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Variations of the Envelope Composition of *Bacillus subtilis* During Growth in Hyperosmotic Medium

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Abstract. The envelope properties of *B. subtilis* cultures grown in LB and LBN hyperosmotic media (LB + 1.5 M NaCl) were compared. Since hypertonic cultures showed a Spo-phenotype, a Spo-mutant grown in LB was also analyzed. LBN cultures showed extensive filamentation and presented different sensitivities toward phage infection (ϕ29 and ϕ105), or antibiotics whose targets are at wall (lysozyme, penicillin G) or membrane level (polymyxin B, phosphonomycin). Results of the biochemical composition revealed that during hyperosmotic growth, the cell wall increased in thickness, and among the membrane lipids, glycolipid and cardiolipin increased in parallel with a decrease in phosphatidylglycerol. The fatty acid composition was also modified, and an increase in saturated straight chain with a decrease of saturated iso-branched fatty acids was observed.
The increase of monounsaturated 18-1 (ω-9) fatty acid was probably related to the absence of sporulation observed in hypertonic media, since its increase has been shown to inhibit the KinA sensor of sporulation. The significance of the other wall and membrane composition variations (and hydrophobic surface properties) in relation to the osmotic adaptation are discussed.

Bacillus subtilis is a Gram-positive, sporulating bacterium, able to adapt to large variations of osmotic strength. To grow in hypersaline media, this bacterium develops different strategies leading to an increase of particular intracellular metabolites and probably to modifications of its envelope, essentially composed of a membrane and a thick wall.

Concerning the content of intracellular metabolite(s) allowing growth in saline media, striking similarities exist among the mechanisms and compounds found in different organisms (plants and bacteria): induction of specific transport systems for adequate osmolytes (K+, proline, etc.), or synthesis of osmoprotectants like glutamate, glycine-betaine, and/or trehalose (polyols) [7, 38] were generally observed. However, osmotic adaptation occurred in at least two steps involving transient and permanent mechanisms. During the first step there was a water efflux, a decrease of the turgor pressure, and the entrance of K+ (a compatible osmolyte). Afterwards, induction of specific mechanisms for transport and/or biosynthesis of osmocompatible solutes allowed growth to continue [7]. However, these osmolytes should fulfill certain conditions such as: (1) to be able to cross the cell membrane quickly; (2) to accumulate in high cellular concentrations without provoking large disturbances in the structure of cellular macromolecules such as DNA and proteins [7, 38].

Uptake and accumulation of osmolytes are among the most important adaptive responses to high osmolarity. Nevertheless, several evidences suggest that cell envelope composition would also play an important role [13, 18, 20, 30]. However, its variation in relation to osmotic adaptation has been scarcely documented. The aim of the present work was to study the variations produced in wall and membrane of *Bacillus subtilis* cultures submitted to osmotic stress. Physiological parameters, such as sensitivity to antibiotics and phages whose primary targets are at the envelope and biochemical determination of some

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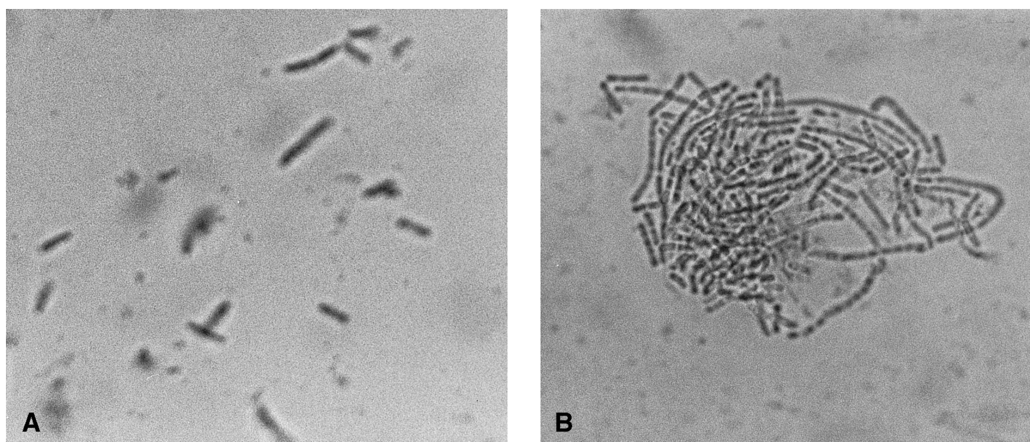

Fig. 1. Observation of *Bacillus subtilis* cultures under optic microscopy. Cultures from YB886 strain grown in LB and LBN were observed under microscope at 1000× and photographed. (A) LB medium; (B) LBN medium.

envelope components (mainly membrane phospholipids and fatty acids), were studied.

