold shock proteins of *Bacillus subtilis*

In *Bacillus subtilis*, three homologues of *E. coli* CspA were identified, CspB, CspC and CspD, which are all cold shock inducible (Willimsky *et al.* 1992; Graumann *et al.* 1996; Graumann and Marahiel 1999b). The presence of at least one of these is essential for *B. subtilis* growth both at an optimal temperature and after a cold shock; the triple deletion mutant is not viable. A hierarchy of importance exists for cellular growth within the *B. subtilis* CSP family, with CspB being of the highest importance (Graumann *et al.* 1997).

CspB and CspC proteins are also induced at the onset of the stationary phase. Deletion of both *cspB* and *cspC* genes leads to lysis of cells during stationary phase (Graumann and Marahiel 1999a). *B. subtilis* CspB was reported to bind to single stranded DNA and RNA (Graumann and Marahiel 1994; Schroder *et al.* 1995). The regulation of *cspB* and *cspC* expression happens at the post-transcriptional level by transient stabilization of their mRNA at a low temperature (Kaan *et al.* 1999). Moreover, the protein conformation of CSPs, that is unstable in the solution, is stabilized by binding to RNA. That might allow additional regulation at the level of protein stability (Graumann and Marahiel 1998).

CSPs are believed to be involved in transcription and /or translation initiation. They appear to function as RNA-chaperones, preventing the formation of secondary structures on RNA, and possibly stabilize RNA-polymerase open complex at low temperatures (Graumann *et al.* 1996; Graumann *et al.* 1997; Mitta *et al.* 1997; Graumann and Marahiel 1998; 1999b; Weber and Marahiel 2002).

Nevertheless, CSPs are not the only proteins induced after the temperature downshift in *B. subtilis*. The increased synthesis of 37 proteins in *B. subtilis* JH642 grown in rich medium after the cold shock (from 37 °C to 15 °C) was reported. The most induced were the cold shock proteins CspB, CspC and CspD. Deletion of *cspB* gene resulted in the

arrest of induction of 15 otherwise cold induced proteins (Graumann *et al.* 1996).

The large group of *B. subtilis* CIPs and CAPs comprises proteins with a broad spectrum of functions: proteins involved in chemotaxis (CheY), sugar uptake (Hpr), protein folding (PPiB, Tig) or general metabolism such as aminoacid synthesis (CysK, IlvC, ThrC, GlnA, LeuC) and glycolysis (TIM, Gap, Fba). This group also includes many ribosome-associated proteins (S6, L7/L12, L10, EF-Ts, EF-Tu, EF-G) that indicates either remodeling of ribosomes and/or increase of their amount in the cell in order to facilitate translation efficiency (Graumann *et al.* 1996; Graumann and Marahiel 1999b; Weber and Marahiel 2002).

Quite recently, two independent genomwide trancriptional profiling studies (using macroarrays) of *B. subtilis* cold shock response were published (Beckering *et al.* 2002; Kaan *et al.* 2002) that reported the cold induction of wide range of genes. Beckering *el al.* used *B. subtilis* strain JH642 grown on minimal medium (supplemented with isoleucine) and the cold shock from 37 °C to 15 °C , whereas Kaan and coauthors employed strain 168, minimal medium with glucose (without Ile) and temperatures 37 °C and 18 °C.

The study of Beckering *et al.* reported around 80 genes induced (more than two fold) 70 minutes after the cold shock (40 of them being of yet unknown function) and around 280 genes repressed (Beckering *et al.* 2002). Kaan with coauthors observed around 50 cold induced genes and the similar number was downregulated after the cold shock (Kaan *et al.* 2002). Among the most induced genes there were found genes encoding cold shock proteins (*cspB*, *cspC*,*cspD*) and fatty-acyl-desaturase (*des*). The other induced genes comprise genes for ribosomal proteins, initiation factor homologues, RNA helicase, gyrase, YtrABC transporter (probably involved in acetoin utilization), manganese ABC transporter and others (Beckering *et al.* 2002; Kaan *et al.* 2002). The induction of a seven-gene operon *ptb-bcd-buk-lpd-bkdA1-bkdA2-bkdB* involved in branched aminoacid (Ile, Val) utilization was reported in the work of Kaan *et al.*, but surprisingly not in the study of Beckering *et al.* (Beckering *et al.* 2002; Kaan *et al.* 2002). Genes encoding proteins involved in aminoacid, purine and pyrimidine biosynthesis and also some genes of glycolysis, citric acid cycle, ATP production and cell wall build-up were down-regulated after the cold shock, most likely because of the slower growth of the cells and overall reduced metabolic activity in low temperature (Beckering *et al.* 2002; Kaan *et al.* 2002). Also many of typical heat shock genes were repressed (Beckering *et al.* 2002).

The synthesis of several proteins of *B. subtilis* that is induced by a low temperature was also reported to be increased by other types of environmental stresses, such as osmotic or heat shock. CheY that regulates the flagellar rotation during chemotaxis, was induced both by cold and heat shock. The synthesis of protein Hpr, that is involved in the phosphoenolpyruvate: sugar phosphotransferase system, was shown to be increased after cold, heat and salt stress in *B. subtilis* (Graumann *et al.* 1996; Aguilar *et al.* 1998; Graumann and Marahiel 1999b).

The regulon of alternative sigma factor σ^B plays an important role in several types of stresses and starvation conditions (Hecker and Volker 2001; Petersohn *et al.* 2001). The induction of this regulon has never been observed after the cold shock; therefore, its role in cold adaptation has never been discussed. However, a recent study by Brigulla *et al.* (2003) reported the increased synthesis of σ^B and induction of its whole regulon during

the continuous growth of *B. subtilis* in a low temperature (15 °C). This chill induction was shown to be completely independent of the classic pathway based on RsbV regulatory protein (usually controlling the σ^B regulon induction under other types of stresses) which suggests the existence of a new pathway for *sigB* gene activation in cold. The deletion of *sigB* gene strongly impaired the growth of *B. subtilis* at 15 °C, but not at temperatures above 16 °C, which indicates that σ^B regulon plays the main role in the cold adaptation to temperatures close to the lower limit of *B. subtilis* growth. However, the analysis of the σ^B regulon members did not reveal any particular proteins that might serve a specific function during continuous growth at low temperatures (Brigulla *et al.* 2003).

3.4.2 Cytoplasmic membrane adaptation to cold

Cytoplasmic membrane fulfills the crucial function of the selective barrier in the bacterial cell. Many types of proteins with a wide range of different functions are associated with the membrane. A number of environmental factors, such as changes in temperature, pH, osmotic and atmospheric pressure, are sensed at the level of cytoplasmic membrane, through which the particular signals are then transmitted to the cell (Fujii and Fulco 1977; Vigh *et al.* 1998). The physical state of the lipid bilayer is critical for all the physiological functions mentioned above. The membrane-bound cellular processes require membrane lipids to be mostly fluid. A decrease in temperature leads to transition of the lipid bilayer from liquid-crystalline (disordered) to gel (ordered) phase. Rigidified membrane is not able to support its physiological functions. Therefore, it is essential for the cell to keep the midpoint of phase transition (the transition temperature) above the actual ambient temperature. The transition temperature is determined by a chemical composition of the particular membrane, above all by the fatty-acyl chains of membrane lipids. The saturated straight fatty-acyl chains are packed much more closely compared to those with double bonds and/or chain branching. The reason for this phenomenon is the steric hindrance brought about by the rigid kink of the *cis* double bond or chain branching (Raudino and Sarpietro 2001). The cross-sectional area occupied by branched fatty acids in monolayer films is significantly larger than that of straight-chain fatty acids (Willecke and Pardee 1971).

The melting points of branched-chain FAs are generally lower than their straight-chain counterparts with the same number of carbons. The FAs of anteiso-series exhibit a melting temperature 25 °C to 35 °C lower than those of non-branched series. Melting points of iso-branched FAs are only slightly lower compared to straight-chain family. However, phospholipids with iso-branched FAs exhibit substantially lower transition temperature than their counterparts with non-branched fatty-acyl chains of the same carbon number (Kaneda 1977; 1991), see Table 2.

Table 2 . Melting point of fatty acids (°C)

Total carbons	non-branched series	iso-branched series	anteiso-branched series
12	44.2		
13	41.5	41.3	6.2
14	53.9	53.3	
15	52.5	51.7	23.0
16	63.1	62.4	
17	61.3	60.2	36.8
18	69.6	69.5	

Reproduced from references: (Kaneda 1977; 1991)

For this reason, the general strategy of membrane adaptation to low temperatures for organisms lacking cholesterol consists of the incorporation of low-melting fatty acids

to their membrane lipids. The fluidity of membranes composed mostly of straight-chain fatty acids is adjusted to the required level by inclusion of monounsaturated fatty acids, whereas organisms with branched fatty acids modulate the levels of different branched FA species (Kaneda 1991; Mansilla *et al.* 2004; Albanesi *et al.* 2009). Due to its membrane composition, *B. subtilis* can apply both aforementioned strategies of membrane fluidity optimization (Suutari and Laakso 1992; Aguilar *et al.* 1998; Klein *et al.* 1999).

The thermal regulation of membrane composition has been widely studied in the model organisms *Escherichia coli* and *Bacillus subtilis*. With the accumulation of knowledge on this topic, it has become more and more evident that these two organisms have evolved totally different mechanisms for achieving the same goal, i.e. adjusting the membrane fluidity to temperature.

In contrast to *E. coli*, *B. subtilis* has separate systems for the synthesis of saturated and unsaturated fatty acid and its regulation. The oxygen-dependent desaturation occurs at the level of saturated fatty-acyl chains formerly incorporated into membrane lipids (Grau and de Mendoza 1993). However, it is not the unique system that allows this bacterium to adjust its membrane composition to low temperatures. *Bacillus subtilis* also takes advantage of its typical membrane lipid composition that is dominated by branched fatty acids. Therefore, the modulation of the ratio anteiso/iso- branched fatty acid species in its membrane lipids provides this bacterium an additional tool for membrane adjustments. Suutari and Laakso (1992) studied the influence of temperature on membrane fatty acid composition of *B. subtilis* (grown in rich cultivation medium). They observed the increased levels of anteiso-branched FAs (anteiso-15:0 and anteiso-17:0) and UFAs in its membranes as the cultivation temperature was reduced. This increase was accompanied by concomitant decrease of iso-branched and non branched FA species (Suutari and Laakso 1992). A similar mechanism is also used by *Listeria monocytogenes* (Annous *et al.* 1997).

Desaturation of membrane lipids is a process that is usually employed as a quick “first aid” when a sudden temperature downshift occurs. In contrast, the change of branching pattern requires *de novo* lipid synthesis and therefore is more convenient for long-term adaptation for low temperatures. Nevertheless, these two mechanisms can substitute for each other to a certain extent.

B. subtilis strain JH642 was reported to poorly survive the cold shock from 37 °C to 15 °C on minimal medium. However, the addition of casaminoacids, isoleucine (Ile) or threonine (Thr) had a cold protective effect and enabled the growth of cold shocked cells. It should be emphasized that this strain is not auxotrophic for Ile or Thr at 37 °C. The effect of Thr was proved to be based on its conversion to Ile. Since α-keto-β-methylvalerate and 2-methylbutyrate, the derivatives of isoleucine, which are intermediates for anteiso-branched fatty acid synthesis, exhibited the same effect, the authors concluded that the principle of cold protection conferred by Ile resides in its function as a precursor of low-melting fatty acids. Indeed, in the membranes of cells supplied with Ile the increased levels of anteiso branched FA were detected 18 hours after the cold shock (Klein *et al.* 1999). Although other *B. subtilis* strains, ATCC6051 and 168, do not require the exogenous Ile supply or related metabolites for cold shock survival, they exhibit the similar FA profiles. This observation is most probably based on their higher biosynthetic capacity for Thr and consequently Ile, compared to strain JH642

(Klein *et al.* 1999). Indeed, the ketol-acid reductoisomerase IlvC and threonine synthase ThrC, involved in Ile and Thr synthesis respectively, were reported to be induced after the cold shock in *B. subtilis* strain JH462 (Graumann *et al.* 1996; Graumann *et al.* 1997). The disruption of *des* gene does not reduce *B. subtilis* exponential growth after the cold in rich medium or minimal medium supplemented with Ile, however, the cells were not able to grow while Ile was absent in the medium (Aguilar *et al.* 1998; Klein *et al.* 1999)

3.5 Anaerobic growth of *B. subtilis*

In the upper layers of soil, which is the natural habitat of *Bacillus subtilis*, fluctuations in oxygen availability, mostly caused by the changing water content, are common. Even though *B. subtilis* is often still generally considered to be obligate aerobe, several recent studies have proved that it is a facultative anaerobe, which is able of both fermentation and anaerobic respiration with either nitrate or nitrite as a terminal acceptor. Both are normally present in high concentrations in soil, being products of nitrification (Nakano and Zuber 1998). However, *B. subtilis* is not capable of forming gaseous products such as NO, N_2O or N_2 and thus is not a denitrifier (Hoffmann *et al.* 1998; Nakano and Zuber 2002).

Genes of anaerobic metabolism are significantly induced by oxygen limitation. The switch between aerobic and anaerobic metabolism is regulated mainly at the transcriptional level and, in some cases, by modulation of enzyme activity (Nakano and Zuber 2002).

A microarray analysis revealed many genes induced under anaerobic conditions. During dissimilatory reduction of nitrate or nitrite the most highly induced genes or regions were *narGHJI, narK*, *fnr* and *hmp*, followed by *nasDEF*, *cydABCD* , *sbo-alb*, *ywiC* and *ywiD* (*arfM*). During fermentative growth these were *lctE*, *lctP* and *alsD*. The details about these genes are given in sections 3.5.1 and 3.5.2. A number of other genes, including those with unknown functions, are influenced by oxygen limitation (Ye *et al.* 2000).

3.5.1 Anaerobic respiration

Anaerobic nitrate reduction in *B. subtilis* was shown to be coupled to energy generation. Nitrite is reduced to ammonia without forming proton gradient and ATP, enhancing the anaerobic growth by serving as an electron sink (Nakano and Hulett 1997; Nakano *et al.* 1998a). Nitrate reductase involved in anaerobic nitrate respiration encoded by the operon *narGHJI* (Hoffmann *et al.* 1995) is membrane bound, whereas nitrite reductase encoded by *nasDE* (Hoffmann *et al.* 1998; Nakano *et al.* 1998a) is soluble. Expression of both is strongly induced by low oxygen tension. The product of gene *moaA* (*narA*), the enzyme that functions in the biosynthesis of molybdopterin, the component of molybdenum cofactor, is indispensable for nitrate respiration in *B. subtilis* (Glaser *et al.* 1995; Nakano and Zuber 1998).

The respiratory nitrite reductase consists of subunits NarG, H, and I. The product of gene *narJ* is required for the assembly of the enzyme. The expression of nitrate reductase is strongly induced by anaerobiosis and the induction is dependent on Fnr anaerobic regulator (Nakano and Zuber 1998), see section 3.6.2. The *narGHJI* operon bears a Fnr-binding sequence within its upstream regulatory region that is essential for its anaerobic activation (Nakano and Zuber 1998; Reents *et al.* 2006) which is further enhanced by the presence of nitrate (Reents *et al.* 2006).

Besides dissimilative nitrate reductase, *B. subtilis* possesses another genetically and biochemically distinct nitrate reductase, encoded by operon *nasBC*, which is responsible

for nitrate assimilation under aerobic nitrogen limitation conditions (Nakano *et al.* 1995; Ogawa *et al.* 1995). On the contrary, the sole nitrite reductase coded by *nasDE* is involved both in anaerobic nitrite ammonification and aerobic nitrite assimilation (Ogawa *et al.* 1995; Hoffmann *et al.* 1998; Nakano *et al.* 1998a).

Genes *nasDE* are part of the *nas* operon (*nasBCDEF*) containing also genes *nasBC* (encoding the above mentioned assimilatory nitrate reductase) and *nasF* (with product involved in siroheme cofactor formation). Two promoters were found to control the transcription of this operon. Transcription from *nasB* promoter is activated during nitrogen limiting conditions (Nakano *et al.* 1995). Transcription from the internal *nasD* promotor occurs when oxygen tension is low, even in the presence of excess nitrogen. The induction of anaerobic nitrite reductase activity is also dependent on ResDE regulatory system (section 3.6.1), but independent on Fnr (Hoffmann *et al.* 1998; Nakano *et al.* 1998a). Nitrite stimulates this anaerobic induction in a ResDE-dependent (Nakano *et al.* 1998a; Nakano and Zuber 1998) and Fnr-dependent (Reents *et al.* 2006) manner. During aerobic growth under nitrogen limitation, *nasDE* transcription is activated by another regulatory circuit, via nitrogen regulator TnrA (Nakano *et al.* 1998a)

The expression of gene *hmp*, encoding flavohemoglobin, was also reported to be induced under anaerobic conditions. This induction requires nitrite (the product of nitrate

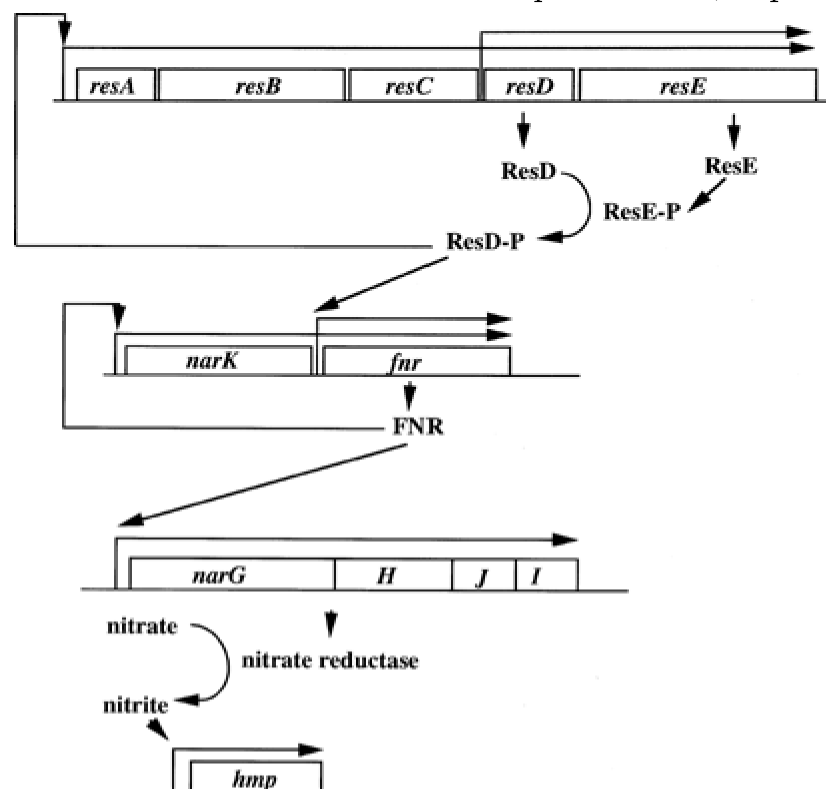


Fig. 9. Regulatory pathway of nitrate respiration in *B. subtilis*. Arrows with lines indicate directions of transcription. Also shown are possible flows of information to induce nitrate respiration. ResD phosphorylated by a ResE kinase activates the transcription of the *resA* operon. ResD-phosphate is also required for the anaerobic induction of fnr transcription from the *fnr*-specific promoter. FNR activates transcription of genes involved in nitrate respiration such as the *narK* and *narG* operons. Nitrite produced from nitrate by nitrate reductase induces *hmp* transcription. Reproduced from reference: (Nakano and Hulett 1997)

reductase) and ResDE two-component system (LaCelle *et al.* 1996). Hmp flavohemoglobin is supposed to function in the detoxification of nitric oxide (NO) that arises from nitrite. NO upregulates *hmp* expression under both aerobic and anaerobic conditions (Moore *et al.* 2004). This NO-stimulated induction of *hmp* happens even in the absence of ResDE, though in lesser extent (Nakano 2002). This ResDE-independent induction in the presence of NO was shown to be mediated by the regulator NsrR (Nakano *et al.* 2006), (see section 3.6.1). The product of *hmp* gene was reported to be important for prolonged survival of *B. subtilis* cells under nitrate respiration conditions (Nakano 2006). The regulatory pathway of *B. subtilis* nitrate respiration is depicted in Fig. 9. Regulatory proteins Fnr and ResDE are discussed more in detail in section 3.6.

3.5.2 Fermentation

In the absence of alternative external electron acceptors, *B. subtilis* can grow by fermentation, using a mixed acid/butanediol pathway, with the main products being lactate, acetate, acetoin and 2,3-butandiol and ethanol (Nakano *et al.* 1997; Cruz Ramos *et al.* 2000), see Fig. 10.

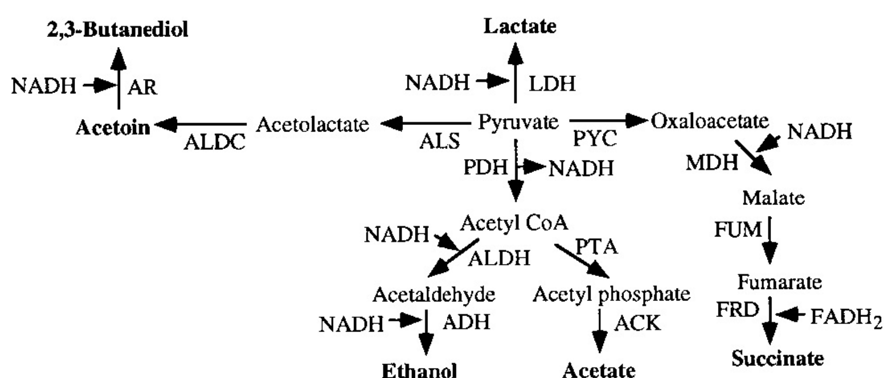


Fig. 10. Fermentation pathways of *B. subtilis*. Enzyme abbreviations: ACK, acetate kinase; ADH, alcohol dehydrogenase; ALDC, acetolacetate decarboxylase; ALDH, aldehyde dehydrogenase; ALS, acetolactate synthase; AR, acetoin reductase; FRD, fumarate reductase; FUM, fumarase; LDH, lactate dehydrogenase; MDH, malate dehydrogenase; PDH, pyruvate dehydrogenase; TA, phosphotransacetylase; PYC, pyruvate carboxylase. Reproduced from reference: (Nakano *et al.* 1997).