Materials and Methods

All the solvents and reagents used were analytical grade compounds.

Bacterial cultures. *Bacillus subtilis* strains were YB886 (*metB5*, *trpC2*, *xin-1*, SPβ^S) and 8E an isogenic *spoOA*::EmR derivative. The standard Luria-Bertani medium (LB), containing 1% tryptone, 0.5% yeast extract, and 0.5% NaCl, or the Schaeffer sporulation medium (D) was employed. Hyperosmotic LBN medium was obtained by adding 1.5 M NaCl to the basal LB. Plates contained 12 g/L Difco agar. Spores were obtained in D medium and stored as indicated [26].

Cultures for lipid analysis were performed in LB or LBN as indicated, grown in aerated bath at 37°C during 24 or 48 h, respectively. Pellets were collected by centrifugation and kept stored at −20°C until their further utilization.

Protoplasts were obtained from pellets suspended in SMM buffer (sucrose 0.5 M, $MgCl_2$ 20 mM in sodium malate 0.02 M, pH 6.5) containing 200 µg/ml lysozyme and incubated 1 h at 37°C with gentle shaking [29]. With LBN cultures, the protocol was modified by adding to the basal SMM buffer 0.5 M NaCl and increasing lysozyme concentration to 4 mg/ml. Once obtained, protoplasts were spun down and membrane extracted as described here below.

Hydrophobicity index. Hydrophobicity was determined by a small modification of the rapid and simple assay method described by Op den Camp [23]. Briefly, culture pellets were thawed, washed twice in PUM buffer (22.2 g $K_2HPO_4{\cdot}3H_2O$, 7.26 g KH_2PO_4, 1.8 g urea, and 0.2 g $MgSO_4{\cdot}7H_2O$ per liter distilled water; pH 7.1), resuspended in the same buffer, and absorbance measured at 550 nm. To 1.2 ml of a turbid bacterial preparation (absorbance at 550 nm 1.0 to 1.5), hexane was added slowly by aliquots of 25 µl until a total volume of 200 µl was reached. Samples were preincubated 30 min at 37°C, mixed uniformly for 120 s, and allowed to stand until phases were separated. The aqueous phase was carefully removed and its absorbance at 550 nm measured. Decrease of absorbance of the aqueous phase was used as a measure of cell surface hydrophobicity as described by the authors.

Lipid extraction. Total lipids were extracted according to Rivas and Luzzati [25] with minor changes. Essentially, culture pellets were thawed, suspended in distilled water, and extracted with 5 volumes of isopropanol. Serial extractions were performed with 5 volumes of isopropanol/chloroform (1:1, vol/vol) and 10 volumes of chloroform/methanol (2:1, vol/vol) (plus 0.84 ml HCl 6*N*/L to improve the lysilPG extraction) [15]. After 16 h at room temperature in the last solvent system, extracts were assembled, filtered, dried under vacuum, reextracted with 5 volumes of chloroform/methanol (2:1, vol/vol) (plus 0.84 ml HCl 6*N*/L), and finally washed with 0.2 volumes of NaCl 0.9% in 0.01*N* HCl [15].

Lipid analysis. Thin layer chromatography (TLC) plates (Silicagel 60 F_{254}, 0.25mm thick) were purchased from Merck. Lipid classes were analyzed by TLC, with the solvent system chloroform/methanol/acetic acid (65:25:6, vol/vol/vol). Phospholipids and glycolipid were detected with iodine vapors and identified by comparison with authentic purified standards and by treatment with specific reagents (ninhydrin, orcinol, and/or ammonium molybdate). Zones of the plates corresponding to each lipid fraction were scraped off and quantified.

Lipid phosphorus was determined according to Bartlett [2], and total carbohydrates were measured as described by Hanson and Phillips [9].