Pyruvate or a mixture of aminoacids stimulate substantially the fermentation of glucose, which is otherwise very ineffective. The reason seems to be the lack of pyruvate formate lyase in *B. subtilis*, which catalyzes formation of acetyl-CoA and formate from pyruvate in *E. coli*. Instead, pyruvate dehydrogenase, PDH, (generating acetyl-CoA from pyruvate), seems to play a key role in *B. subtilis* fermentation, as mutants lacking PDH activity produce hardly any fermentation products (Nakano *et al.* 1997). In fact, pyruvate is necessary for anaerobic induction of PDH coding region *pdhABCD* (Ye *et al.* 2000).

During fermentative growth the NADH produced in glycolytic pathway is reoxidized by conversion of pyruvate to fermentation products. Lactate dehydrogenase

catalyzing formation of lactate is indispensable for both fermentative and respiratory anaerobic growth of *B. subtilis.* The operon *lctEP*, encoding lactate dehydrogenase (*lctE*) and lactate permease (*lctP*) is strongly induced by low oxygen levels (Cruz Ramos *et al.* 2000); however, its induction is significantly lowered when the nitrate is present in the medium (Cruz Ramos *et al.* 2000; Marino *et al.* 2000; Ye *et al.* 2000). This way, energetically less efficient fermentation process is decreased in the presence of nitrate respiration. The nitrate-mediated repression of lactate dehydrogenase is dependent on *fnr*-controlled expression of nitrate reductase (Marino *et al.* 2000; Reents *et al.* 2006). Interestingly, Cruz Ramos and coworkers (2000) did not find any change in the fermentation product spectrum when nitrate was added to fermenting cells.

The conversion of pyruvate to acetoin and 2,3-butanediol is catalyzed by α-acetolactate synthase (encoded by *alsS*), α-acetolactate decarboxylase (*alsD*) and acetoin reductase. Acetyl-CoA (formed from pyruvate) is converted to acetate in two-step reaction catalyzed by phosphotransacelytase and acetate kinase; this is the energetically most efficient fermentation pathway. Ethanol is produced from pyruvate by function of aldehyde dehydrogenase and alcohol dehydrogenase (Cruz Ramos *et al.* 2000; Nakano and Zuber 2002).

Genes for enzymes involved in fermentation are induced without direct involvement of Fnr (section 3.6.2), whereas the ResD-ResE two-component transduction system (section 3.6.1) is required for fermentation in *B. subtilis* (Nakano *et al.* 1997; Cruz Ramos *et al.* 2000).The expression of *alsSD* and *lctEP* was shown to be partially dependent on redox regulator ArfM (section 3.6.3) and subjected to AlsR regulator, which was suggested to respond to intracellular pH (Cruz Ramos *et al.* 2000; Marino *et al.* 2001). Phosphoacetyl transferase Pta (involved in acetate formation) is crucial for all types of anaerobic growth, whereas *alsSD* operon (acetoin and 2,3-butandiol production) is dispensable (Cruz Ramos *et al.* 2000). It was shown recently that regulation of *lctEP* transcription is dependent on nitrate reductase activity and mediated by YdiH repressor (Larsson *et al.* 2005; Reents *et al.* 2006), see section 3.6.4.

Acetoin (3-hydroxy 2-butanone) can be secreted out of the cell and later serve as a source of carbon when other carbon sources are exhausted. The protein AcuB involved in acetoin utilization was reported to be induced in anaerobiosis (Clements *et al.* 2002). The YtrABC transporter, probably also involved in reutilization of acetoin (Yoshida *et al.* 2000), is induced after cold shock. Acetoine is of potential interest for cold adaptation, because it is easily converted to 2,3-butanediol; polyols have been described as cryoprotectants (Beckering *et al.* 2002).

3.5.3 Adaptation of metabolic pathways to anaerobiosis

Under anaerobiosis, the basic metabolic pathways of the cell are also affected. For example, in *E. coli* many of glycolytic enzymes are induced under anaerobic conditions, especially during fermentation (Smith and Neidhardt 1983). In *B. subtilis*, pyruvate dehydrogenase is induced under anaerobic conditions, especially under conditions of fermentation (Ye *et al.* 2000; Clements *et al.* 2002).The production of Krebs cycle is also regulated in response to oxygen availability. Especially during fermentative growth, in which NADH is not reoxidized by the respiratory chain, the Krebs cycle enzyme activity

is lowered and the cycle is transformed from a cyclic pathway to two oppositely oriented halfways and functions to supply biosynthetic precursors. Synthesis of 2-keto-glutarate dehydrogenase is replaced by fumarate reductase to allow reductive production of succinyl coenzyme A (Nakano and Zuber 2002). During anaerobic growth with nitrate, citrate synthase and aconitase activities are substantially reduced (independently on Fnr or ResDE), due to transcriptional repression of genes *citB* and *citZ* (Nakano *et al.* 1998b) and succinyl-CoA synthetase production (SucD) is induced (Clements *et al.* 2002).

A proteomic study identified 14 proteins responding to changes in oxygen tension and to anaerobic stress. From these proteins FbaA (fructose 1,6-bisphospate aldolase) and PdhD (E3 subunit of pyruvate dehydrogenase) and YwfI (unknown function) were induced by general anaerobiosis, Hmp (flavohemoglobin of unknown fuction) anaerobically in the presence of nitrite, LctE (lactate dehydrogenase) during fermentation, and proteins GlpK (glycerol kinase) and YjlD (potential NADH dehydrogenase) were anaerobically repressed. Seven other genes were induced by rapid shift to strict anaerobiosis (Marino *et al.* 2000).

As shown by a microarray analysis, genes induced under anaerobiosis include, among others, genes for cytochromes, genes involved in iron uptake and transport and production of antibiotics. Besides genes with known functions, several yet uncharacterized ORFs were shown to be affected during anaerobic conditions, some of them being induced by anaerobiosis in general, others under specific anaerobic conditions, i.e. nitrate or nitrite respiration or fermentation (Ye *et al.* 2000).

Both these studies were performed in a rich cultivation medium, which allows growth of *B. subtilis* by both anaerobic respiration and fermentation. A proteomic study performed in defined medium (Clements *et al.* 2002) revealed proteins induced exclusively by nitrate respiration or stimulated by anaerobic fermentation. The first group comprises proteins Hmp (flavohemoglobin), FeuA (iron-binding protein) and YtkD (mutator homolog); in the second group there are SucD (succinyl-CoA synthetase subunit), PyrR (involved in pyrimidine biosynthesis), TrpC (involved in tryptophan biosynthesis) and YwjH (transaldolase of pentose phosphate cycle). Several proteins were induced at both anaerobic condition tested: AcuB (acetoin utilization protein), PdhC (E2 subunit of pyruvate dehydrogenase), YdjL (2,3-butanediol dehydrogenase) and YvyD (unknown function, similar to ribosomal protein) (Clements *et al.* 2002). However, it must be kept in mind that 2D electrophoresis technique, used for both proteomic experiments cited above, often fails to detect membrane proteins (such as, for example, membrane bound nitrate reductase).

Anaerobic stress in soil is usually connected with the changes of water content which brings about also other type of fluctuations in the environment, such as changes in osmolarity, pH or availability of nutrients. Therefore it is quite probable that some anaerobic genes may be regulated in coordination with some other stress genes. However, so far, little direct evidence has been reported. Gene *ftsH*, which was shown to be essential for both nitrate respiration and fermentation, is also required for *B. subtilis* survival after heat treatment and under high osmolarity, for entry to sporulation and for secretion of exoproteins (Lysenko *et al.* 1997; Nakano *et al.* 1997). Also, anaerobic metabolism and stationary growth share some similarities in regulatory circuits (Ye *et al.* 2000).

3.6 Regulation of anaerobic metabolism

In the complex regulation of anaerobic metabolism of *B. subtilis* genes, *resD*, *resE* and *fnr* play the key roles that constitute a regulatory cascade. Recently, two novel members of this network *arfM* and *YdiH* have also been identified and characterized.

3.6.1 ResD-ResE two-component system

The two-component signal transduction system encoded by genes *resE* and *resD* that consists of the sensor kinase ResE and its cognate response regulator ResD was reported to be essential for anaerobic nitrate respiration of *B. subtilis* (Sun *et al.* 1996b). Although expression of *resDE* is markedly induced during anaerobiosis, this system was proved to play the essential role in global regulation of both aerobic and anaerobic respiration and various other central cellular processes, such as the response to phosphate starvation (Sun *et al.* 1996a; Sun *et al.* 1996b; Nakano *et al.* 2000b).

The kinase ResE, in response to a yet unknown signal, autophosphorylates and subsequently activates the ResD response regulator through transphosphorylation (Zhang and Hulett 2000). ResE functions both as kinase and phosphatase during aerobic growth, but its phosphatase activity, which seems to be regulated by oxygen availability, is reduced under anaerobic conditions (Nakano *et al.* 1999; Nakano and Zhu 2001). This leads to an accumulation of phosphorylated ResD (ResD~P) under oxygen limitation and subsequent induction of ResD-controlled genes (Nakano *et al.* 2000b; Nakano and Zhu 2001).

The sensor kinase ResE is composed of an N-terminal signal input domain and a C-terminal catalytic domain. The N-terminal domain, consisting of two transmembrane subdomains and a large extracytoplasmic loop, is connected to C-terminal kinase domain via PAS subdomain and HAMP linker. The PAS subdomain was shown to be indispensable for signal transduction. The extracytoplasmic subdomain likely serves as a second signal-sensing subdomain which enhances the effect of the PAS subdomain (Baruah *et al.* 2004).

ResD is a monomer in solution, in either phosphorylated or unphosphorylated state (Zhang and Hulett 2000). Asp57 was proved to be the phosphorylated residue of ResD (Geng *et al.* 2004). Activation of ResD seems to be somehow connected with glycolytic pathway as mutation is 3-phosphoglycerate kinase (and subsequent accumulation of glycolytic intermediate, probably 1,3-phosphoglycerate) was able to suppress the absence of ResE kinase in *B. subtilis* (Nakano *et al.* 1999).

Genes that encode this two-component system (*resE* and *resD*) constitute a larger operon, together with upstream essential genes *resABC* encoding products involved in cytochrome c biogenesis (Sun *et al.* 1996b; Le Brun *et al.* 2000; Erlendsson *et al.* 2003). Two promoters controlling the transcription were identified within *resABCDE* operon. Weak intergenic promotor located upstream of *resD* gene drives the transcription of *resDE*. The products of *resDE* genes are then required for transcription of the whole *resABCDE* operon that starts on the stronger promotor located upstream of *resA* gene (Sun *et al.* 1996b).

Besides the induction of its own operon, ResDE system was reported to influence the expression of several other anaerobically induced genes. These are: *nasDEF*, encoding nitrite reductase (Nakano *et al.* 1998a), *fnr*, encoding anaerobic gene regulator (Nakano *et al.* 1996), *hmp*, encoding flavohemoglobin (LaCelle *et al.* 1996), *lctE*, encoding lactate dehydrogenase (Cruz Ramos *et al.* 2000) and *sbo-alb*, encoding cyclic bacteriocin subtilosin and an enzyme required for its production (Zheng *et al.* 1999; Nakano *et al.* 2000a). Also, some genes encoding the components of the respiratory chain are dependent on ResDE, such as *ctaA* and *ctaBCDEF*, encoding genes involved in heme A and cytochrome caa3 oxidase synthesis (Sun *et al.* 1996b; Liu and Taber 1998) and *petCDE* (also named *qcrABC*) encoding menaquinol:cytochrome c oxidoreductase (Sun *et al.* 1996b). These observations have been supported by microarray assay of ResDE mutant (Ye *et al.* 2000).

ResD was shown to bind specifically to the sequences residing upstream of *hmp*, *nasD*, *fnr* and *ctaA* genes. An analysis of ResD-binding regions of these genes revealed a certain sequence similarity; the sequence TTTGTGAAT has been detected, that is suggested to be the ResD-binding motif (Nakano *et al.* 2000b; Zhang and Hulett 2000). *hmp* and *nasD* promoters contain multiple binding sites for ResD, whereas *fnr* has only one. Phosphorylation stimulates binding (probably cooperative) of ResD to the promoter regulatory regions of *hmp* and *nasD* genes, but not to that of *fnr* (Nakano *et al.* 2000b). Five ResD monomers were shown to bind tandemly to upstream regions of *hmp* and *nasD* promoters. Although the phosphorylation has stimulating effect on ResD activity, the unphosphorylated ResD is also able to activate the transcription of ResDE-dependent genes under oxygen limitation, though to a lesser extent (Geng *et al.* 2004). Phosphorylated ResD (ResD~P) was shown not to bind directly to *fnr*. Instead, it interacts with the C-terminal domain of α-subunint of RNA polymerase which facilitates cooperative binding of ResD~P and RNAP and thus icreases *fnr* transcription iniciation (Geng *et al.* 2007). The promoter of *ctaA* gene contains three binding ResD that differs in their discrimination between ResD and ResD~P for binding (Zhang and Hulett 2000).

The anaerobic induction of operon *yclJK*, encoding a two-component system of unknown function (Ye *et al.* 2000) that is strictly dependent on ResDE system (Hartig *et al.* 2004) was also reported. ResD directly interacts with *yclJ* promoter and phosphorylation of ResD by its sensor kinase ResE significantly stimulates this binding (Hartig *et al.* 2004). A comprehensive microarray analysis of uncharacterized two-component systems in *B. subtilis* identified several candidate genes which could be regulated by *yclJK* two-component system (Kobayashi *et al.* 2001) However, a later study by Hartig *et al.* (2004) did not confirm any of them as target genes of *yclJK* in physiological conditions, hence the role of this two-component system in anaerobic regulation remains unsolved.

It needs to be emphasized that the interaction of ResDE system with genes that were reported to be ResDE-dependent is not always direct. In certain cases it happens via other proteins of the anaerobic regulatory pathway (e.g. Fnr) or their chemical products (e.g. nitrite). For full induction of ResDE-controlled genes, oxygen limitation is required but not sufficient – nitrate, nitrite or NO is also needed. Recent studies showed that full induction of ResDE-regulated gene in the presence of nitrate requires NsrR regulator (Nakano *et al.* 2006).

Although the mechanisms by which ResE-ResD system controls its target genes have been elucidated to the great extent, the character of the signal that it perceived by the sensor still remains mostly unknown. One candidate suggested was NADH and its accumulation in the cell (Nakano *et al.* 1999). However, the recent study of Nakano suggests that NO plays an important role in ResDE signal transduction pathway and that ResDE regulon is not only activated by the oxygen limitation but is also stimulated by the presence of NO. Hence, the authors suggested that the signal sensed by the ResE is either NO itself or a signal derived from NO (Nakano 2002). Later, the NO-sensitive transcriptional repressor NsrR (formerly YhdE) was characterized, which negatively regulates ResDE-dependent transcription in the absence of nitrate. NO, generated from nitrite, is a direct effector of NsrK that modulates its activity rather than its amount in the cell (Nakano *et al.* 2006). NsrK is a DNA-binding protein carrying $[4Fe\text{-}4S]^{+}$ cluster. Interaction of NO with this cluster results in formation of dinitrosyl-iron complexes that is likely to result in weakened DNA-binding activity and hence derepression of NsrK-regulated genes (Yukl *et al.* 2008). Indeed, putative NsrR-binding sites were identified in the promotors of genes *hmp* and *resD* (Nakano *et al.* 2006). However, the problem of the generation of NO in *Bacillus subtilis*, which is not a denitrifying bacterium, has not yet been fully understood.

3.6.2 The anaerobic regulator Fnr

As mentioned above, ResDE system is required for anaerobic transcription of another anaerobic regulator gene, *fnr*, and is also essential for nitrate respiration of *B. subtilis* (Cruz Ramos *et al.* 1995; Nakano *et al.* 1996).

Its product is a global redox regulator Fnr, a member of the CAP family of transcriptional regulators possessing characteristic DNA-binding domain that is indispensable for nitrate respiration in *B. subtilis.* Fnr acts as a transcriptional regulator controlling the expression of its target genes in response to anaerobiosis. Its activity is supposed to be modulated by oxygen via mechanism involving iron bound to clustered cystein residues (Cruz Ramos *et al.* 1995; Nakano *et al.* 1996). In *E. coli*, NO reacts with the [4Fe-4S] cluster of Fnr.

B. subtilis fnr is a second gene of bicistronic operon also containing gene *narK*, required for nitrite extrusion (Cruz Ramos *et al.* 1995; Nakano *et al.* 1996). Transcription of *fnr* is strongly activated by oxygen limitation and driven from two promotors; *narK* promotor is Fnr-dependent while the the *fnr* promotor is not. Intergenic, Fnr-specific promotor is activated by ResDE regulatory system. Fnr, once synthesized, enhances its own expression as well as that of *narK* from Fnr-dependent *narK* promotor (Cruz Ramos *et al.* 1995; Nakano *et al.* 1996). However, the transcriptional stimulation of narK by Fnr occurs only when oxygen is limiting (Nakano *et al.* 1996)

Genes *narG*, *narK*, *arfM,* that were found to be dependent on Fnr, have highly conserved potential Fnr-binding site (consensus sequence TGTGA-N_6-TCACA/T) in their promoter regions (Cruz Ramos *et al.* 1995). This potential binding site was also identified in the promoter region of gene *ywcJ*, the product of which is putative second nitrite transporter (Cruz Ramos *et al.* 1995). The same Fnr-like boxes, together with additional potential Fnr-binding site were found in the promoter region of genes *lctEP* and *alsD*

involved in fermentation, which are induced by anaerobiosis, but repressed by the presence of nitrate. However, their participation a direct Fnr-mediated anaerobic induction of *lctEP* and *alsD* has been ruled out (Cruz Ramos *et al.* 2000). The position of these Fnr-like boxes is different from those found in directly Fnr-activated genes (Reents *et al.* 2006) and Fnr seems to influence these genes indirectly, via Fnr-dependent induction of *arfM* (Cruz Ramos *et al.* 2000), see section 3.6.3.

The Fnr regulon has been recently analysed and 37 genes, including 10 operons, were found to be affected by the mutation of *fnr* (Reents *et al.* 2006). Fnr-dependent genes were found to fall into four groups.

Genes of group 1 that are induced anaerobically (under conditions of both fermentative and growth and anaerobic nitrate respiration) by direct binding Fnr to their promotor comprise *narK, fnr*, *narGHJI* and *arfM* (Reents *et al.* 2006).

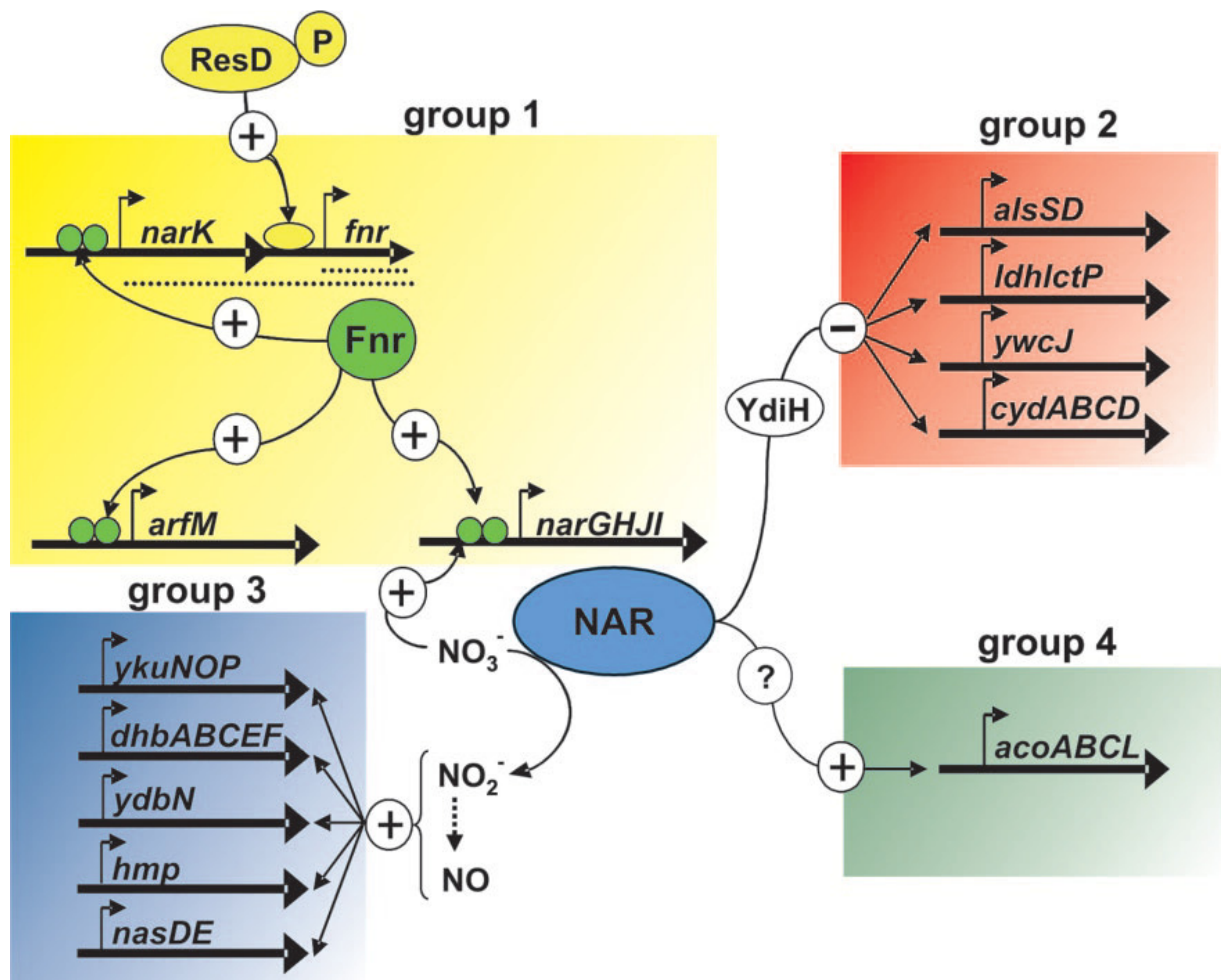


Fig. 11. Regulatory model for Fnr function during the transition to anaerobic growth conditions. See details in the text. Reproduced from reference: (Reents *et al.* 2006).

Group 2 includes anaerobically induced genes *lctEP*, *alsSD*, *cydABCD* (terminal oxidase cytochrome bd) and *ywcJ*, which are repressed in the presence of nitrate in an Fnr-dependent manner. In contrast to the other genes in this group, the *cydABCD* promoter does not contain an obvious Fnr box. The Fnr-dependent repression of this gene group in the conditions of nitrate respiration was shown to be mediated by anaerobic

induction of nitrate reductase and a novel redox-sensing repressor YdiH (Larsson *et al.* 2005; Reents *et al.* 2006).

The expression of genes of group 3, *dhbABCEF* (2,3-dihydroxy benzoate synthesis), *hmp*, *nasDEF* and several genes with products of unknown function, is strongly induced under nitrate-respiring conditions. Promoters of these genes do not contain any Fnr-binding sites (Reents *et al.* 2006) and the expression of most of them is dependent on the presence of nitric oxide (NO) (Nakano 2002). Nitrosylation of Fnr leads to its lower binding to promoter of gene *hmp* and thus to its derepression in the presence of NO (Cruz-Ramos *et al.* 2002).

The last group, group 4, contains only one operon *acoABCL*, involved in acetoin utilization, the anaerobic induction of which is completely dependent of Fnr, but no obvious Fnr-binding site was detected in the corresponding promoter region (Reents *et al.* 2006). Based on these observations, the following regulatory model for Fnr function during transition to anaerobic growth conditions was suggested (Reents *et al.* 2006), see Fig. 11. Fnr directly induces transcription of group 1 genes, including genes encoding nitrate reductase. By regulation of nitrate reductase formation and via YdiH repressor, Fnr mediates the repression of group 2 genes that are mostly involved in fermentation. Nitrite produced by nitrate reductase is converted to NO that serves as a second messenger and stimulates the expression of group 3 genes, including genes of nitrite reductase operon. Group 4 is induced by Fnr also indirectly, via nitrate narGJHI induction and possibly other yet unidentified protein (Reents *et al.* 2006). However, the model proposed by Reents and coworkers does not take into account the role of ArfM in the regulation of several anaerobic genes that was reported by Marino *et al.* (2001) and which is discussed in the next section (3.6.3).

3.6.3 Anaerobic regulatory protein ArfM

ArfM is a recently identified component of anaerobic regulatory cascade (Cruz Ramos *et al.* 2000; Marino *et al.* 2001). This gene, originally named *ywiD*, which is located in the 5'region of *narGHJI* operon, was shown to be induced in the condition of anaerobic respiration (Ye *et al.* 2000) and contains highly conserved potential FNR-binding site (Cruz Ramos *et al.* 2000). This gene was shown to be involved in anaerobic induction of *nasDE*, *hmp*, *lacEP* and *alsSD* genes. *arfM* is expressed exclusively anaerobically in complete dependence on intact *fnr* gene. Presence of nitrite was reported to reduce its expression by 50 % (Marino *et al.* 2001). The sequence TGTGA-N_6-TCACT identified in the promoter region of *arfM* has been verified as a Fnr-binding box indispensable for *arfM* anaerobic induction. Also, *arfM* negatively regulates its own expression (Marino *et al.* 2001). The complex regulatory pathway involved in the induction of gene expression under low oxygen tension was proposed (see Fig. 12).

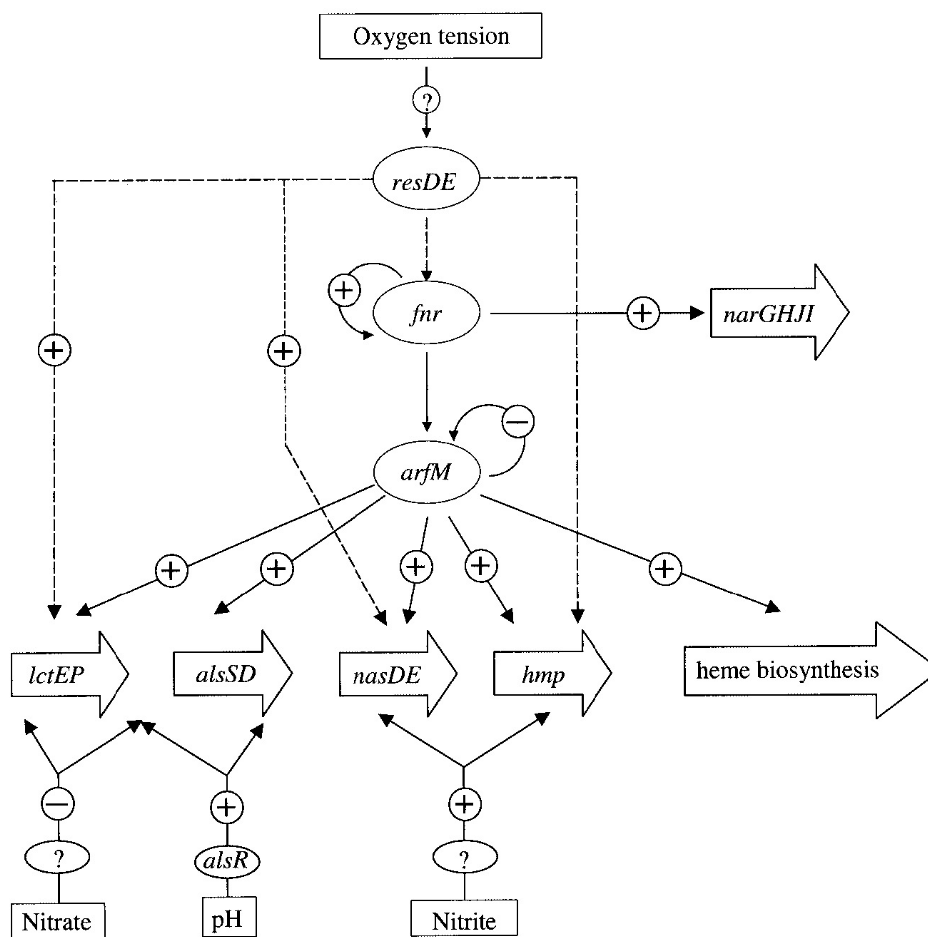


Fig. 12. Regulatory cascade involved in the induction of gene expression under low oxygen tension conditions in *B. subtilis*. Reproduce from reference: (Marino *et al.* 2001).

3.6.4 Redox-sensing repressor YdiH

Bacillus subtilis ydiH gene encodes a protein homologous to redox-sensing repressor Rex that regulates the expression of cydABCD operon of *Streptomyces coelicolor*. The DNA-binding activity of Rex was shown to be controlled in response to change of the cellular NADH/NAD^+ ratio (Brekasis and Paget 2003). Based on analogy with Rex proteins of other Gram-positive bacteria, *B. subtilis* YdiH was suggested to serve also as a redox sensor regulated by the levels of NADH and NAD^+ that acts as a negative regulator of transcription of *cydABCD* operon encoding terminal oxidase cytochrome bd (Schau *et al.* 2004) and also of genes *lctEP*, *alsSD* and *ywcJ* (Larsson *et al.* 2005; Reents *et al.* 2006). YdiH was shown to bind to the promoter region of *cydA* (Schau *et al.* 2004) and *ywcJ* (Reents *et al.* 2006) overlapping the putative non-functional Fnr-binding sites that are present also in promoters of gene *lctE* and a*lsS* (Cruz Ramos *et al.* 2000). YdiH is active as repressor under the conditions that enables reoxidization of NADH to NAD^+ (aerobic respiration or anaerobic respiration of nitrate). When NADH accumulates, the YdiH is inactivated and the genes that it controls are derepressed which leads to the production of cytochrome bd, lactate dehydrogenase, α-acetolactate decarboxylase and synthase and YwcJ (Larsson *et al.* 2005; Reents *et al.* 2006). YdiH then represents the link in coordination of nitrate respiration and fermentation at the transcriptional level.

4 Material and methods

4.1 Experimental microorganisms

4.1.1 Bacterial strains

Bacillus subtilis, a model Gram-positive bacterium, was used for experiments carried out within this work. *B. subtilis* strains employed in the experiments are listed in Table 3. Two new *B. subtilis* strains M19 and FL1 were constructed for the purpose of this study and further experimental work; details of construction are given in sections 4.1.4.1 and 4.1.4.2. *E. coli* strain DH5α *(deoR endA1 gyrA96 hsd R17 (rk⁻, mk⁺) recA1 relA1 supE44 thi-1 (lac ZYA-arg F) U169 φ80lacZ M15 F⁻λ⁻)* from laboratory stock was used as an auxiliary microorganism for multiplication of constructed plasmids.

Table 3. ***Bacillus subtilis*** **strains used in experiments**

strain	relevant characteristic	antibiotic resistance*	source/reference
168	trpC2 (wild type)	-	laboratory stock
M19	168 *amyE*::P*des-lacZ*	Cm^5	constructed in this work
JH642	trpC2 pheA1 (wild type)	-	laboratory stock
AKP3	JH642 *amyE*::P*des-lacZ*	Cm^5	Aguilar *et al.* 2001
AKP4	JH642 *des*::*Km^r^* *amyE*::*Pdes-lacZ*	Cm^5Km^5	Aguilar *et al.* 2001
AKP21	JH642 *desKR*::*Km^r^* *amyE*::*Pdes-lacZ*	Cm^5Km^5	Aguilar *et al.* 2001
CM31	JH642 *desΩ*pAG19(*des-gfp*) *amyE*::P*des-lacZ*	Cm^5 Sp^{50}	C. Mansilla (personal communication)
FL1	JH642 *desKR*::*Km^r^* *amyE*::*Pdes-lacZ*	$Cm^5Km^5Ery^1Lin^{25}$	constructed in this work

* see abbreviation used for antibiotics in Table 5. and Table 6.

4.1.2 Storage of bacterial strains

For long-term storage, overnight liquid culture of the particular strain was mixed 1:1 with 30 % [v/v] glycerol and stored at -70 °C. *B. subtilis* strains that were frequently used for experiments were stored in the form of spores, see section 4.1.3.

4.1.3 Preparing spore preserves of *B. subtilis* strains

1. Sporulation agar was prepared as follows:

- solution A:

NaCl	1 g/L
$MgSO_4 \cdot 7 H_2O$	0.25 g/L
Nutrient agar (Difco)	23 g/L

- solution B:

$FeSO_4 . 7 H_2O$	10^{-3} M
$MnCl_2 . 4 H_2O$	10^{-2} M
$CaCl_2 . 2 H_2O$	1 M

After sterilization and cooling to ca. 50 °C, 1 liter of solution A was mixed with 1 ml of solution B and poured into sterile Petri dishes (Ø 9 cm). Solidified agar plates were left to dry in a sterile box for 4 days.

2. Petri dishes with sporulation agar were inoculated with 100 µl of the overnight culture of the particular *B. subtilis* strain (grown on complex medium) and cultivated 4 days at 40 °C.
3. To each plate, 2 ml of sterile distilled water were added. The bacterial biomass was resuspended and then removed to the sterile centrifugation tube. The biomass was washed three times with distilled water and finally the pellet was resuspended in 15 % [v/v] glycerol, divided in small volumes in microtubes, froze in liquid nitrogen and stored at -20 °C. Each tube was thawed and used only once.
4. Prior to an experiment, the spores were activated by heating at 70 °C for 15 min and then used for inoculation of an overnight culture.

4.1.4 Construction of new *B. subtilis* strains

Two new *B. subtilis* strains were constructed in this study. Details are given below. Plasmids used in construction are listed in Table 4.

Table 4. Plasmids used for construction of *B. subtilis* strains M19 and FL1

plasmid	characteristic	source/reference
pDG1661	plasmid designed to integrate a *lacZ* fusion into *B. subtilis 168* chromosome at the *amyE* locus	Bacillus genetic stock centre
pJB01	plasmid pDG1661 containing 660bp region upstream of translational start of *des* gene	constructed in this work
pAG47	integration vector (*thrC locus*) bearing the 3.2 kbp DNA fragment, containing the *desKR* operon under xylose-inducible promoter	Aguilar *et al.* 2001
pET28b+	auxiliary vector in this work	Novagene
pJB1	3.2 kb fragment from pAG47 containing *PXyl–desKR* cloned into pET28b+	constructed in this work
pJB2	pJB1 with modified *desK* sequence ("flash1" mutation)	constructed in this work
pJB3	pAG47 with modified *desK* sequence ("flash1" mutation)	constructed in this work

4.1.4.1 Construction of *Bacillus subtilis* strain M19

Strain M19 (*Bacillus subtilis* 168 *amyE::Pdes-lacZ*) bearing the fusion of *des* promoter with *lacZ* in *amyE* locus, was constructed according to the protocol of Aguilar *et al.* (1998). Chromosomal DNA of strain 168 (section 4.4.2) was used as a template for PCR reaction using oligonucleotides 'desprom1' (GTTTGGAATTCACCCCTCAAGTGAGTGGAGC) and 'desprom2' (TAGTTAGGATCCTCTCATTGTGTGTCTCGGTT) as primers to amplify the 660 bp region upstream of translational start of *des* gene and introduce the restriction sites (underlined) for EcoRI and BamHI restriction endonucleases (see sequences in section 8). The fragment obtained was digested with EcoRI and BamHI and cloned into integrational vector pDG1661 (Guerout-Fleury *et al.* 1996) purchased from Bacillus Genetic Stock Centre (strain ECE112). The resulting plasmid pJB01 was transformed in *E. coli* DH5α competent cells (section 4.5.1) for amplification, isolated, linearized with BstEII and used for transformation of *B. subtilis* 168 competent cells (section 4.5.3).

B. subtilis transformants were selected for chloramphenicol resistance (5 μg/ml) and the loss of spectinomycin resistance (100 μg/ml). The success of the double-crossover event was also controlled by streaking the obtained transformants on the LB plates containing starch and consequent staining by Lugol's iodine solution. The transformants with integration into *amyE* locus lost the ability to lyse starch and no clearing zones were found around their colonies. Furthermore, the correct sequence of the construct was confirmed by sequencing.

4.1.4.2 Construction of *Bacillus subtilis* strain FL1

Strain FL1 (JH642 *desKR::Km^r amyE::Pdes-lacZ thrC*::[*Pxyl-desK*-desR*]) bears gene *desK* with modified sequence (*desK**) together wild type *desR* under the control of xylose-inducible promoter (*Pxyl*) in *thrC* locus. The DesK sequence modification consisted of the introduction of the Cys-Cys-X-X-Cys-Cys motif into the protein sequence (see section 8) that is required for binding of FLASH fluorescent probe.

For construction of this strain, integration plasmid pAG47 bearing the 3.2 kbp DNA fragment, containing the *desKR* operon under xylose-inducible promoter (Aguilar *et al.* 2001) was modified as follows.

Plasmid pAG47 was digested by BamHI restriction endonuclease and the fragment containing *desKR* under the control of xylose-inducible promoter was cloned via BamHI restriction site into plasmid pET28b+ (Novagen) yielding plasmid pJB1. (The need to use pET-28b(+) (5.4 kbp) as an auxiliary plasmid arose because of the excessive length of the pAG47 (8.5 kbp) and especially because of the lack of information about its whole sequence.) The second longer fragment of the vector pAG47 (pAG47 "scaffold"), that resulted from BamH1 digestion, was stored and used later, see below.

Plasmid pJB1 was used as a template for mutagenic PCR, using oligonucleotides 'Fwd-PstI' (GGCTGCAGGAATTCTTAC) and 'flash-BssSI' (GTTCACGAGCTGCACAGCAGGGATCACAGCATATCAATTTTCTTGCTAAGTC) as primers to amplify a 2091 bp fragment and to introduce the desired mutation into *desK* (in red). The primers contained the restriction sites for PstI and BssSI (underlined) which naturally occurs in the pJB1 sequence. The resulting DNA fragment "desK-FL1" was isolated and

digested with PstI and BssSI. Plasmid pET-28b(+) (containing two BssSI restriction sites) was partially digested by a low concentration of BssSI and the fragment corresponding to the linearized plasmid (8567 bp) was isolated and digested with PstI. The fragment of the length corresponding to 6476 bp was isolated from the agarose gel and ligated with PstI and BssSI digested fragment "desK-FL1". The ligation product was transformed to *E. coli* DH5α competent cells and the transformants were selected for ampicilin (100 μg/ml) resistance. Several plasmids isolated from the amplicilin-resistant transformants were checked by sequencing using oligonucleotide 'desk-rev' (TTCCTTTAAGCACATGCTC) as primer to confirm the occurrence of the desired "flash" mutation in *desK*. The resulting plasmid with the correct sequence was named pJB2.

Plasmid pJB2 was digested by BamH1 endonuclease and the fragment (3147 bp) was ligated again with the pAG47 "scaffold" fragment to yield the plasmid designated pJB3. Plasmid pJB3 was used for transformation of *B. subtilis* AKP21 competent cells. *B. subtilis* transformants were then selected for erythromycin (1 μg/ml) and linkomycin resistance (25 μg/ml).

4.2 Cultivation media

Several different cultivation media were used in this work. Details of their use for particular experiments are given where individual experiment protocols are described.

4.2.1 Media with the same mineral basis and different carbon source

The series of three cultivation media with the identical mineral basis and different carbon source was used for experiments described in section 5.1.

Mineral basis:

NH_4Cl	2 g/l
NaCl	3 g/l
$Na_2HPO_4 . 12H_2O$	6 g/l
KH_2PO_4	3 g/l
$MgSO_4 .7 H_2O$	0.25 gl
D-L glutamic acid	0.25 g/l

Complex medium (CM): mineral basis supplemented with glucose 5 g/l, beef extract (Difco) 1.5 g/l, yeast extract (Oxoid) 1.5 g/l and peptone 5 g/l.

Mineral medium with glucose (MMGlu): mineral basis supplemented with glucose 5 g/l.

Mineral medium with glycerol (MMGlyc): mineral basis supplemented with glycerol 2.5 g/l.

Aminoacids trypthophan (Trp) and phenylalanin (Phe) were added in concentration of 50 mg/l to mineral media (MMGlu and MMGlyc) according to the growth requirements of used strain.

4.2.2 LB medium and LB medium with glucose and nitrate (LBGluNO$_3$)

LB medium (Luria broth) was prepared from powder supplied by Sigma-Aldrich (product number L3522, 22 g/l). For some experiments, it was supplemented with glucose (5 g/l) and KNO_3 (2 g/l). This medium was used in experiments described in section 5.2.

4.2.3 Agar plates

For preparation of agar plates the required liquid medium was solidified by addition of 2.5 % [w/v] of agar powder (Difco). Antibiotics were added as selection markers when necessary after cooling the medium to ca. 45 °C. The medium was poured into sterile Petri dishes. Solidified plates were left to dry in a sterile box.

4.2.4 Antibiotics

Antibiotics were added to cultivation media for selection of *E. coli* or *B. subtilis* with desired genotype or plasmid. See concentrations used in Table 5. and Table 6.

Table 5. Antibiotics used for *B. subtilis* selection

antibiotics	abbrev.	stock solution (mg/ml)	final conc. in medium (µg/ml)
chloramphenicol	Cam^5	50 (in ethanol)	5
kanamycin	Kan^5	50 (in water)	5
spectinomycin	Spc^{50}	50 (in water)	50
erythromycin	Ery^1	10 (in ethanol)	1
lincomycin	Lin^{25}	25 (in water)	25

Table 6. Antibiotics used for *E. coli* selection

antibiotics	abbrev.	stock solution (mg/ml)	final conc. in medium (µg/ml)
kanamycin	Kan^{60}	50 (in water)	60
ampicilin	Amp100	100 (in water)	100

4.2.5 Sterilization

Media, solutions and plastic pipette tips were autoclaved for 20 minutes at 121 °C (101.5 kPa). Glassware was sterilized by dry heat at 160 °C for 3 hours. Temperature sensitive solutions, such as antibiotics stock solutions, were sterilized by filtration.

4.3 Cultivation

4.3.1 Aerobic cultivation

B. subtilis cultures were grown in sterile Erlenmayer flasks filled with cultivation medium (to a maximum of 1/5 of their volume) and covered with aluminum foil cap. Flasks were temperated in a heated/cooled orbital shaker (170 rpm).

4.3.2 Anaerobic cultivation

Anaerobic cultivation was performed in closed screw-cap bottles (250ml) filled to the top with medium. Bottles were temperated in a water bath and the aggregation of bacteria was prevented by mild stirring using magnetic stirrer.

4.3.3 Cold shock

In particular experiments, the cultures were rapidly shifted from the higher temperature to the lower one (exact temperatures are indicated where particular experiments are described). Such shifts were performed in the orbital shaker with temperated water bath (aerobic cultivation) or in an unshaken temperated water bath (anaerobic cultivation). Temperature downshifts were performed in the exponential phase of culture growth at OD_{450} ca. 0.2.

4.3.4 Growth curve and growth rate calculation

The growth of cultures was followed by measuring the optical density (OD_{450}) at 450 nm using spectrophotometer Beckman DU 530 with glass 10 mm cuvette. Distilled water was used for reference and the background signal of colored cultivation medium was always subtracted from the final value. Culture samples with OD_{450} higher than 0.3 were appropriately diluted. Doubling time was calculated from the measured OD_{450} values. Cultures were aseptically sampled at intervals depending on the growth rate, usually at least twice within the doubling time.

4.4 DNA manipulation techniques

4.4.1 Horizontal agarose electrophoresis

Horizontal agarose gel electrophoresis was used for control of DNA samples, separation of different DNA fragments and/or their purification. The concentration of agarose was 0.4 % or 1 % (depending on the size of analyzed DNA). TAE buffer (40 mM Tris-acetate, 1m M EDTA) was used. Ethidium bromide (1 % [w/v] solution) was added directly into the agarose gel in a final concentration of 0.5 μg/ml. Electrophoresis was performed using the MSMINI device (Cleaver), the size of the gels was either 7×7 or 7×10 cm. DNA samples were loaded in Loading Dye Solution (Fermentas) and Gene Ruler™ DNA-Ladder Mix (Fermentas) was always run with samples as a size standard. The voltage of 60V was applied for 1 hour (or longer if necessary). For visualization, the UV-transiluminator was used.

When necessary, the desired DNA fragments separated by electrophoresis were cut from the agarose and isolated gel using a GeneJet™ Gel Extraction Kit (Fermentas).

4.4.2 Isolation of *B. subtilis* chromosomal DNA

The following protocol was used to obtain the *Bacillus subtilis* 168 chromosomal DNA that was used as template in PCR reaction for amplification of *des* promoter fragment.

Solutions:

- TE buffer (10 mM Tris-Cl pH8, 1 mM EDTA pH 8)
- lysis buffer (25 % sacharose w/v, 50 mM Tris-Cl pH 8, 1 mM EDTA pH 8, lysosyme 1 mg/ml)
- proteinase K solution (20 mg/ml dissolved in TE buffer)
- 10 % (w/v) SDS
- phenol
- chloroform
- isoamylalcohol
- ribonuclease A solution (1 mg/ml)
- 10 M LiCl
- 70 % (v/v)ethanol

1. The culture of *B. subtilis* (grown on LB medium) in the late exponential phase of growth was used for DNA isolation. Cells from 40–50 ml of culture were pelleted by centrifugation (10 min, 20000 × g) and washed by 10 ml TE buffer.
2. The pellet was resuspended in 1 ml of lysis buffer, incubated for 30 min at 37 °C and then cooled in an ice bath.
3. 50 μl of proteinase K solution and 200 μl of 10% SDS were added and the content of the tube was gently mixed by slow inversion.

4. The tube was incubated for 20 min in an ice bath and then for 2 hours at 55 °C.
5. 1 volume of phenol/chloroform/isoamylalcohol solution was used for extraction. The phases were separated by centrifugation (ca. 20 min, 20000×g, 4 °C). The upper viscous phase was removed to the clean tube and extracted by 1 volume of chloroform. The phases were separated by centrifugation (ca. 20 min, 20000×g, 4 °C)
6. The upper viscous phase was removed to the clean tube.
7. RNase in final concetration 80 μg/ml was added, then the incubation for 2 hours at 37 °C followed.
8. Chromosomal DNA was precipitated by 2 volumes of isopropanol with 1/10 of 10 M LiCl, washed by 70 % ethanol and dried overnight at laboratory temperature.
9. Finally it was dissolved in TE buffer and stored at -4 °C until use.

4.4.3 Polymerase chain reaction

The polymerase chain reaction (PCR) was used for DNA fragments amplification as detailed below. The reaction was performed in the cycler DOPPIO (VWR).

PCR for *Pdes* fragment amplification (strain M19 construction)

- **reaction** (in 50 μl volume)

  5 μl - PCR buffer 10× (Fermentas)
  5 μl - dNTP mix 2mM each (Fermentas)
  2 μl $MgCl_2$ (25 mM)
  0.3 μl primer I (0.1 mM)
  0.3 μl primer II (0.1 mM)
  300 ng chromosomal DNA template
  0.25 μl Taq polymerase 5 U/μl (Fermentas)
  deionized water up to 50 μl

- **temperature cycle**

initial heating	95°C	4 min	1×
denaturation	95°C	30 s	
annealing	62°C	30 s	30×
elongation	72°C	45 s	
final extension	72°C	7 min	1×

PCR for *desK* fragment amplification (FL1 strain construction)

- **reaction** (in 20 µl volume)

  10 µl – 2x Phusion Master Mix with HF buffer *(Finnzymes)
  1 µl primer I (0.01 mM)
  1 µl primer II (0.01 mM)
  1 ng of plasmid DNA
  deionized water up to 20 µl
  *contains 0.04 U/µl Phusion DNA polymerase, 2x Phusion HF buffer , 400 µM of each dNTP

- **temperature cycle**

initial heating	98°C	30 s	1×
denaturation	98°C	10 s	
annealing	60°C	30 s	30×
elongation	72°C	15 s	
final extension	72°C	10 min	1×

4.4.4 Plasmid DNA miniprep

The following protocol was used for the isolation of recombinant plasmid from transformed *E. coli* cells.

Solutions:

- PEB – plasmid extraction buffer (25 mM Tris, 50 mM glucose, 10 mM Na_2EDTA)
- ALM – alkaline lysis mix (0.2N NaOH, 1 % (w/v) SDS)
- 3 M potassium acetate
- isopropanol
- 70 % and 95 % ethanol (v/v)

1. 5 ml of overnight *E. coli* culture (grown in LB medium) were used. Cells were pelleted by centrifugation (5 min, 20000 × g) and resuspended in 0.2 ml of PEB in a microtube.
2. 0.4 ml of freshly prepared ALM was added, the suspension was gently mixed by inversion and incubated on ice for 10 min.
3. 0.2 ml of 3 M potassium acetate was added and the content of the tube was again mixed by inversion and incubated on ice for 10 min.
4. The precipitate was separated by centrifugation (10 min, 20000×g, 4 °C) and the supernatant was transferred to the clean tube.

5. DNA was precipitated by addition of 0.8 volume of 95 % ethanol (30 min at -20 °C) and pelleted by centrifugation (10 min, 10000×g, 4 °C).
6. Pellet was washed with 70 % ethanol and vacuum dried. Finally, it was dissolved in TE buffer and stored at -20 °C until use.

Alternatively, the commercial product GeneJet™ Plasmid Miniprep Kit (Fermentas) was used for plasmid preparation.

4.4.5 Large scale preparation of plasmid DNA

The following protocol was used for isolation of higher amounts of plasmid pAG19, pET28 and pJB1 from *E. coli* cultures.

Solutions:

- PEB – plasmid extraction buffer (25 mM Tris, 50 mM glucose, 10 mM Na_2EDTA)
- ALM – alkaline lysis mix (0.2 N NaOH, 1% SDS)
- lysosyme solution (25 mg/ml in PEB)
- 3 M potassium acetate
- isopropanol
- 70 % ethanol

1. A fully grown overnight culture of *E. coli* strain (grown on LB medium) bearing the required plasmid was used for isolation. Cells from 500-1000 ml of culture were pelleted by centrifugation (10 min, 20000 × g), washed by distilled water and resuspended in 4 ml of PEB.
2. 1 ml of lysosyme solution was added and the incubation at RT for 10 min followed.
3. 10 ml of freshly prepared ALM was added and the mixture was incubated on ice for 10 min.
4. 7.5 ml of 3 M potassium acetate was added, the content of the tube was gently mixed by inversion and the tube was incubated on ice for 10 min.
5. The precipitate was removed by centrifugation (15 min, 20000 × g, 4 °C). The supernatant was filtered through the syringe membrane (Millipore HAWP 025 00, pores Ø 0.45 μm).
6. DNA was precipitated by 0.6 volume of isopropanol (10 min at RT) and pelleted by centrifugation (10 min, 10000×g, 4 °C). Pellet was washed by 70 % ethanol and vacuum dried. Finally, it was dissolved in TE buffer and stored at -20 °C until use.

4.4.6 Digestion with restriction endonucleases

Restriction endonucleases were purchased from Fermentas. Digestion was usually performed in 20 μl volume. The general composition of the reaction mixture is given below:

- 2 µl of recommended restriction endonuclease buffer 10× (Fermentas)
- restriction endonuclease, according to the recommendation of the supplier (Fermentas)
- DNA (0.1- 4 µg)
- deionized water up to 20 µl

The incubation temperature and time varied for different enzymes; the recommendations of the supplier were followed.

4.4.7 Ligation protocol

Ligation was performed in 20 µl volume:

- 2 µl T4 DNA ligase buffer 10×, containing ATP (Fermentas)
- 1 µl T4 DNA ligase 1U/µl (Fermentas)
- linear vector 20-100 ng
- insert DNA of 1:1 to 5:1 molar ratio over vector

The reaction mixture was incubated for at least 1hour at 22 °C.

4.5 Competent cells and transformation

4.5.1 Preparation of *E.coli DH5α* competent cells

Solutions:

- LB medium
- 0.1 M CaCl
- 30 % (v/v) glycerol

1. 50 ml of LB medium was inoculated by *E. coli DH5α* and aerobically grown overnight (16 hours) at 37 °C.
2. 1 ml of overnight culture was used to inoculate 100 ml of LB in a 250 ml flask the next morning and the culture was grown for 3hrs at 37 °C.
3. The cells were pelleted by centrifugation in pre-cooled tubes (10 min, 2200×g, 4 °C), gently resuspended in 10 ml of cold 0.1M CaCl, incubated on ice for 20 mins and centrifuged again (10 min, 2200×g, 4 °C).
4. The supernatant was discarded and the pellet was gently resuspended in 5 ml of cold 0.1M CaCl.
5. Finally, 5 ml of 30% glycerol was added and cells were aliquoted in microtubes, frozen in liquid nitrogen and stored at -80 °C.

4.5.2 Transformation of *E. coli DH5α* competent cells

100µl of *E. coli* competent cells (section 4.5.1) was added to a microtube with 1–2µl

of plasmid (ca. 100 ng of DNA) or the whole volume of a ligation reaction (section 4.4.7). A negative control tube with no added DNA was also prepared.

Cells were incubated for 30 min on ice, then heat shocked for 2 min at 42 °C and put back on ice. 900 µl of LB medium was added to each tube that was then incubated at 37 °C for 30 min (without shaking). Several 150 µl aliquots of both transformed cells and negative control were spread on LB selective plates and grown overnight at 37 °C.

4.5.3 Preparation of *Bacillus subtilis* competent cells

Solutions:

- 5× concentrated minimal salts (pH 7.0):

$(NH_4)_2SO_4$	10.0 g/l
K_2HPO_4	74.0 g/l
KH_2PO_4	27.0 g/l
sodium citrate	9.5 g/l
$MgSO_4.7H_2O$	1.0 g/l

- 20 % (w/v) glucose
- 2 % (w/v) casaminoacids
- tryptophan (2 mg/ml)

- <u>minimal medium</u> (100 ml): minimal salts 20 ml, glucose 2.5 ml, casaminoacids 1 ml, tryptophan 1 ml
- <u>starvation medium</u> (100 ml) : minimal salts 20 ml, glucose 2.5 ml

1. 10 ml of <u>minimal medium</u> in a 100 ml flask was inoculated with desired *B. subtilis* strain and cultivated for 18 hours at 37 °C.
2. 1.4 ml of this culture was inoculated into 10 ml of fresh pre-warmed <u>minimal medium</u> and cultivated for 3 hours at 37 °C.
3. 11 ml of <u>starvation medium</u> were added and cultivation continued for 2 hours at 37 °C (at maximal aeration). At this moment the cells are fully competent and should be used for transformation.

(For longer storage sterile glycerol to final concentration 10 % (v/v) can be added and cells can be quickly frozen in liquid nitrogen and stored at -70 °C. The loss of competence is usually less than two fold.)

4.5.4 Transformation of *Bacillus subtilis* competent cells

100 µl of *B. subtilis* competent cells (section 4.5.3) was added to a microtube with 1-10 µl of circular plasmid (0.2 – 0.5 µg of DNA) or the whole volume of a ligation reaction (section 4.4.7). A negative control tube with no added DNA was also prepared.

Cells were incubated for 20 min at 37 °C in a water bath. Then 500 µl of LB

medium was added to each tube which was then incubated at 37 °C for 90 min (with shaking). 100 μl aliquots of both transformed cells and negative control were spread on LB selective plates and grown overnight at 37 °C.

4.6 DNA sequencing

DNA Sequencing was performed in the Laboratory of DNA sequencing (Faculty of Science, Charles University) using 3130 Genetic Analyzer (Applied Biosystems).

4.7 Membrane isolation

B. subtilis cytoplasmic membranes were isolated using the enzymatic method (Poole 1993). The orientation of membrane vesicles acquired with this method is right-side-in.

Solutions:

- 50 mM phosphate buffer, pH 8
- 50 mM Tris-Cl buffer, pH 7
- 150 mM K^+- EDTA
- 500 mM $MgSO_4$
- deoxyribonuclease I (DNase) solution (1 mg/ml dissolved in 50 % (w/v) glycerol, 20 mM Tris-HCl, pH 7.5 and 1 mM MgCl2 , stored at -20 °C)
- ribonuclease (RNAase) solution (1 mg/ml dissolved in 10 mM Tris-HCl pH 7.5 and 15 mM NaCl , boiled 15 min at 100 °C, stored at -20 °C
- 100 mM PMSF (phenyl-methyl-sulphonyl-fluoride in isopropanol)
- lysozyme

1. Cells from exponentially growing culture (OD_{450} ca. 0.5) were harvested by filtration (using membrane filters Pragopor 5, pores Ø 0.6 μm) and washed 3 times on the filter with phosphate buffer (temperated to the temperature of cultivation).
2. The biomass was recovered from the filter and resuspended properly in phosphate buffer (using 10 ml of buffer for each 400 ml of original culture).
3. To each 10 ml of suspension 60 μl of PMSF solution, 10 μl of DNase solution, 10 μl of RNase solution and 3 mg of lysosyme were added and mixed well. Then $MgSO_4$ to final concentration 10 mM was added.
4. The suspension was incubated at the same temperature as was the one used for prior cultivation until the protoplasts were formed. The formation of the protoplast was checked under the microscope. The usual time for protoplasts to form was 20-30 min for 37–40 °C and 70-90 min for 20–25 °C incubation temperature.
5. K^+-EDTA was added to final concentration 15 mM and incubated for 1 minute. Then $MgSO_4$ to final concentration 10 mM was added again.
6. Suspension was centrifuged for 1 hour at 25000×g (4 °C), the sediment was finally

resuspended in Tris-Cl buffer, divided in small aliquots in microtubes, frozen in liquid nitrogen and stored at -70 °C.

The Pierce BCA Protein Assay kit (Pierce Biotechnology) was used for protein determination. BSA was used to construct the standard curve.

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.

4.8 Lipid isolation

Solutions:

- 60 mM phosphate buffer pH 7.4
- methanol
- chloroform

1. Cells from 500-1000 ml of exponentially growing culture (OD_{450} ca. 0.5) were harvested by filtration (using membrane filters Pragopor 5, pores Ø 0.6 μm) and washed 3 times on the filter with phosphate buffer (temperated to the temperature of cultivation).
2. The biomass was recovered from the filter and resuspended properly in a small amount of buffer (2 ml) 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 hours.
3. 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-Cl buffer (50 mM, pH 7.0) was added and multilamellar liposomes were formed by vigorous shaking of the tube. Large unilamellar vesicles of a mean size of 400 nm were prepared by repeated extrusion of multilamellar vesicles using the LiposoFast Basic apparatus (Avestin) with a polycarbonate membrane filter of 400 nm pore diameter (Avestin), as previously described (MacDonald *et al.* 1991).

4.9 Fluorescence anisotropy measurement

The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH, Sigma) in acetone was

added at a final concentration of 1 μM to the *Bacillus subtilis* membrane vesicles or to the unilamellar liposomes from *B. subtilis* lipids prepared as described above. The volume of each 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) equipped with DataMax software and polarization accessory, with excitation and emission wavelengths of 360 nm and 430 nm, respectively. The temperature of the sample was checked with an accuracy of 0.1 °C directly in the cuvette.

The background fluorescence of non-labeled samples did not exceed 2 % of the experimental values. The steady-state anisotropy was calculated according the following formulas

$$r_{ss} = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \qquad I_0 = I_{\parallel} + 2I_{\perp}$$

where $I_{\parallel}$ and $I_{\perp}$ represent the intensity of parallel and perpendicular polarized emission respectively, and I_0 the total intensity of fluorescence (Lakowicz 1999).

4.10 Fatty acid analysis

0.3 mg of isolated whole cell lipids was an amount sufficient for identification of the fatty acid (FA) profile of *B. subtilis* under different growth conditions. Fatty acid analysis was performed at the Institute of Soil Biology, Biology Centre of the Academy of Science of Czech Republic in České Budějovice (Czech Republic).

1. The concentrated lipids were subjected to a mild alkaline methanolysis by dissolving them in 1 ml methanol-toluene mixture (1:1 [v/v]) and 1ml of 0.2 M KOH, and heating for 15 min at 37 °C. Then, 2 ml H_2O and 0.3 ml of 1 M acetic acid were added.
2. The resulting fatty acids methyl esters (FAME) were extracted twice with 2 ml of a hexane-chloroform mixture (4:1 [v/v]).
3. The FAs (in the form of FAME) were separated by gas chromatography (Agilent 6850, Agilent Technologies, USA) with a 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).

4.11 Differential scanning calorimetry (DSC)

Experiments that involved DSC measurements were performed during my repeated short term stays (in the years 2004, 2006 and 2007) at the Department of Plant Physiology and Biochemistry, Faculty of Biochemistry, Biophysics and Biotechnology at Jagellonian University in Kraków, Poland.

Solutions:

- buffer (1 mM EDTA, 10 mM HEPES, 50 mM KCl, pH 7)

Dry lipid films prepared as described above (section 4.8) were suspended in a buffer at a concentration of about 1 mg of lipids per ml. Unilamellar liposomes were prepared as described in section 4.8.

DSC experiments were performed using a CSC Model 6100 Nano II Differential Scanning Calorimeter (Calorimetry Sciences Corporation). 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 of 1 °C min^{-1} for both 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 determination 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). 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.

4.12 Colorimetric determination of nitrites

The concentration of nitrites in the culture was determined using a colorimetric assay based on determination of reddish-purple coloration produced by 1-aminonaphthalene with diazotized 4-aminobenzenesulfonic acid as described previously by Rider and Mellon (1946).

Solutions:

- acetone
- 0.6 % [w/v] sulfanilic acid
- 0.6 % [w/v] α-naphthylamine
- 2 M Na-acetate

1. 1-ml samples of analyzed culture were taken, mixed with 3 ml of acetone and diluted with distilled water to 25 ml to prepare a colorimetric sample for assay.
2. 1–3 ml of this sample (V) were mixed with 1 ml of sulfanilic acid and 3 ml of distilled water and incubated for 7 min at room temperature.
3. 1 ml of α-naphthylamine and 1 ml of Na-acetate was added and the solution was diluted with distilled water to 50 ml
4. The absorbance of the resulted solution at 520 nm (A_{520}) was measured.

5. The nitrite concentration c [µmol NO_2^-/ml] in the sample was calculated according the formula:

$$c = \frac{25 \times A_{520}}{V \times 0.7}$$

where A_{520} represents the absorbance at 520 nm and V [ml] the volume of the colorimetric sample. (The calculation is based on the standard curve that was measured with standard solution of KNO_2.)

4.13 β-Galactosidase activity assay

β-Galactosidase activity was analysed according to Miller (1972) with some modifications for Gram-positive *Bacillus* cells.

Solutions:

- Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM ß-mercaptoethanol)
- o-nitrophenyl-ß-D-galactopyranoside (ONPG), 4.5 mg/ml in water
- 10 mg/ml lysosyme
- 10 % [v/v] Triton X
- 1.2 M Na_2CO_3

1. The samples of the culture were taken during growth, the cultivation medium was removed by centrifugation (1 min, 25 000×g) and the samples were frozen in liquid nitrogen and stored at -20 °C until assayed.
2. For the β-galactosidase activity assay cell samples were diluted in Z-buffer and optical density at 525 nm (OD_{525}) was measured.
3. Aliquots (50–250 µl) were then taken, the volume was adjusted to 730 µl with Z-buffer and 10 µl of lysozyme solution was added, samples were thoroughly mixed and incubated at 37 °C for 20 min.
4. Then 10 µl of TritonX solution and 100 µl of ONPG solution were added, samples were again mixed and incubated at 28 °C until yellow color 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.
5. 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:

$$MU = \frac{A_{420}}{OD_{525} \times T \times V_{alq}}$$

where T [min] represents time of the reaction and V_{alq} [ml] the volume of the sample aliquot.

Each cell suspension sample was analyzed in triplicate.

4.14 SDS polyacrylamide gel electrophoresis (SDS PAGE) and Western blotting

SDS PAGE and Western blot techniques were used to detect the presence of Des-GFP protein in aerobic and anaerobic cultures of *B. subtilis* strain CM31.
1 ml-aliquots of culture were harvested, centrifuged, and frozen in liquid nitrogen. The pellets were resuspended in the lysis buffer (Schujman *et al.* 2001), with addition of 160 µl of buffer per OD_{450} unit of the original culture. A 20-µl volume of the cell resuspension was disrupted by incubation with lysozyme (final concentration 500 µg/ml) for 30 min at 37 °C followed by 5 min of boiling in the presence of the loading buffer. The final volume of the sample was 40 µl (20 µl of resuspended cells, 1 µl of lysozyme solution, 19 µl of loading buffer). A 20-µl aliquot from each sample was fractionated by SDS gel electrophoresis in a 12 % acrylamide gel. Proteins were blotted onto a PVDF membrane and detected using anti-GFP monoclonal mouse antibody, secondary goat anti-mouse IgG conjugated to horseradish peroxidase and Pierce® ELC Western Blotting substrate. The blots were exposed to film and developed using an ECL Fome Optimax X-ray Film Processor.

Solutions for sample preparation:

- lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol)
- lysosyme 10 mg/ml in water
- loading buffer (0.125 M Tris, 4 % [w/v] SDS, 20 % [v/v] glycerol, 0.02 % Bromphenol Blue)

Solutions for SDS PAGE :

- 30% acrylamide/bis-acrylamide stock solution (ratio 37:1) (Sigma) (AA)
- stacking gel buffer 0.5 M Tris/HCl, pH 6.8
- separation gel buffer 1.5 M Tris/HCl, pH 8.8
- 10 % sodium dodecyl sulphate (SDS)
- 10 % ammonium persulphate (APS) (Serva)
- N,N,N',N'-tetramethylethylenediamine (TEMED) (Serva)
- SDS PAGE electrode buffer (25 mM Tris-Cl, 200 mM glycine, 0.1 % [w/v] SDS)

The gels (100 × 100 × 0.75 mm) were prepared according to the following protocols:

- <u>4 % stacking gel (5 ml)</u>: 0.65 ml of AA solution, 1.25 ml of stacking gel buffer, 3.05 ml distilled water, 50 µl of SDS, 25 µl of APS and 5 µl of TEMED.

- <u>12% separation gel (10 ml)</u>: 4ml of AA solution, 3.75 ml of separation gel buffer, 3.35 ml distilled water, 100 µl of SDS, 50 µl of APS and 5 µl of TEMED.

Vertical electrophoresis was performed in Omni Page Mini system (Cleaver), filled SDS PAGE with electrode buffer. The initial current of 20 mA was increased to 30 mA after the samples had fully entered the gel. The ECL Plex Fluorescent Rainbow markers (Amersham) were used as molecular weight standards.

Solutions and chemicals for Western blotting and detection:

- polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore)
- Gel-blotting paper GB005 (Whatman)
- Anode buffer I (0.3 M Tris, 10 % [v/v] methanol, pH 10.4)
- Western-blot Anode buffer II (25 mM Tris, 10 % [v/v] methanol, pH 10.4)
- Western-blot Cathode buffer (25 mM Tris, 40 mM glycin, 10 % [v/v] methanol, pH 9.4)
- PBS (4.3 mM Na_2HPO_4, 0.14 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl)
- blocking buffer (PBS with 0.05 % Tween20 and 1 % bovine serum albumin)
- primary antibody: mouse anti-GFP IgG (Roche)
- secondary antibody: goat anti-mouse IgG, peroxidase conjugated (Thermo Scientific)
- luminol-based chemiluminiscent substrate for peroxidase (Pierce® ECL Western Blotting substrate)

Semi-dry system transfer was used for blotting and performed according to the protocol described in the user guide for Immobilon-P membrane (Millipore) supplied by the manufacturer (also available at http://www.millipore.com/userguides/tech1/pr02531). A current of 1.2 mA per 1 sq. cm of the membrane was applied for 1 hour. The membrane was then blocked by incubation for 1 hour in the blocking buffer, incubated for 1 hour in PBS with primary antibody (0.4 μg/ml) and washed twice with PBS (10 min). Then, it was incubated for 1 hour in PBS with secondary antibody (0.1 μg/ml) and again washed twice with PBS (10 min). For detection the Pierce® ECL Western Blotting substrate was used. The detection was performed according to the protocol supplied by the manufacturer (also available at http://www.piercenet.com/Objects/View.cfm?type=File&ID=9F4AFB94-DC07-4528-8BFB-C380D872A680).

5 Results and discussion

5.1 *Bacillus subtilis* membrane cold adaptation under different nutrition conditions

This part of my work aimed to investigate the cold adaptation of *Bacillus subtilis* 168 in different nutrient conditions. I compared the adaptation of the membrane in *B. subtilis* grown in three different cultivation media at 40 °C, 20 °C and after the cold shock from 40 to 20 °C. To characterize the chemical and physical changes in the membranes I employed fatty acid analysis, fluorescence anisotropy measurement, differential scanning calorimetry and reporter gene induction. The data described and discussed in section 5.1 were published in the journal Biochimica Biophysica Acta - Biomembranes (Beranova *et al.* 2008); see the fulltext in Appendix (page 108).

5.1.1 Growth of *Bacillus subtilis* strain 168 in different cultivation media

The adaptation to the optimal (40 °C) and suboptimal (20 °C) cultivation temperature (T_c) of *Bacillus subtilis* strain 168 was studied in three different cultivation media containing the same mineral basis and differing in the carbon source: complex medium with glucose (CM) and mineral media with either glucose (MMGlu) or glycerol (MMGlyc), see section 4.2.1. The optical density was measured at 450 nm (OD_{450}). After

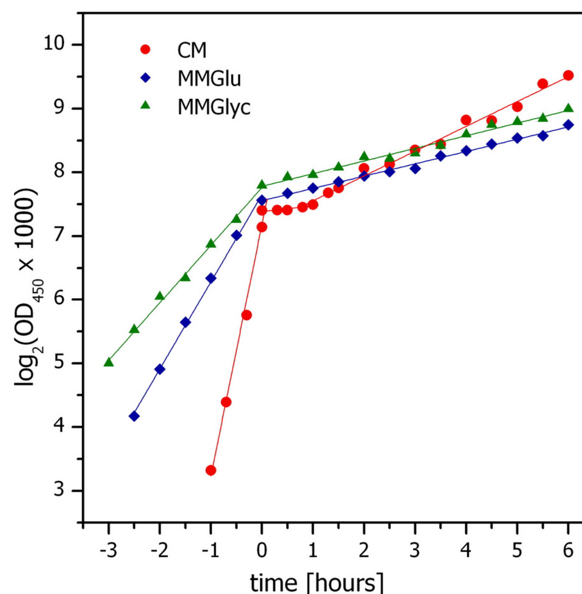


Fig. 13. Growth of *B. subtilis* 168 in different cultivation media at 40 °C and after the cold shock from 40 to 20 °C. *B. subtilis* cultures were grown in complex medium with glucose (CM), mineral medium with glucose (MMGlu) and mineral medium with glycerol (MMGlyc). The time of transfer is identified 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.

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 the rapid transfer from 40 to 20 °C. The growth curves were measured for each medium and generation times were calculated. At T_c 40 °C, the generation times of *Bacillus subtilis* growing in CM, MMGlu and MMGlyc medium were 15, 44 and 66 min, respectively. At T_c 20 °C, generation times increased to 186, 311 and 312 min in CM, MMGlu and MMGlyc, respectively.

After the rapid temperature downshift from 40 °C to 20 °C, two types of growth behavior were observed, see Fig. 13. Cells grown in complex medium (CM) ceased to grow after the temperature shift. After 60 minutes their growth was restored with the generation time corresponding to their steady-state exponential growth at the temperature of 20 °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 20 °C.

The growth lag following the temperature downshift in complex medium was earlier shown to be caused by membrane rigidization, as benzylalcohol, which is the membrane fluidizing agent, was able to shorten it substantially (Konopasek *et al.* 2000). The fact that this growth pause was not observed in either of the mineral media tested, suggested that the nutrient conditions substantially influence the cold adaptation of *B. subtilis* cells. Mineral media seem to provide the cells with certain features that make them better prepared for the negative effects of temperature decrease. Therefore, in the experiments described further, I investigated the membranes of these cells in more detail.

5.1.2 Fatty acid composition of membrane lipids

The changes in the chemical composition of the bacterial membrane underlie the adaptation of its properties to different environmental conditions. In my experiments, I focused on the composition of the fatty-acyl chains in membrane lipids that are known to have the most prominent effect on the membrane fluidity.

The lipids isolated from cultures of *B. subtilis* (strain 168) grown in CM, MMGlu and MMGlyc media at both cultivation temperatures, T_c 40 °C and T_c 20 °C, were subjected to comparative fatty acid (FA) analysis using gas chromatography. The results are summarized in Fig. 14.

The profiles were compared in attempt to a) reveal the influence of the carbon source on the membrane composition at the optimal and suboptimal temperature and b) define the character of cold adaptation with respect to cultivation medium. (The cultivation of cells in CM medium at 40 °C represented the standard optimal growth situation, with which the others were compared.) In the complex medium CM, at both T_c 40 °C and T_c 20 °C, the membrane lipids contained mostly branched fatty acids that contribute to more than 80 % of the total. The FA profiles are dominated by i-15:0, a-15:0, i-17:0 and a-17:0 FAs. The main difference between CM lipids from T_c 40 °C and T_c 20 °C lies in the increased levels of anteiso-branched fatty acids at lower cultivation temperature that was accompanied by concomitant decrease of iso-branched and straight-chain FA species. This trend correlates with the data published earlier by other authors (Svobodova *et al.* 1988; Suutari and Laakso 1992).

The cells grown at Tc 40 °C in MMGlu medium showed an increased level of i-16:0 and a-17:0 and decreased level of the straight-chain n-16:0 FAs (when compared with CM at 40 °C) that could be interpreted as the membrane fluidization of these cells.

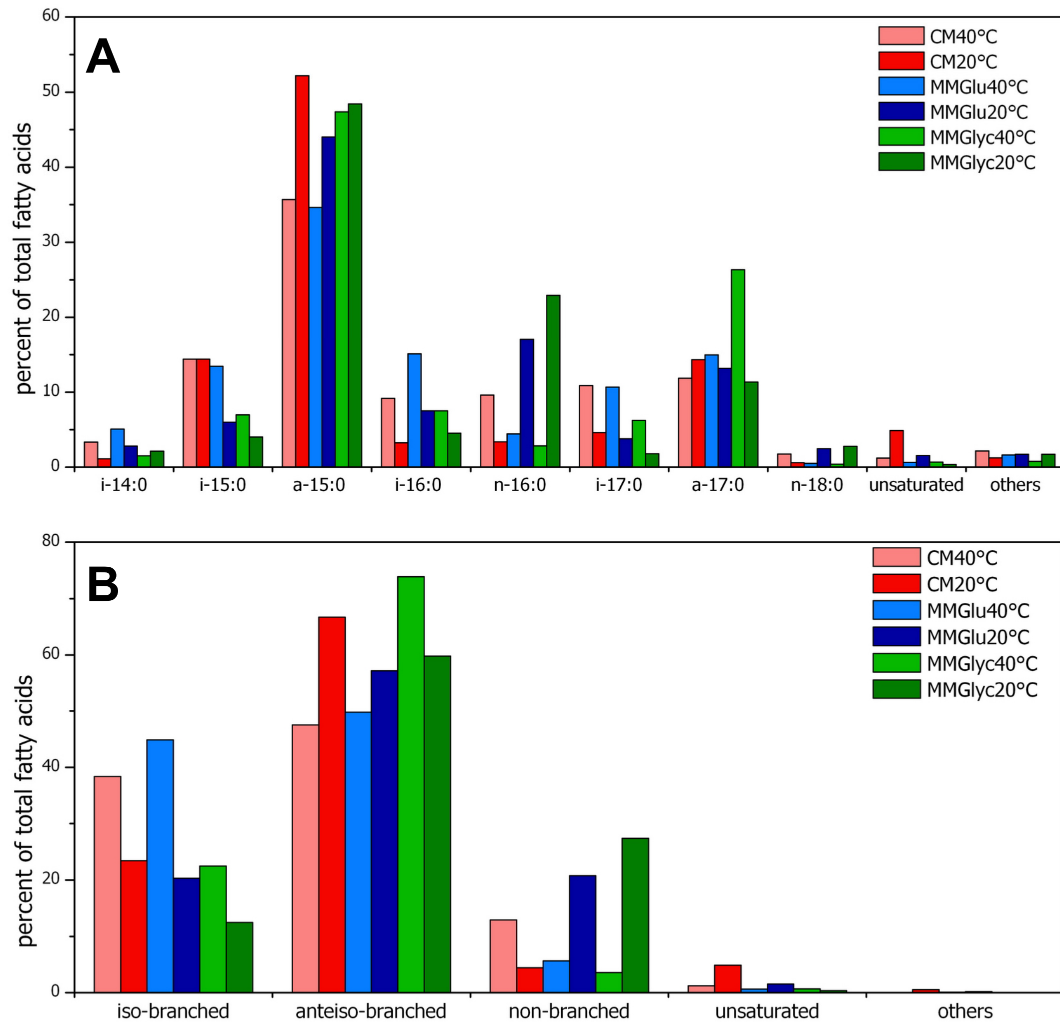


Fig. 14. The effect of growth conditions on the composition of *B. subtilis* membrane fatty acids. *B. subtilis* 168 cells were grown in three different media at a constant temperature of 40 °C or 20 °C and harvested in the mid-log phase (at OD_{450} of 0.5–0.6) for lipid isolation and fatty acid analysis. From a complete fatty acid profile recorded for each FA sample the proportions of the most prominent species (**A**) and main structural fatty acid types (**B**) are presented. The data shown are the averages of at least two independent experiments (cultivation, membrane isolation, and fatty acid analysis). **Abbreviations**: 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. The prefixes i-, a-, n- denotes the respective branching pattern for iso-, anteiso- and non-branched fatty acids, respectively.

The analysis of lipids of the cells grown in MMGlyc at Tc 40 °C revealed a quite unusual fatty acid profile, one that displayed obvious differences in levels of almost all major FA types (in comparison with that of CM cells from Tc 40 °C). The characteristic

and striking feature of MMGlyc (T_c 40 °C) membranes was the very high level of low-melting a-15:0 and a-17:0 fatty acids, 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 (Svobodova *et al.* 1988; Suutari and Laakso 1992) and not for those cultivated near their growth temperature optimum as in this case. My results therefore indicate that even at the optimal temperature the choice of cultivation medium has apparently substantial influence on membrane chemical (and also biophysical, see further) characteristics.

The extent of cold adaptation in different nutrient conditions can be estimated by comparing the fatty acid profiles recorded for cells cultivated in the particular medium at T_c 20 °C and T_c 40 °C. As written above, in CM medium low temperature induced the incorporation of low-melting species of FAs in the membrane lipids. The level of anteiso-branched FA increased by more than 15 % at T_c 20 °C and also a higher amount of unsaturated FAs (that was very low at T_c 40 °C) was detected.

In MMGlu medium, the levels of particular FA species were different from that of CM, however the pattern of changes brought about by the low temperature was rather similar. Cells grown in MMGlu at 20 °C displayed a marked decrease of all iso-branched species (i-15:0, i-16:0 and i-17:0) and an increase of a-15:0 FA. The level of a-17:0 remained almost unchanged. However, I found a remarkable increase of straight-chain FA types (both n-16:0 and n-18:0), which was not found in CM. Compared to that, the respective adaptation of MMGlyc lipids was very different. Low temperature induced the decrease in levels of both branched FA groups and synchronous increase of straight-chain FA types. While the level of a-15:0 remain almost unchanged, the proportion of a-17:0 drastically decreased at T_c 20 °C. The amount of straight-chain n-16:0 increased by more than 20 % at the lower cultivation temperature.

As seen from results presented in this section, nutrient conditions were obviously shown to essentially influence the membrane chemical composition of *B. subtilis*. Nevertheless, the basic question arose of how these chemical changes are reflected on biophysical level. Therefore, I employed the DPH fluorescence anisotropy measurement and differential scanning calorimetry technique to investigate this aspect of membrane adaptation.

5.1.3 Membrane physical properties measured by DPH fluorescence anisotropy

The measurement of steady state fluorescence anisotropy (r_{ss}) of a fluorescent probe, which falls within the methods of fluorescence spectroscopy, is often used for studies of biological membranes (Lentz 1989). For this work, I used the membrane probe

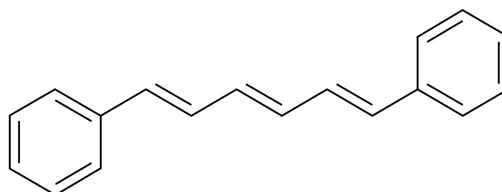


Fig. 15. The structure the fluorescent probe 1,6-diphenyl –1,3,5-hexatriene (DPH)

1,6-diphenyl-1,3,5-hexatriene (DPH). Due to its structure (Fig. 15), DPH molecules intercale into the membrane bilayer along the fatty-acyl chains of the lipids, adopting the position perpendicular to the membrane plane. The excitation and emission maximum wavelengths of DPH are 350 and 428 nm, respectively. The rotation of the probe within the lifetime of the excited state is influenced by the physical properties of the membrane and is reflected in the value of DPH fluorescence anisotropy. Therefore, this method allows us to estimate the physical impact of chemical changes in membranes, as this parameter reflects roughly the membrane fluidity. If the membrane is rigid, the r_{ss} values are higher than those obtained for a more fluid membrane. The general concept of membrane adaptation to cold presumes that in lower temperatures the chemical composition is adjusted to increase the membrane fluidity. Measurements on isolated lipids reflect the properties that result solely from lipid composition, while measurement on isolated membranes encompasses also the influence of membrane proteins that generally have rigidifying effect.

Measurements were performed with DPH-labeled lipid and cytoplasmic membrane vesicles which were derived from *B. subtilis* cells cultivated at 40 °C and 20 °C in three media tested (CM, MMGlu, MMGlyc). Fig. 16 shows the r_{ss} DPH values that were measured over the temperature interval from 10 °C to 45 °C.

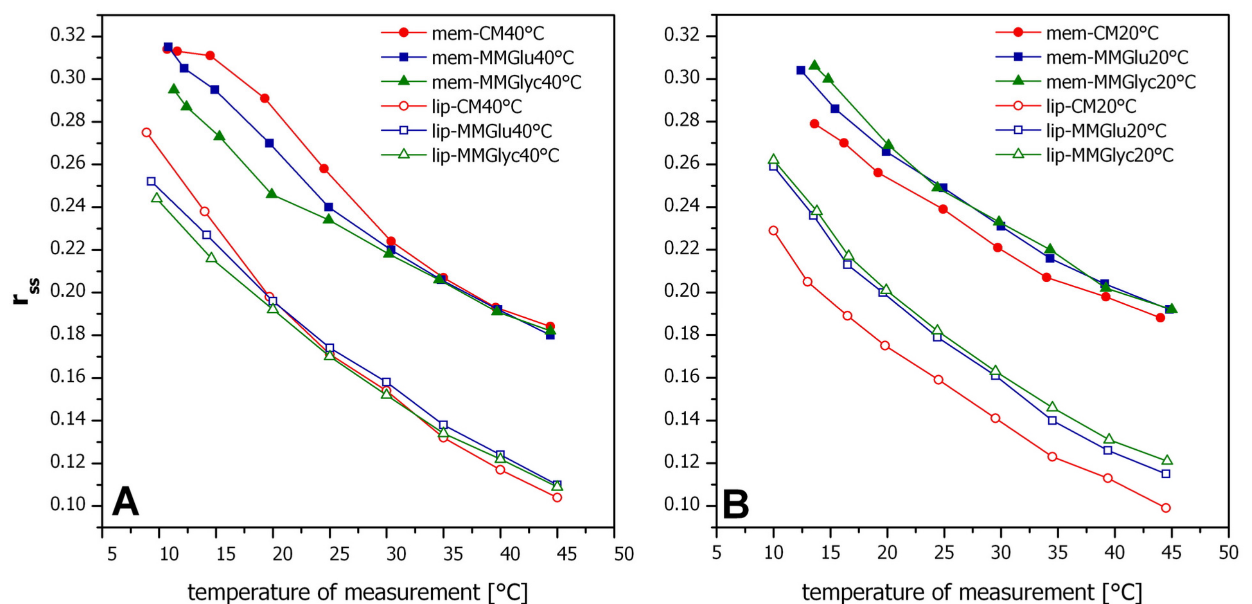


Fig. 16. Temperature dependence of DPH fluorescence anisotropy in cytoplasmic membranes and membrane lipids isolated from *B. subtilis* cultivated in different media at 40 °C (A) and 20 °C (B). Cytoplasmic membranes or membrane lipids were isolated from *B. subtilis* strain 168 cells grown in different media at a constant temperature of 40 °C or 20 °C and labeled with DPH. 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. **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.

The extent of membrane adaptation can be assessed when the r_{ss} values are compared, that were obtained for the temperature of measurement (T_m) equal to temperature of cell cultivation (T_c). For T_m=T_c= 40 °C the same r_{ss} values were found for all three types of membranes tested (Fig. 16A). This indicates that *B. subtilis* 168 grown at 40 °C maintained the same membrane fluidity independent of the growth rate, carbon source or the presence of branched fatty acid precursors. With lipid samples the DPH anisotropy measurements provided very similar results (Fig. 16A). However, at lower measurement temperatures (T_m 10–25 °C) diverging r_{ss} values were obtained, showing the highest, medium and lowest r_{ss} values for CM, MMGlu and MMGly cytoplasmic membranes, respectively (Fig. 16A). Hence the membranes of cells grown on CM were the most rigid ones in this temperature interval. Measurements with lipid vesicles at lower temperatures showed qualitatively similar but lesser r_{ss} differences between lipid samples. This correlates very well with the observation that the lag phase occurred after the temperature downshift to 20 °C occurred only in complex medium (Fig. 13)

For T_c 20 °C, a different data trend was found. The r_{ss} values for membrane samples derived from CM were lower than those of MMGlu or MMGlyc ones, at any temperature of measurement (Fig. 16B). These differences were even more prominent when r_{ss} was measured in DPH-labeled lipid vesicles. This observation implies that at T_c 20 °C, the cells grown in CM medium maintained the most fluid membranes. The differences between the two mineral media tested were not substantial, showing slightly higher values (less fluid membranes) for MMGlyc membranes and at the lower measurement temperatures (Fig. 16B).

The technique of fluorescent anisotropy measurement provides valuable data about membrane physical features, however as an indirect method it has its limitation and the interpretation of results can not always be straightforward (Konopasek *et al.* 2000). In further experiments, I investigated the transition temperatures of membrane lipids by direct method of differential scanning calorimetry.

5.1.4 Transition temperature of membrane lipids measured by differential scanning calorimetry

Differential scanning calorimetry (DSC) is thermodynamic technique that allows us to study the thermotropic phase behaviors of lipids in model or biological membranes, without the introduction of exogenous probe molecules. The principle of DSC is based on simultaneous controlled heating of a studied sample and a reference sample (usually the corresponding solvent) so that the temperature difference between them is zero. If the sample undergoes a thermally-induced event, the detector senses the temperature difference between the sample and reference cell and supplies more or less heat to the sample cell to hold its temperature equal to that of reference. The recorded parameter in DSC measurement is thus the excess specific of differential heat as a function of temperature (McElhaney 1986). Primarily, DSC is used to study the thermally-induced transition of lipid bilayers between ordered crystalline-like state and disordered, fluid-like state. For pure lipids this transition is sharp and symmetrical, whereas for biological

membranes, which are typically of heterogeneous composition, phase transition is rather broad and often asymmetric. It is generally acknowledged that the adaptive changes in membrane compositions are aimed to maintain the temperature of lipid transition (T_t) below the temperature of environment (cultivation). While the DSC technique seems to be an ideal instrument for T_t determination it was employed exactly for this purpose.

DSC measurements were performed on membrane lipid vesicles. Transition temperature for each sample was estimated from a respective thermogram (shown in Fig. 17) as the temperature corresponding to the maximal height of the transition peak. The samples were measured along the temperature interval from 2–55 °C (only relevant data are shown in Fig. 17). Because the calorimeter used is not able to operate below the freezing temperature of the sample, it was not often possible to record the whole width of the transition peak.

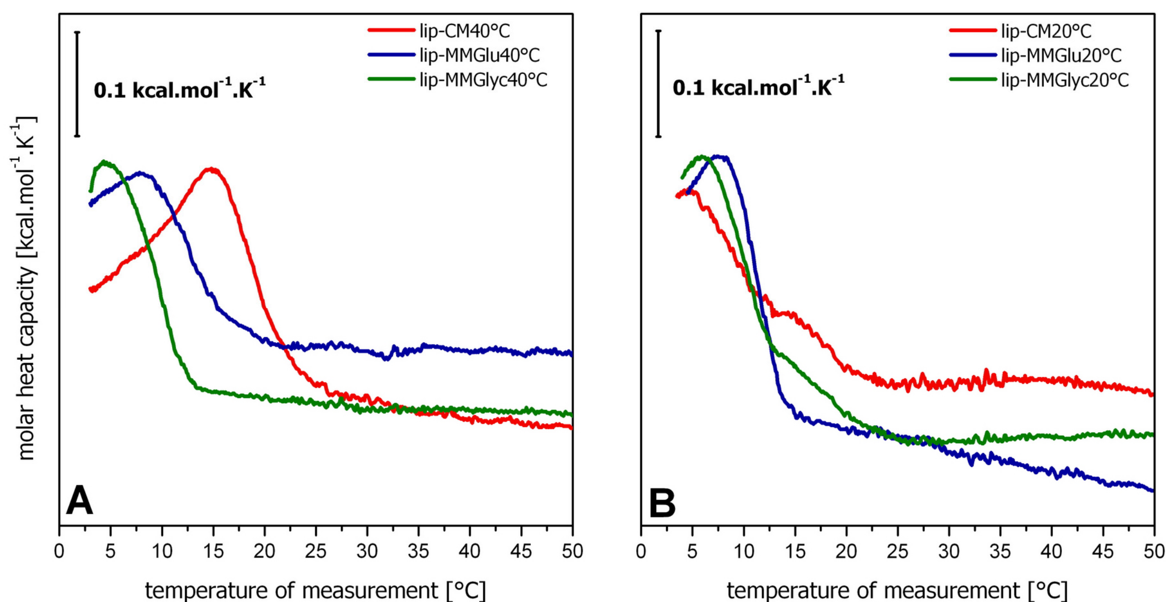


Fig. 17. DSC thermograms of membrane lipids isolated from *B. subtilis 168* cells cultivated in different media at 40 °C (A) and 20 °C (B). Shown here are the representative heating curves obtained for unilamellar liposomes from bacterial lipids. Scans were collected at a heating rate of 1 °C min^{-1} from 2 to 70 °C; only relevant parts of the curves from 4 to 50 °C are shown. Calculated phase transition temperatures T_t 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.

For lipid samples isolated from cells cultivated at 40 °C in CM, MMGlu and MMGly medium, the T_t were located at 14.4, 7.6 and 4.5 °C, respectively (Fig. 17A, Table 7). The T_t values obtained for the lipids isolated from all three cultures grown at 20 °C were lower. For CM, MMGlu and MMGlyc medium they were found at 3.9, 6.6. and 5.2 °C, respectively (Fig. 17B, Table 7).

All of these results correlate with the data obtained from fluorescent anisotropy measurement. This indicates that in T_c 40 °C the membranes of cells grown in mineral

media possessed more fluid membranes than those of complex medium, which favored them after the temperature downshift (no lag phase occurred). During long-term cultivation at T_c 20 °C the situation was just the opposite, with CM cells showing the most fluid membranes.

Table 7 Transitions temperatures of lipids of *B. subtilis* cultivated on different media

cultivation medium	T_c 40 °C	T_c20 °C	difference
CM	14.4 °C	3.9 °C	10.5 °C
MMGlu	7.6 °C	6.6 °C	1.0 °C
MMGlyc	4.5 °C	5.2 °C	-0.7 °C

T_c – cultivation temperature

As evident, for all lipid samples their respective T_t values were located well below the temperature of cultivation. The difference of T_t values acquired for the compared cultivation temperatures (Table 7) can also serve as a good measure of the extent of adaptation. Based on these results, it can be concluded that the most profound adaptation was found in CM medium where T_t of membrane lipids decreased by 10.5 °C at T_c 20 °C (compared to T_c 40 °C). For MMGlu the difference was just small, about 1 °C and for MMGlyc even the increase by 0.7 °C was detected.

5.1.5 Construction of *Bacillus subtilis* strain M19

In *Bacillus subtilis*, the induction of synthesis of fatty acid desaturase (Des) was shown to be an important short-term adaptation mechanism for membrane fluidization after a cold shock (Klein *et al.* 1999; Aguilar *et al.* 2001). Therefore, I wanted to attest whether the different nutrient conditions influenced the induction of its coding gene (*des*). As all the previous experiments in our laboratory were carried out on the *Bacillus subtilis* strain 168 *(trpC2 sfp0)*, there was a need to have a strain that would allow us to monitor the *des* promoter activity under different cultivation conditions on the background of strain 168. For this reason I constructed the strain named M19 bearing the fusion of the promoterless reporter gene *lacZ* to the *des* promoter (*Pdes*) in non-essential *amyE* locus (Fig. 18). In this strain, the activity of *Pdes* leads to synthesis of β-galactosidase which can be assayed colorimetrically. See details of the strain construction in section 4.1.4.1.

This strain was used in experiments testing the activity of the *des* promoter after the cold shock (see section 5.1.6).

Fig. 18 The schematic sequence of the *B. subtilis* strain M19 bearing modified *amyE* locus. Legend: amyE´, ´amyE – 5' and 3' portion of gene encoding α-amylase, respectively; *Pdes* – *des* promoter; *spoVG-lacZ* – promoterless gene for β-galactosidase; *cat* – gene encoding chloramphenicol acetylase

5.1.6 The induction of desaturase after the cold shock

To determine whether the induction of fatty acid desaturase after a cold shock was dependent on the cultivation medium, *B. subtilis* strain M19 cells were cultivated at 40 °C in CM, MMGlu or MMGlyc medium, until the culture reached OD_{450} of 0.2, then transferred to 20 °C and their β-galactosidase activity was assayed for 6 hours after the 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. 19). These results indicate that the membrane FA composition of cells growing in mineral media that resulted in higher membrane fluidity after the cold shock did not lower or prevent the maximum fatty acid desaturase induction.

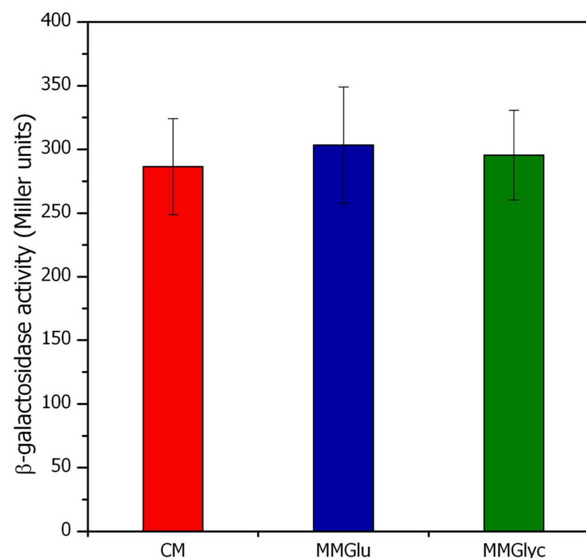


Fig. 19 Effect of the growth conditions on *Pdes-lacZ* expression after the cold shock. Fatty acid desaturase induction after the cold shock in different cultivation media was expressed in terms of β-galactosidase activity in *B. subtilis* M19 strain. Cells were cultivated at 40 °C either in complex 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 the cold shock) as described in Materials and methods. The results shown are means of three independent experiments with standard error bars.

5.1.7 Discussion I

The objective of the experiments described above was to illuminate the effect of medium composition on cytoplasmic membrane characteristics and the extent of their changes induced during adaptation to an optimal and suboptimal temperature in *B. subtilis*. The membrane composition and, hence, its physical properties are surely substantially influenced by the spectrum and abundance of various chemical compounds that are available to growing cells. It is generally recognized that bacterial cells adjust their membrane fluidity to retain it within a certain range that supports growth at a given temperature (McElhaney 1984). However, the suitable physical state can result from many different patterns of chemical compositions; some of those could possibly provide further advantage in the adaptation of the cell to cold or other environmental stresses. In my experiments, I tested 3 cultivation media for *B. subtilis* growth that are supposed to induce 3 different types of catabolic metabolism and different growth rates; rich complex medium with glucose, minimal cultivation medium with effective carbon and energy source, glucose and poor minimal cultivation medium with glycerol.

Earlier, DPH fluorescence polarization studies were performed on Gram-negative *E. coli* cultivated at 37 °C in various growth media which provided a wide range of distinct growth rates and the authors observed crucial differences in membrane fluidity between tested *E. coli* cultures. The cells grown in rich media, i.e. those with the shortest doubling times, exhibited the most fluid membranes with the lowest order parameter (Zaritsky *et al.* 1985; Parola *et al.* 1990). Inspired by these results, in my work I tested whether this strategy is also employed by *Bacillus subtilis,* which – in contrast to intestinal commensal *E. coli* – is more likely to encounter the temperature fluctuations in its natural soil habitat.

The growth of *B. subtilis* 168 in complex medium at 40 °C resulted in fatty acid profile (shown in Fig. 14) that is in accord with the results published earlier for *Bacillus subtilis* (Svobodova *et al.* 1988; Konopasek *et al.* 2000). The transition temperature T_t of membrane lipids of these cells was found to be 14.8 °C, i.e., more than 25 °C below the cultivation temperature which is in agreement with the generally accepted concept of membrane adaptation to low temperatures (McElhaney 1984).

However, when cells were grown in mineral media, their membrane FA composition and related membrane biophysical features differed substantially, which indicates the relevance of medium composition in membrane adaptation. In glycerol medium at T_c 40 °C, the proportion of anteiso-branched fatty acids was unusually high and probably reached the possible limit (nearly 75 % of total, see Fig. 14A). Such a high level was reported for *Bacillus subtilis* cultivated in a complex medium at 15 °C (Suutari and Laakso 1992). Indeed, this unusual FA profile provided the lipids of MMGlyc cells with the very low transition temperature of 4.3 °C. This was a value of more than 10 °C lower than T_t of cells grown on CM at 40 °C (T_t 14.8) and almost identical to the value obtained for cold adapted CM cells (T_t 4.6 °C). In mineral medium with glucose, MMGlu, the cells also produced a higher proportion of branched fatty acids at T_c 40 °C (in comparison with complex medium, CM), but the difference was not as prominent as for

MMGlyc (Fig. 14A). Nevertheless, even these amounts of low-melting FA types were enough to lower T_t value of MMGlu lipids for 7 degrees (compared to CM cells) to 7.8 °C. These results demonstrate the important influence of cultivation medium composition on membrane chemical and physical features during the growth of *B. subtilis* at a constant optimal temperature.

Klein and coworkers observed that the presence of isoleucine, which serves as a precursor for anteiso-branched FA synthesis, is essential for *B. subtilis* strain JH642 membrane adaptation after the cold shock in mineral medium with glucose (Klein *et al.* 1999). However, the authors found substantial differences in the requirements for isoleucine when they compared their *B. subtilis* strain JH642 with the strain 168 (sometimes also referred as Marburg strain) that was used in my work. Strain JH642 (*pheA1 trpC2 sfp0*) exhibited strong isoleucine dependence while strain 168 (*trpC2 sfp0*) not. They supposed that strain 168 possesses higher biosynthetic capacity for threonine (from which isoleucine is derived in biosynthetic pathway) than JH642. My present results strongly support this hypothesis, as *Bacillus subtilis* 168 cells grown at T_c 40 °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. In fact, the levels of anteiso-branched fatty acids were even higher in mineral media used than in the complex medium.

Unfortunately, the information available about the mechanisms that regulate the FA synthesis in *B. subtilis* in dependence on carbon source is scarce. Therefore, one can only speculate at the moment as to why *B. subtilis* cells grown at T_c 40 °C preferred to synthesize the highest proportion of anteiso-branched FA in nutrient-poor MMGlyc medium, where the least favorable conditions for the branched anteiso-FA synthesis can be intuitively expected. The increased proportion of anteiso-branched FAs should arise either from the mechanism of the primer selection by the FabH1 and FabH2 iso-enzymes, the condensing enzymes involved in the first step of fatty acid elongation, or from the levels of the primer precursors. 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 (Choi *et al.* 2000), however, no direct evidence has been given so far. Nevertheless, it cannot be a temperature-dependent mechanism that is responsible for membrane fluidization in the case of cultivation in mineral media at the optimal growth temperature. Thus, it is most probably the increased level of the precursors for anteiso-branched fatty acids that leads to the high level of a-15:0 and a-17:0 FA observed in the membranes of cells grown in glycerol medium at T_c 40 °C. However, further experiments will be needed to test the hypothesis that the growth near the temperature optimum in minimal medium with glycerol (or glucose) enhances threonine and/or isoleucine synthesis in *Bacillus subtilis* strain 168.

Having found that at the optimal temperature the choice of cultivation medium has apparently substantial influence on membrane chemical and biophysical characteristics, I studied further how the growth in different media affected the adaptation for the low cultivation temperature.

The data published earlier regarding low-temperature induced changes in fatty acid compositions of *B. subtilis* membrane are in general agreement with my results. In

low temperatures, this bacterium tends to increase the contents of low-melting branched fatty acid species in its membrane lipids, namely those of anteiso-branched group (Svobodova *et al.* 1988). Also, Suutari and Laakso demonstrated that the proportion of anteiso-branched fatty acids increased almost proportionally with decreasing cultivation temperature in complex medium (Suutari and Laakso 1992). In my work, with cells grown in complex medium, I obtained the correlating results indicating the membrane fluidization at the low cultivation temperature. The adjustment of lipid fatty acid composition for growth at the suboptimal temperature led to higher membrane fluidity and the shift of transition temperature (T_t) down for more than 10 °C.

However, the cells cultivated in mineral media at T_c 20 °C exhibited lower contents of fluidizing a-15:0 FA in their membrane compared to cells from complex medium. In MMGlyc, the overall proportion of anteiso-branched FAs was even lower at T_c 20 °C than at T_c 40 °C. Consequently, the proportion of non-branched FAs increased markedly at T_c 20 °C in both mineral media. Considering the hypothesis of Choi and coworkers that the condensing enzyme of FA synthesis FabH prefers anteiso-branched primers for its substrate at low temperatures (Choi *et al.* 2000), these results suggest that at a suboptimal temperature the synthesis of branched-FA precursors is rather insufficient.

A further aim of my study was to correlate the changes in membrane chemical composition with measurable biophysical parameters. Despite the fact that DPH fluorescence anisotropy (r_{ss}) as a measure of membrane fluidity was shown to have some limitations (Konopasek *et al.* 2000) the r_{ss} measurements employed in this work provided results consistent with those of our DSC measurements which proves for their biological relevance. The lipid samples with higher melting temperatures also exhibited higher DPH fluorescence anisotropy values at lower temperatures of measurement. The only exception was observed in the case of slightly higher DPH values for MMGlyc in comparison with MMGlu samples (T_c 20 °C, both membranes and lipids) while T_t for MMGlyc lipids was slightly lower.

The r_{ss} values (Fig. 16), validated by DSC measurement (Fig. 17), therefore indicate that the membranes of cells grown at T_c 40 °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. Hence, the cells grown in mineral media (T_c 40 °C) shifted to the low cultivation temperature (T_c 20 °C) might be therefore better prepared for performing their membrane-bound activities after cold shock than those of CM that are much closer to the lipid phase transition. This conclusion is strongly supported by the fact that cells in mineral media restored their growth immediately after the temperature downshift from 40 °C to 20 °C, while cells cultured on complex medium needed 60 min to recover (Fig. 13). Such growth lag had to be caused by membrane rigidization because the length of the lag could be shortened by the addition fluidizing agent benzylalcohol (Svobodova *et al.* 1995; Konopasek *et al.* 2000).

Compared to experiments in *E. coli*, described by Parola and colleagues, and discussed above (Zaritsky *et al.* 1985; Parola *et al.* 1990), the situation in *Bacillus subtilis* was obviously different. Unlike *E. coli*, *B. subtilis* cells cultivated and measured at 40 °C exhibited the same DPH anisotropy values regardless on cultivation medium (growth rate)

and despite the substantial differences in the fatty acid composition of membrane phospholipids. Nevertheless, it must be noted that the authors cited used a constant temperature of 37 °C (equal to cultivation temperature) for fluorescence polarization measurement, while I used measurement along the temperature scale which provides more information about the membrane characteristics. Indeed, in my experiments the differences between the compared cultures were the best detectable at lower measurement temperatures, while at the T_m close to cultivation temperature the divergences were only slight. Our results show that the different membrane composition, which resulted from the growth in different media, provides the cells with physical membrane features that are very similar at the temperature of growth (i.e. 40 °C) but very different when the ambient temperatures decreases.

However, neither of the unique membrane properties of cells grown in glycerol medium prevented the induction of fatty acid desaturase after cold shock. The maximum activity of *Pdes*-controlled β-galactosidase was the same in all three media tested (Fig. 19). For full desaturase induction, therefore, a sufficient abrupt change of the membrane fluidity, induced by cold shock, was more critical rather than its initial value.

In terms of T_t adjustment for different cultivation temperatures, the largest extent of cold adaptation of *B. subtilis* membrane was found in CM medium, where T_t decreased from 14.8 °C at T_c 40 °C to 4.6 °C at T_c 20 °C. In mineral media, T_t values were already very low at T_c 40 °C (7.8 and 4.5 °C for MMGlu and MMGlyc, respectively) and at T_c 20 °C decreased only slightly (in MMGlu medium) or even decreased (on MMGlyc) compared to T_c 40 °C (Fig. 17). I presume, therefore, that at lower cultivation temperatures the nutritionally poorer medium, especially the one with glycerol as sole carbon source, 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.

5.2 *Bacillus subtilis* membrane cold adaptation during anaerobic growth

The aim of this part of my work was to investigate how a lack of oxygen influences the adaptation of *B. subtilis* membrane to a low temperature. To this end, I compared the membrane adaptation of the in *B. subtilis* grown on complex cultivation medium at 37 °C, 25 °C and after the temperature downshift from 37 to 25 °C. I focused on fatty acid composition of membrane lipids, the membrane physical properties and the role and function of fatty acid desaturase Des, which needs oxygen for its function, under anaerobic conditions.

The following experiments were performed on the *B. subtilis* strains AKP3, AKP4, AKP21 and CM31 derived from wild type strain JH642 (see details in section 4.1.1). These strains were generously provided by the laboratory of prof. Diego de Mendoza (Universidad Nacional de Rosario, Rosario, Argentina), where I stayed for 2 months in 2005 and performed some of the experiments.

The results obtained were published in the Journal of Bacteriology (Beranova *et al.* 2010), see the fulltext in Appendix (page 108).

5.2.1 Growth and nitrite production of *Bacillus subtilis* strain AKP 3 in aerobic and anaerobic conditions

The adaptation of *B. subtilis* to suboptimal temperature (25 °C) was investigated during its aerobic and anaerobic growth in complex LB medium supplemented with glucose and nitrate, (see detail of cultivation in section 4.3).

After inoculation to the starting OD_{450} (0.005 and 0.05 for aerobic and anaerobic culture, respectively), the cells of *B. subtilis* strain AKP3 were grown at 37 °C. During the mid-exponential phase of growth (OD_{450} of 0.15–0.2) the growing cells were shifted to 25 °C.

The growth curves were measured for each culture and generation times were calculated. For 37 °C the generation times were 23±3 min and 64± 9 min for aerobic and anaerobic conditions, respectively. After the temperature downshift to 25 °C, the cultures attained doubling times of 72 ± 4 min and 160 ± 17 min for aerobic and anaerobic conditions, respectively. No lag phase was observed after the downshift in either the aerobic or the anaerobic culture (Fig. 20A). To verify whether the anaerobic growth conditions used in our experiment are able to support the effective anaerobic nitrate respiration of *B. subtilis*, the production of nitrite from nitrate was examined. As can be seen in Fig. 20B, the nitrite production in the anaerobic culture was stable both before and after the transfer from 37 °C to 25 °C, while in aerobic culture supplemented with nitrate, no nitrite production was observed. The nitrite levels shown in Fig. 20B do not represent the net nitrite formation from nitrate because the nitrite produced from *B. subtilis* grown under anaerobic conditions was shown to be utilized for subsequent conversion into ammonia (Hoffmann *et al.* 1998)

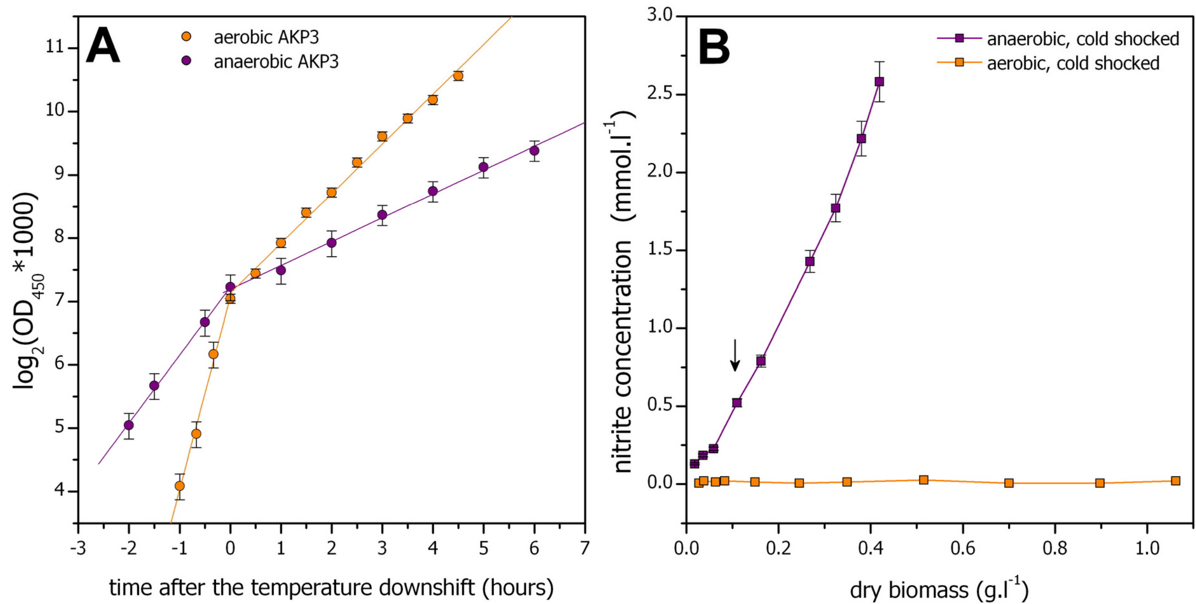


Fig. 20. Growth and nitrite production of *B. subtilis* strain AKP3 after the temperature downshift under aerobic and anaerobic conditions. *B. subtilis* cultures were grown aerobically or anaerobically in LB medium with glucose and nitrate. After inoculation, the cells were grown at 37 °C and transferred to 25 °C at an OD_{450} of 0.13 to 0.16. (**A**) Growth under aerobic or anaerobic conditions. The time of the transfer is identified as the zero point on the time scale. (**B**) Nitrite concentration in downshifted cultures grown anaerobically or aerobically. The time of transfer is indicated by the arrow. Data represent the means ± standard errors of the means from five (A) or two (B) independent experiments

5.2.2 Fatty acid profiles

To reveal the membrane adaptation on the chemical level, we compared the fatty acid profiles of aerobically and anaerobically grown *B. subtilis* AKP3 cultures at stable cultivation temperatures 25 and 37 °C and at different intervals after cold shock from 37 °C to 25 °C (see Fig. 21 for details). Longer time intervals were used for *B. subtilis* anaerobic culture due to its longer doubling time at 25 °C. The results are shown in Fig. 21. The trend in the patterns of fatty acid composition, which was basically similar when aerobic and anaerobic systems were compared, indicates the stepwise adaptation for lower cultivation temperature.

The level of low-melting anteiso-branched FA increased and concurrently the level of iso-branched FA decreased with low temperature in both systems (Fig. 21). However, some differences were found in the levels of i-15:0 and a-17:0 fatty acids. In the aerobic system the level of i-15:0 FA increased after the cold shock and during the long-term growth at lower temperature (25 °C). During the anaerobic growth its level almost did not change. On the contrary, the level of a-17:0 was increased at low temperature only in the anaerobic culture. The increase of major fluidizing fatty acid a-15:0 at the lower temperature was less pronounced in anaerobic system than in aerobic one. There was also no decrease of n-16:0 in anaerobic cells grown at 25 °C probably due to the fact

that this substrate could not be used for desaturation in anaerobic conditions (see sections 5.2.4 and 5.1.6). Anaerobic cultures exhibited slightly higher levels of anteiso- , lower levels of iso- and higher levels of non-branched fatty acids when compared with aerobic ones (Fig. 21A).

As for the unsaturated FA levels after the cold shock, in aerobic conditions, I observed the marked increase of unsaturated fatty acids levels reaching roughly 5 % of total FAs 2 hours after cold shock. In anaerobic conditions the proportion of unsaturated

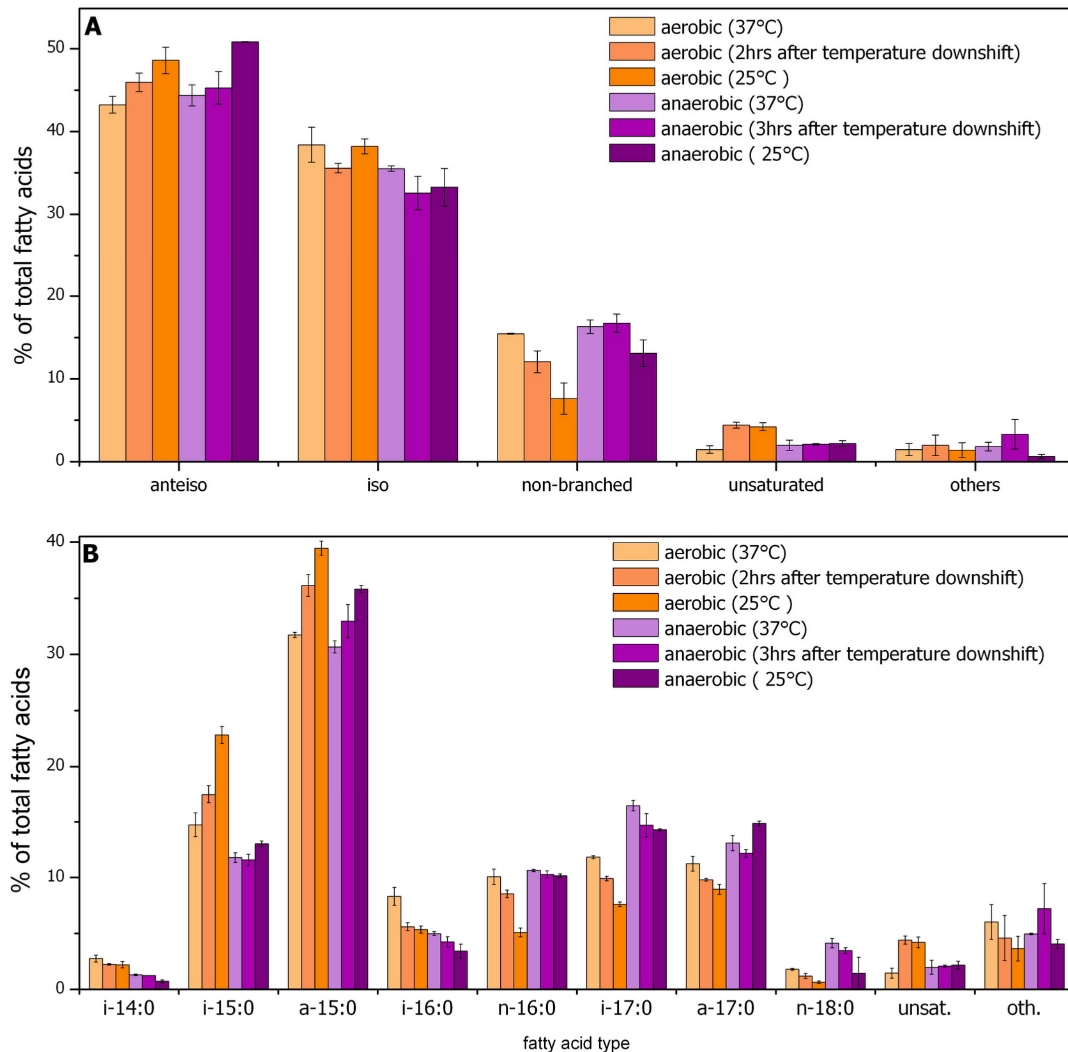


Fig. 21. The effect of anaerobic respiration on cold adaptation of membrane fatty acid composition in *B. subtilis* AKP3. The cells were grown under aerobic and anaerobic conditions at the constant temperature of 37 °C or 25 °C and harvested in the mid-log phase (OD_{450} of 0.5 to 0.6) for lipid isolation and fatty acid analysis. Parallel cultures were subjected to the temperature downshift (from 37 °C to 25 °C) in the mid-log phase and harvested after the temperature shift as indicated in the legend. A complete FA profile was recorded for each sample, and the proportions of the main structural types (**A**) and the most prominent species (**B**) are shown. The data presented are the averages of two independent experiments (cultivation, membrane isolation, and fatty acid analysis). **Abbreviations**: unsat. – unsaturated fatty acids; oth. – others. The prefixes i-, a-, and n- indicate the respective branching patterns for iso-, anteiso-, and non-branched FAs, respectively.

fatty acids remained stable around 2 % (Fig. 21). In both aerobic and anaerobic cultures, the adaptation of fatty acid content for lower temperature was noticeable within 1-2 generation times after cold shock.

5.2.3 Membrane physical properties measured by DPH fluorescence anisotropy

To estimate the impact of changes in fatty acid composition on membrane physical properties, the DPH fluorescence anisotropy technique was employed. The membranes from aerobic and anaerobic cultures of *B. subtilis*, both from T_c 37 °C and T_c 25 °C, were labeled with the membrane fluorescence probe DPH and DPH fluorescence anisotropy (r_{ss}) was then measured over the temperature (T_m) interval from 5 to 45 °C. Results are shown in Fig. 22. At T_c 37 °C, the r_{ss} values are very similar when aerobic and anaerobic cultivation were compared. This indicates that the lack of oxygen does not substantially influence the membrane fluidity of *B. subtilis* at the growth temperatures near the

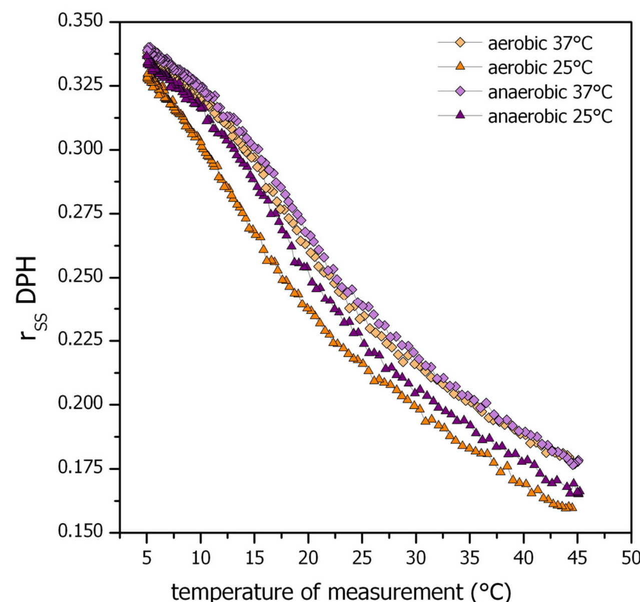


Fig. 22. DPH fluorescence anisotropy (r_{ss} DPH) measured in cytoplasmic membranes isolated from *B. subtilis* cultivated aerobically and anaerobically at a constant temperature. Cytoplasmic membranes were isolated from *B. subtilis* AKP3 cells grown aerobically or anaerobically at a constant temperature of 37 °C or 25 °C (mid-exponential phase cells, OD_{450} of 0.5 to 0.6) and were labeled with DPH. The steady-state fluorescence anisotropy of DPH embedded in cytoplasmic membrane vesicles was measured along the temperature scale (T_m) as indicated on the X-axis.

optimum. For both aerobic and anaerobic system, the r_{ss} values for the membranes from cells cultivated at T_c 25 °C were lower than the ones measured in the membranes from T_c 37 °C. This observation indicates a fluidization of the membranes of cold-adapted cells. However, the difference in the extent of cold adaptation was found between aerobically and anaerobically grown cells. From the obtained data, the extent of the adaptation can be estimated if we compare the distance between the r_{ss} curves obtained for membranes in

comparison at a reference temperature. Generally, the higher this distance, the more effective the adaptation is in terms of membrane physical properties reflected by fluorescence anisotropy measurement. In case of aerobically cultivated cells, the distance between r_{ss} data curves obtained for reference (T_c 37 °C) and cold-adapted (T_c 25 °C) membranes at the T_m=25 °C (i.e. the temperature for which the adapted membranes were retailored) is slightly higher than the one corresponding to anaerobic culture. Thus, the conclusion can be drawn that under the oxygen-limiting conditions *B. subtilis* was able to fluidize its membrane at lower temperature but the extent of fluidization was lower than that of aerobic conditions.

5.2.4 Induction of fatty acid desaturase synthesis after cold shock under aerobic and anaerobic conditions

The FA profiles analysis shown above verified the assumption that unsaturated fatty acids (UFAs) are not newly formed under the conditions favoring anaerobic respiration and, therefore, the short-term mechanism of rapid membrane fluidization is blocked. In further experiments I tested whether in such conditions desaturase, while unable to function properly, is still synthesized and regulated by the DesK-DesR two-component system similarly as in aerobic conditions (Aguilar *et al.* 1999; Aguilar *et al.* 2001). For this purpose we employed *Bacillus subtilis* strains bearing *Pdes-lacZ* fusion in *amyE* locus that allowed monitoring of *des* promoter induction: AKP3 with wild type desaturase and AKP4 with insertion mutation in desaturase gene. Previous work showed that the transcriptional activity of the *des* promoter was inhibited by addition of unsaturated fatty acids, with oleic acid having the most prominent effect (Aguilar 2001). The authors presumed that exogenous UFA lowered the requirements for desaturation due to fluidization of cytoplasmic membrane. In this work I tested the effect of oleic acid on *Pdes* activity in both aerobic and anaerobic conditions. To verify that in anaerobic conditions this induction is also exclusively controlled by DesK-DesR two-component system we used strain AKP21 with nonfunctional DesK-DesR system.

Wild type strain (AKP3) and strain with disrupted *des* (AKP4) were cultivated aerobically or anaerobically at 37 °C, transferred to 25 °C and their β-galactosidase activity was assayed for 5 hours after the temperature shift. To study the effect of fluidization by exogenously added UFA, parallel cultures were supplemented with oleic acid (n-18:1Δ9) in final concentration 5 μM at the moment of the cold shock.

Under aerobic conditions (Fig. 23A), β-galactosidase expression in strain AKP3 started with approx. 30-minute delay after cold shock and reached the maximum level of induction in 2 hours. Then the rate of synthesis decreased and β-galactosidase activity was stabilized. The final optical density reached by all four cultures was not higher than 1.4 (not shown). When AKP3 culture was supplemented with oleic acid, β-galactosidase activity was induced by cold shock similarly as in culture without UFA addition but its synthesis was suppressed earlier and more effectively.

In the *des* mutant strain AKP4, the stable *Pdes* induction was inhibited only if the external UFA was added. This pattern results from the inability of AKP4 to produce UFAs and to down-regulate the *des* promoter induction. However, the decrease in

β-galactosidase activity induced by oleic acid in AKP4 did not reach the level obtained for AKP3 with UFA (Fig. 23A). The more effective inhibition of *Pdes* induction in the case of the AKP3 strain can be explained by higher fluidization of membrane due to combined effect of desaturase activity and exogenous UFA.

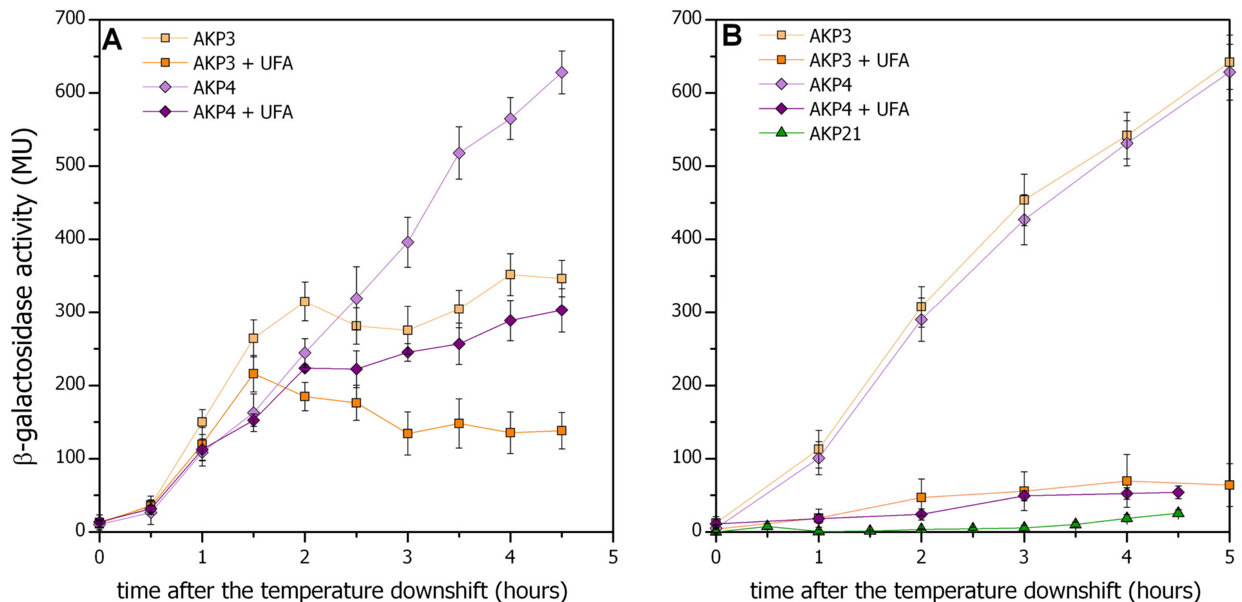


Fig. 23. The effect of anaerobic respiration on the cold-induced *des* expression and on membrane fluidization by external oleic acid. Induction of *Pdes*-controlled β-galactosidase synthesis was followed in the AKP3 (*des*$^+$ *Pdes-lacZ*), AKP4 (des$^-$ *Pdes-lacZ*), and AKP21 (*desKR*$^-$; *Pdes-lacZ*) strains after the temperature downshift from 37 °C to 25 °C (performed at OD_{450} of 0.15 to 0.2). Panels (**A**) and (**B**) show data from experiments under aerobic and anaerobic growth conditions, respectively. The addition of 5 μM oleic acid at the moment of the temperature shift is indicated as "+UFA". Data represent the means ± standard errors of the means from three independent experiments.

Under anaerobic conditions (Fig. 23B), I observed similar patterns of P*des* induction for the two strains. In AKP3 and AKP4 cultures without exogenously added UFAs, a stable increase of β-galactosidase activity was observed which was almost identical to that found in the aerobic AKP4 system. In anaerobic AKP3 and AKP4 cultures supplemented with UFA we observed the strongest decrease of β-galactosidase activity.

To verify that this induction under anaerobic conditions is also exclusively controlled by the DesK-DesR two-component system, we used strain AKP21, which has a nonfunctional DesK-DesR system. In this mutant strain no β-galactosidase expression was observed.

These results showed that induction of *des* expression after the cold shock occurred both in aerobic and anaerobic conditions despite the fact that Des synthesis without available oxygen could not support any fluidization. Under both conditions, *des* expression was regulated by the two-component system DesK-DesR and could be affected by the isothermal membrane fluidity changes.

5.2.5 Detection of Des by Western-blot

Besides the quantification of *des* promoter activity using *lacZ* reporter, the synthesis of desaturase in anaerobic conditions was also tested by Western blot analysis using monoclonal anti-GFP antibody. This strategy was employed because the specific antibody against the membrane bound Des protein was not available.

B. subtilis strain CM31 bearing isotopic Des-GFP fusion was cultivated in aerobic or anaerobic conditions at 37 °C and then transferred to 25 °C. In aerobic or anaerobic system the samples for analysis were taken at 1, 2, 3 or 2, 3 and 4 hours after cold shock, respectively (Fig. 24). From the results presented in Fig. 24 is clearly visible that Des is synthesized in both aerobic and anaerobic system after the cold shock.

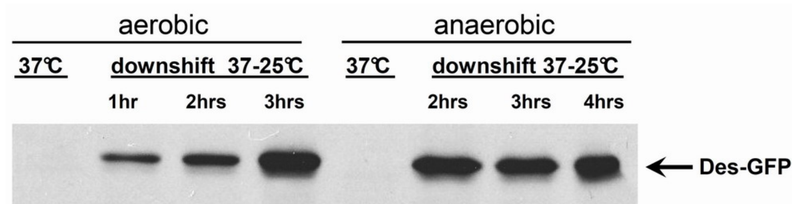


Fig. 24. The expression of Des-GFP under aerobic and anaerobic conditions at 37 °C and after the temperature downshift. Strain CM31, bearing the Des-GFP fusion, was cultivated aerobically and anaerobically at 37 °C and then shifted to 25 °C. The samples for immunoblot analysis were taken at 1-hour intervals after temperature downshift, as indicated, and analyzed as described in Materials and Methods.

5.2.6 The effect of oxygen on the desaturase activity

The findings resulting from Western-blot experiments raised the question whether the Des protein synthesized under anaerobic conditions (that do not support its activity) is somehow affected in its natural function when oxygen is again provided to cells.

Therefore, I studied the effect of the rapid transfer from anaerobic to aerobic conditions on *Pdes* induction after the temperature downshift. This approach allowed us to confirm whether it is indeed the desaturase fluidizing activity that negatively regulates *des* expression under aerobic conditions.

The AKP3 strain was cultivated anaerobically at 37 °C and was then transferred to 25 °C and β-galactosidase activity was assayed. After a substantial increase of β-galactosidase activity, i.e. 2 h after the temperature downshift, the culture was transferred into aerobic conditions. Fig. 25 shows that *Pdes* induction was inhibited shortly after the shift to aerobic conditions. Most probably, the high level of desaturase that was accumulated during anaerobic growth resulted in the effective fluidization after the transfer to aerobic conditions and consequently blocked further *des* transcription.

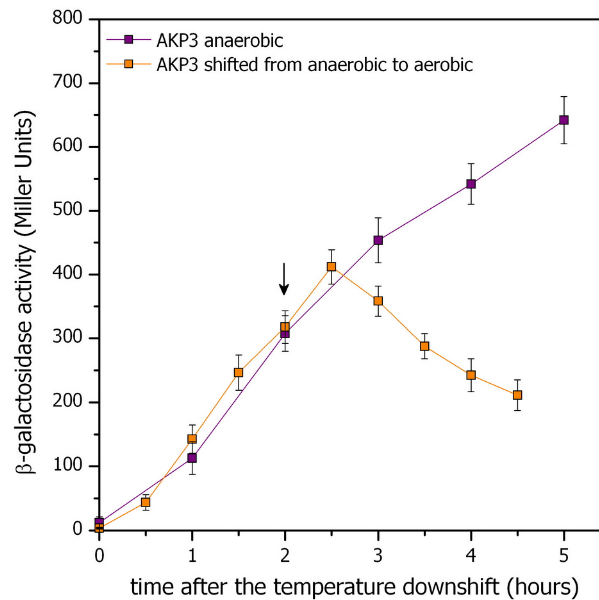


Fig. 25. The effect of transfer from anaerobic to aerobic conditions on the induction of *Pdes*. LB medium was inoculated with B. subtilis AKP3 (starting OD_{450} of 0.05) and grown anaerobically, and the induction of *Pdes*-controlled β-galactosidase synthesis was followed continually after the temperature downshift (performed at an OD_{450} of 0.15). Two hours after the temperature downshift (arrow), the culture was transferred into aerobic conditions. The AKP3 strain, which was cultivated under anaerobic conditions, was used as the control. Data shown represent the means ± standard errors of the means from three independent experiments.

5.2.7 Discussion II

Although *Bacillus subtilis* is still generally considered to be obligate anaerobe, this bacterium is in fact able of effective anaerobic growth (Nakano and Zuber 1998). Also, it can be expected that oxygen limitation is a frequent event in the soil environment, together with temperature fluctuations. Therefore, the research on *B. subtilis* cold adaptation under anaerobiosis is surely of biological relevance.

For my experiments, I used complex medium (LB) with glucose, supplemented with nitrate that enables *B. subtilis* to perform nitrate respiration. As seen from Fig. 20A, *B. subtilis* strain JH642 under anaerobic conditions at 37 °C growths on this medium with generation time which is more than double than that attained for this strain in the presence of oxygen. After the temperature downshift the generation times of both cultures increased, but no growth lag was observed for either. This indicates that the adaptation for low temperature is immediate.

The production of nitrite detected during the anaerobic growth (Fig. 20B) confirmed that the level of oxygen in the culture was low enough to allow for synthesis of dissimilatory nitrate reductase and shift of the energetic metabolism to anaerobic mode.

The observations discussed above (section 5.1.7) demonstrated that the available carbon source and consequently activated metabolic pathways considerably influence the

membrane composition, most probably via the levels of different FA precursors. Therefore, I expected that a fundamental change in metabolic network, which accompanies anaerobic growth, would also affect the membrane composition and its other features in a substantial way. Apart of chemical and physical characterization of cold-adapted membranes, I focused on the role of oxygen-dependent fatty acid desaturase Des in this process under oxygen limiting conditions. I presumed that anaerobic conditions would abolish the activity of FA desaturase.

I compared the membranes of *B. subtilis* cultured aerobically and anaerobically at stable temperature (37 °C and 25 °C) and subjected to the temperature downshift from 37 °C to 25 °C. The fatty acid profiles obtained (Fig. 21) clearly indicate that under oxygen-limiting conditions *B. subtilis* is not able to synthesize new unsaturated fatty acids and that therefore the short-term mechanism of rapid membrane fluidization cannot be employed after the temperature shift. However, even in the cold-treated aerobic culture the UFA level was rather low, comprising approx. 5 % of total cellular fatty acids. Such low proportions of UFAs seem to be a characteristic feature of strain JH642. Comparable low levels of UFAs in *B. subtilis* JH642 grown aerobically on mineral medium after the temperature downshift were reported by Altabe and coworkers (Altabe *et al.* 2003). The authors found that an increase in the production of UFAs requires overproduction of response regulator DesR, in the absence of the sensor kinase DesK. Therefore, anaerobically grown *B. subtilis* employs for membrane fluidization the mechanism based exclusively on an increased anteiso/iso- ratio of branched-chain fatty acids. When grown aerobically, this organism additionally exploits an increased level of unsaturated fatty acids after the temperature downshift. At stable low temperature, however, its membrane is fluidized by an increased anteiso/iso- ratio similarly as under anaerobic conditions. The general trend, leading to an increase of anteiso- and decrease of iso-branched FA levels with the low temperature, was the same in anaerobic and aerobic cultures, however, when the FA profiles are seen more in detail some differences could be observed. In addition to low levels of UFAs, the anaerobic cultures differed from aerobic ones by the proportion of non-branched fatty acids that was almost unaffected by cultivation temperature. The straightforward explanation for this result could be based on the known mechanism of FA desaturation in *B. subtilis* that takes place on fatty-acyl chains incorporated to membrane lipids. Non-branched fatty-acyl chains are mostly used as substrates for desaturation and this is the most probable reason why their levels decrease concomitantly with the increase of UFA proportions (as can be observed for aerobic cultures in Fig. 21).

Anaerobic cultures exhibited slightly higher overall levels of anteiso- and lower levels of iso-branched FA. These differences were only small, but they appear more pronounced when we focus on the levels of individual fatty acid types falling into those two respective groups. For example, while in aerobic cultures the major iso-branched fatty acid was i-15:0, in anaerobic cultures it was i-17:0. Also, the level of a-17:0 was higher in the anaerobic culture and was increased when the temperature was lower, while in aerobiosis it decreased with the temperature drop. Together with other minor differences in FA acid composition that can be seen on Fig. 21B these observations clearly show that the general goal of membrane cold adaptation, which consists in the lowering of the lipid transition temperature by incorporation of low-melting fatty-acyl chains into lipid

molecules, can be achieved by various individual patterns of chemical composition.

However, when I correlated the differences in chemical composition with biophysical parameter, represented here as DPH fluorescence anisotropy, I found that the anaerobically cultivated *B. subtilis* seemed to have slightly lower capacity for compensation of membrane fluidity. The extent of adaptation for 25 °C compared with that for 37 °C was quantified according to the method of Cossins and Sinensky (Cossins and Sinensky 1984) as the homeoviscous efficacy (HE). HE values of 40 and 30 % were found for aerobic and anaerobic conditions, respectively. That indicates the nitrate respiration-connected metabolism represents certain disadvantage in membrane adaptation to cold which is, however, not fundamental.

Although the regulation mechanisms involved in the adaptation of *B. subtilis* to a low cultivation temperature have been elucidated to a considerable extent, almost no data are available that would link the adaptation to cold with the adaptation to a limited oxygen supply. During anaerobic growth, the metabolism of *B. subtilis* undergoes a necessary remodeling that involves several hundreds of genes, including those involved in basic metabolic pathways (Ye *et al.* 2000). The tricarboxylic acid branch of the Krebs cycle was shown to be reduced during anaerobiosis (Nakano and Zuber 1998). The related changes in key metabolites available in the cell are therefore quite likely to have a substantial influence on the biosynthesis of FAs. The complex cultivation medium (LB with glucose), which was used for our experiments, provided sufficient amino acids for branched FA synthesis. From the differences in FA composition that were found between aerobically and anaerobically grown cells at 37 °C, it can be clearly concluded that the regulation of biosynthetic pathways leading to branched FA formation also depends on the type of energy metabolism. Despite the fact that the detailed mechanism of such regulation has yet to be elucidated, my experiments clearly shows that in anaerobiosis, the different strategy of cold adaptation observed at the level of FA composition resulted in a lesser adaptation of membrane fluidity.

The experiments relating to the monitoring of desaturase gene (*des*) transcription provided the interesting results showing that the promoter of the *des* gene is activated by membrane rigidization stimulus via DesK-DesR regulatory loop even under the conditions which block the enzymatic activity of its product. Desaturase is produced after a temperature downshift in anaerobic conditions and the feedback inhibition, which in the presence of oxygen shuts off its expression when the optimal membrane fluidity is restored, does not function. The reason seems solely to be the inability of Des to anaerobically produce UFA that would fluidize the membrane, as the addition of external UFAs immediately lowers the activity of *des* promoter not only during aerobic (Aguilar *et al.* 2001) but also during anaerobic growth.

The detection of Des labeled by GPF protein using the Western blot technique verified that the *des* promoter activation, detected as β-galactosidase activity in strains bearing *lacZ* reporter gene fused to *Pdes*, indeed reflected also the production of the desaturase protein. Without oxygen, Des is continuously produced and accumulated in the cold-treated cells without any obvious physiological reason. The adaptation of membrane composition to lower temperature thus has to be accomplished by alternative mechanism (i.e. increasing of level of low-melting branched FA species). However, when oxygen is provided, its effect on the des regulatory pathway is very quick. Results

presented in Fig. 25 show that the renewed oxygen supply results in rapid repression of *Pdes*. I presume that the desaturase accumulated in the anaerobic cells starts to act with oxygen available and immediately desaturates fatty acids. In consequence, the feedback regulatory pathway down-regulates *des* transcription.

With this knowledge, it can be concluded that the control mechanism of FA desaturase synthesis is the same as that under aerobic conditions, in spite of the fact that the enzyme is not functional under anoxic conditions.

5.3 Construction of *Bacillus subtilis* strain FL1

In our further studies we would like to focus on the membrane fluidity sensor DesK and the mechanism of sensing the changes in the membrane physical state. We would like to employ the fluorescent probe 4´, 5‘-bis (1, 3, 2-dithioarsolan-2-yl) fluorescein, also called FLuorescein Arsenical Helix binder, abbreviated as FLASH. This small (70 Da), membrane-permeant ligand is able to bind an α-helical tetracysteine motif Cys-Cys-X-X-Cys-Cys, where Cys stands for cystein residue and X for any other aminoacid (Griffin *et al.* 1998). The excitation and emission spectrum of FLASH partially overlap which provides the possibility for occurrence of the fluorescence resonance energy transfer between two closely located molecules of FLASH (homo-FRET). Homo-FRET results it fluorescence depolarization and thus in the decrease of fluorescence anisotropy. We plan to use this effect in the study of the signaling mechanism of DesK, namely the possible dimerization.

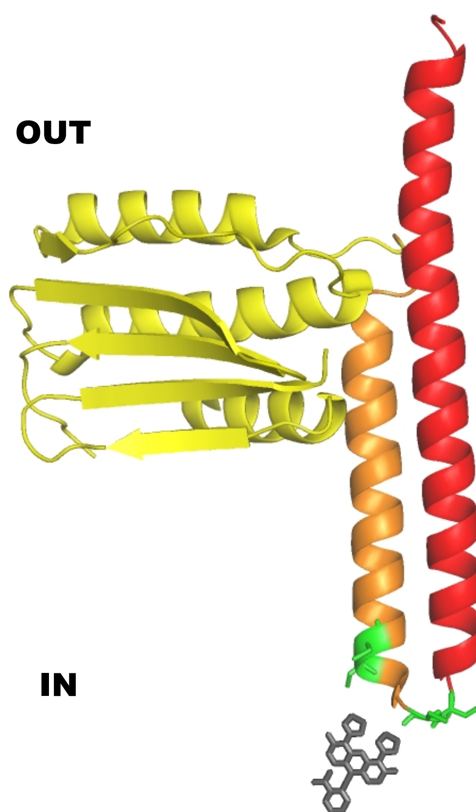


Fig. 26 The cytoplasmic domain of membrane sensor DesK of *B. subtilis* strain FL1 (ribbon model). Cystein residues (FLASH binding site) introduced into the sequence by site directed mutagenesis are showed in green, the molecule of FLASH probe is depicted in grey. **Color coding:** red - helix α1; orange - helix α2; yellow - C-terminal ATP binding domain. OUT - direction to the membrane, IN - direction to the cell interior.The graphic model was created in the software PyMol Molecular Graphic Systems using the data published by (Albanesi *et al.* 2009) and available in Protein Data Bank (PDB ID code 3EHH).

To this end, I tried to introduce the FLASH-binding motif into the aminoacid sequence of DesK. Based of the recently published structure of cytoplasmic domain of DesK (Albanesi *et al.* 2009), we designed its localization as shown in Fig. 26, into the bend of N-terminal 2-helix hairpin. The original sequence -Tyr-Lys-Asp-Pro-Glu-Gln- was changed to -Cys-Cys-Asp-Pro-Cys-Cys- (see the whole gene and protein sequences in supplementary data, section 8). To this end I used the plasmid pJB3 (see section 4.1.4) for transformation of *B. subtilis* strain AKP21 (JH64 *desKR::Km*^{r} *amyE:Pdes-lacZ*). The resulting strain FL1 contains the genes *desK* with modified sequence and *desR* under the control of xylose-inducible promoter in the *thrC* locus.

This strain will be used for further studies conducted in our laboratory. It will be employed in the investigation of the DesK signalling mechanism; the FLASH probe, binded to DesK molecules, should allow for monitoring of DesK dimerization.

6 Summary

Part I - *Bacillus subtilis* membrane cold adaptation under different nutrition conditions

- The nutrient conditions influence substantially the membrane fatty acid profiles and membrane biophysical parameters both at the optimal (40 °C) and suboptimal (20 °C) growth temperatures.
- The growth of *B. subtilis* in mineral media at 40 °C resulted in the fatty acid composition that provided the cells with more fluid membranes, compared to those cultivated in rich medium, which favored the former after the temperature downshift. Cells grown at 40 °C in nutrient-poor mineral medium with glycerol, exhibited very unusual fatty acid profiles with very high levels of anteiso-branched and straight-chain fatty acids.
- At the cultivation temperature of 20 °C, the cells grown in complex medium incorporated higher amounts of low-melting fatty acids into their membranes, which resulted in lowering the lipid transition temperature by more than 10 °C. The cold adaptation of cells cultivated in mineral media was not so pronounced, compared to complex medium; however, those cells already exhibited very fluid membranes at 40 °C.
- *Bacillus subtilis* 168 is able to synthesize sufficient levels of branched fatty acid precursors during growth in all three media tested; however, this ability seems to be reduced to a certain extent when it is cultivated at 20 °C in nutrient-poor medium.
- The induction of fatty acid desaturase after the temperature downshift was not affected by the composition of cultivation medium.

Part II - *Bacillus subtilis* membrane cold adaptation during anaerobic growth

- A lack of oxygen does not substantially influence the membrane fatty acid composition and its fluidity of *B. subtilis* at at the near-optimum temperature.
- Under both aerobic and anaerobic conditions, *B. subtilis* employed the same long-term strategy of membrane cold adaptation, i.e. increasing the levels of low-melting fatty acid types in membrane lipids.
- The cells cultivated under the oxygen-limiting conditions at 20 °C exhibited slightly higher values of DPH fluorescence anisotropy (compared to those grown aerobically), which indicates that the extent of fluidization was lower than in aerobic conditions.
- After cold shock, *des* gene transcription is induced and Des protein synthesized under anaerobic conditions, despite the lack of oxygen disabling the proper function of desaturase.
- No new unsaturated fatty acids were synthesized in anaerobically grown cells after cold shock.

Part III - Construction of *Bacillus subtilis* strain FL1

- A new *B. subtilis* strain FL1 was constructed within this work. FL1 bears a binding motif for FLASH fluorescence probe, introduced by substitution in the sequence of membrane fluidity sensor DesK. This strain will be used for conformation studies employing time-resolved fluorescence spectroscopy in further projects.

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8 Supplementary data

<u>The sequence of the *Bacillus subtilis* gene *des* and its upstream region, sequences and locations of the primers used for construction of *Pdes-lacZ* fusion strain M19</u>

<u>Primer sequences:</u>
(restriction sites for EcoRI and BamHI endonucleases are shown in magenta/red and underlined):

<u>desprom1</u> (forward primer, with EcoRI restriction site):
5´-GTTTGG/AATTCACCCCTCAAGTGAGTGGAGC-3´

<u>desprom2</u> (reverse primer, with BamHI restriction site):
5´-TAGTTAG/GATCCTCTCATTGTGTGTCTCGGTTC-3´
3´-ATCAATC/CTAGGAGAGTAACACACAGAGCCAAG-5´

EcoRI G/AATTC
BamHI G/GATCC

<u>Sequence of *des* and its upsteam region</u>:

color coding:
yocD oper reading frame (blue) – intergene segment (black) – *des* (red); primer binding sites (green, undelined); nucleotids given in **black boldface** were changed in order to introduce the restriction sites)

aggagtattttctttcttgctgcgcttcagacgatccgtttg**a**aattcacccctcaagtgagtggagc
gatgatcgctggtttttggatcaagaaaacaggcgttttatcctaataacggccctgttgtaatcca
agaaggttatgcggaaggtactttaattggcggaaacttatgcacgcttaatttgctgcagggaacgg
agtattttcccgaaacagaacatactattttattgattgaagatgattatatgtcagacatccatatg
tttgaccgcgatctgcaatcactcatccacctccccgctttttcacatgtaaaggcgattttgatcgg
cagatttcagaaagcatcaaacgtatcaatagatcttgtaaaagccatgatcgaaacaaaaaaagaat
tatccggcatcccgatcatcgcaaatataaatgccggacatacctcgccaattgccacgttccctata
ggaggaacatgcaggattgaagctatttcgggtacatcacgaatatggattgataaacattaatcagc
ttgtaaattttttacaagctttttagcgcaatcggctatgcatgccgcacgagacatgacaaatgt
catataggaggcatgatgtgtgctactacaaaagacttctctcattagcgtatactgaaccgagacac
acaatgagaggat**a**ct**t**actatgactgaacaaaccattgcacataaacaaaaacagctgacaaagcaa
gtcgctgcatttgctcagcctgaaacaaaaaacagcctgattcagcttttaaacacgtttatcccatt
cttcggcctatggtttcttgcttatctcagcctcgatgtctcctatcttcttacgttagcattaacgg
tgattgccgcaggttttctgacaagaattttatcatcttccatgactgctgccatcaatctttttc
aaacaaaaacgctataaccacattctcggttttctgacaggtgtcctgactttattcccgtatcttca
atggcagcacagccattcgattcatcatgcaactagcagcaatctggataaacgcggaacaggagaca
tctggatgttaacagtaaacgaatataaagctgcatccagacgaacaaagcttgcatacagactttat
agaaacccgtttatcatgtttattctcggaccgatttatgtttttctgatcacgaaccgttttaacaa
aaaaggcgcaagacgcaaggaacgtgtaaacacataccttacgaatctggcaattgtcgcgttggctg
ccgcttgctgtctgatctttggctggcaatcgtttttactggtgcaaggcccgatatttctgatttca
ggttcaatcggtgtttggctgttttatgtgcagcatacctttgaagattcttattttgaagcggatga
aaactggagctacgttcaggctgctgttgaaggcagctcattttataaactcccgaaactgcttcaat
ggctaacaggcaatattggttaccaccacgttcatcatttgagtccaaaggtgcctaactataagctt
gaagttgctcatgaacatcacgaaccattaaaaaacgtaccgacaatcaccttaaaaacaagcctgca
atcacttgcgttccgtctatgggatgaagataacaaacagtttgtgtcatttcgggctataaaacata
tacctgtaagccttccgcctgattcaccagaaaaacagaagctgcggaagaatgcc

The sequence of the *Bacillus subtilis* gene *desK* (wild type) and the location of the primers used for construction of strain FL1

Primer sequences:

restriction sites for BssSI and PstI endonucleases is given in blue/red and underlined, changed codons are highlighted in yellow

BssSI	C/TCGTG
	GAGCA/C
Pst	CTGCAG

Fwd-Pst (forward primer, with PstI restriction site)

5´- GGCTGCA/GGAATTCTTAC - 3´

flash-BssSI (reverse primer, mutagenic, with BssSI restriction site)

5´- GTTCACGA/GCTGCACAGCAGGGATCACAGCATATCAATTTTCTTGCTAAGTC -3´

3´- CAAGTGCT/CGACGTGTCGTCCCTAGTGTCGTATAGTTAAAAGAACGATTCAG -5´

desK (wild type) sequence:

color coding:

flash-BssSI primer binding site - magenta, underlined; changed codons - higlighted in yellow, BssSI restriction site - blue

```
atgattaaaaatcattttacatttcaaaaactaaacgggattacgccctatatatggacgatattttt
catcctccccttctactttatatggaaatcatcatctacatttgttattattgtcggcatcattttga
cgctttttatttttttcggtatatcgatttgcttttgtctcaaaaggctggaccatttatttgtgggga
ttttttattaattggcatttcaaccgcctccattactctgttcagttatatttattttgctttctttat
tgcttattttattggaaacattaaggaacgcgtcccttttcatattttatattatgtccatttaataa
gcgcggccgtcgcagccaatttcagtctcgtattaaaaaggaattctttctgacacaaattcctttt
gtcgttattaccctcatcagcgcaattttattgcccttcagtataaaaagccgcaaggagcgcgaacg
acttgaagaaaagctcgaggatgcaaatgaacggattgcagaactggtaaaattagaagaacgtcagc
gaattgcccgcgatctccatgatacgcttgggcaaaagctttctcttattggtttaaaaagcgactta
gcaagaaaattgatatacaaagatcccgaacaagcagctcgtgaactgaaaagtgttcagcaaacagc
gcgaacttctttaaatgaagtaagaaaaatcgtttcctctatgaagggcatccggctcaaggatgaat
tgatcaacatcaaacaaattctcgaagcagctgacattatgtttatctatgaagaagagaaatggccc
gagaatatctcattgctgaatgaaaacattttgagcatgtgcttaaaggaagctgtcacaaatgtcgt
caaacacagccaggctaaaacttgccgagttgacattcagcagctctggaaggaagttgtgattacag
tgtctgacgatggaacattcaaaggagaagaaaattcctttttcaaaaggacatggcttactcgggatg
agagaacggcttgagtttgcaaacggaagccttcacatcgataccgaaaacgggaccaagcttaccat
ggcaattcctaataattcaaaa
```

The amino acid sequence of the *Bacillus subtilis* protein DesK (wild type) and the location of aminoacid residues changed in FL1 strain

Mutated sequence (highlited in yellow): YKDPEQ → CCDPCC

```
MIKNHFTFQKLNGITPYIWTIFFILPFYFIWKSSSTFVIIVGIILTLLFFSVYRFAFVSKGW
TIYLWGFLLIGISTASITLFSYIYFAFFIAYFIGNIKERVPFHILYYVHLISAAVAANFSLV
LKKEFFLTQIPFVVITLISAILLPFSIKSRKERERLEEKLEDANERIAELVKLEERQRIARD
LHDTLGQKLSLIGLKSDLARKLIYKDPEQAARELKSVQQTARTSLNEVRKIVSSMKGIRLKD
ELINIKQILEAADIMFIYEEEKWPENISLLNENILSMCLKEAVTNVVKHSQAKTCRVDIQQL
WKEVVITVSDDGTFKGEENSFSKGHGLLGMRERLEFANGSLHIDTENGTKLTMAIPNNSK
```

9 Appendix - Publications

<u>Beranová, J.</u>, M. Jemioła-Rzemińska, D. Elhottová, K. Strzałka and I. Konopásek (2008). "Metabolic control of the membrane fluidity in *Bacillus subtilis* during cold adaptation." <u>Biochimica et Biophysica Acta - Biomembranes</u> **1778** (2): 445-453.

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.

doi:10.1016/j.bbamem.2007.11.012

Beranová, J., M. C. Mansilla, D. de Mendoza, D. Elhottová and I. Konopásek (2010). "Differences in Cold Adaptation of Bacillus subtilis under Anaerobic and Aerobic Conditions." Journal of Bacteriology **192**(16): 4164-4171.

Abstract:

Bacillus subtilis, which grows under aerobic conditions, employs fatty acid desaturase (Des) to fluidize its membrane when subjected to temperature downshift. Des requires molecular oxygen for its activity, and its expression is regulated by DesK-DesR, a two-component system. Transcription of des is induced by the temperature downshift and is decreased when membrane fluidity is restored. *B. subtilis* is also capable of anaerobic growth by nitrate or nitrite respiration. We studied the mechanism of cold adaptation in *B. subtilis* under anaerobic conditions that were predicted to inhibit Des activity. We found that in anaerobiosis, in contrast to aerobic growth, the induction of des expression after temperature downshift (from 37 °C to 25 °C) was not downregulated. However, the transfer from anaerobic to aerobic conditions rapidly restored the downregulation. Under both aerobic and anaerobic conditions, the induction of des expression was substantially reduced by the addition of external fluidizing oleic acid and was fully dependent on the DesK-DesR two-component regulatory system. Fatty acid analysis proved that there was no desaturation after des induction under anaerobic conditions despite the presence of high levels of the des protein product, which was shown by immunoblot analysis. The cold adaptation of *B. subtilis* in anaerobiosis is therefore mediated exclusively by the increased anteiso/iso ratio of branched-chain fatty acids and not by the temporarily increased level of unsaturated fatty acids that is typical under aerobic conditions. The degrees of membrane fluidization, as measured by diphenylhexatriene fluorescence anisotropy, were found to be similar under both aerobic and anaerobic conditions.

doi:10.1128/JB.00384-10