Fatty acid analysis. Total lipid extract was suspended in 2 ml of a fresh solution of KOH 10% in ethanol and digested during 45 min at 80°C; 1 ml of distilled water was added and extracted three times by shaking with 2 ml of hexane. The upper organic phase (insaponifiable) was discarded. The aqueous layer was acidified with 0.5 ml of concentrated HCl and extracted twice with 2 ml hexane. Free fatty acids were dried under a nitrogen stream and submitted to methyl esterification with 2 ml of 2% H_2SO_4 in methanol at 60°C for 120 min under a nitrogen atmosphere. Then, 2 ml of chloroform and 0.67 ml of distilled water were added. The aqueous phase was discarded, and the organic phase was washed twice with distilled water. After washing, the organic phase was evaporated under a nitrogen stream, resuspended in hexane, and aliquots containing fatty acid methyl esters were injected into an RC8 Shimadzu Gas Chromatograph with an Omegawax 250 column (30m × 0.25mm, 0.25 µm film) [37].

Results

Physiological and morphological evidences. One of the first observations indicating that during growth in hypertonic medium *B. subtilis* varies its envelope composition was the extensive filamentation presented by its cultures (Fig. 1). This finding was confirmed by the behavior of

Table 1. Sensitivity to antibiotics and phages of *B. subtilis* cultures grown in different media

Antibiotic[a]	LB[a]	LBN[a]
Penicillin G		
10 µl (0.16 mg/ml)	2.0	3.0
Tetracycline		
10 µl (10 mg/ml)	2.8	3.2
Phosphonomycin		
10 µl (35 mg/ml)	1.5	1.0
Polymyxin B		
10 µl (10 mg/ml)	3.0	0.3
Lysozyme		
10 µl (20 mg/ml)	1.4	0.6
Phage[b]		
Φ29	S	R
Φ105	S	R

[a]Bacteria grown in LB or LBN (LB+ 1.5 M NaCl) were spread onto these same solid media. A few µl of antibiotic was added as indicated in the table, and the plates were incubated 24–48 h. The halo of inhibition was measured, and the diameter in cm recorded in the table. The antibiotic concentrations were previously chosen as those giving the maximum halo at the minimum concentration.
[b]After inoculation of one drop of a phage suspension to a lawn of bacteria and further incubation for 24 h at 37°C, a halo of lysis was observed; S or R stands for the presence or absence of lysis respectively.

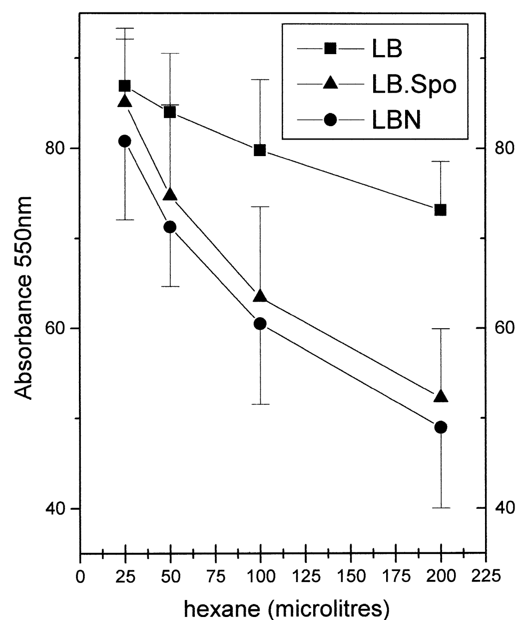

Fig. 2. Hydrophobicity index of *Bacillus subtilis* cultures. LB and LBN cultures from YB886 (LB and LBN) and an LB culture from 8E (LB8E) were submitted to succesive extractions with hexane, as indicated in Materials and Methods. Absorbance at 550 nm from the different aliquot extractions was recorded. Values represent individual determinations ± SD.

cultures submitted to phages or antibiotics whose primary targets were at the envelope level. Sensitivity to penicillin G and to the detergent polymyxin B (acting at the membrane level), to lysozyme and phosphonomycin (acting at the wall level), all showed different efficiency with cultures grown on LB and LBN. In addition, phages ϕ29 and ϕ105, a virulent and a temperate phage of *B. subtilis*, were both unable to lyse LBN cultures (Table 1). Moreover, it is worth noting that protoplasts from LBN cultures cannot be easily obtained; in particular, lysozyme concentration would be increased at least 10 times (see Materials), suggesting that the cell wall thickness was larger. Altogether, these results indicated that important variations in envelope composition (membrane and cell wall) had occurred.

Biochemical characterization of the envelope. Since *B. subtilis* grown in hyperosmotic medium is unable to sporulate, this strain culture condition will be considered similar to Spo$^-$ phenocopies. However, a SpoOA$^-$ strain (carrying a *spoOA* mutation) did not support growth in hyperosmotic media (osmosensitive) [26]. Thus, variations involved in the osmotic adaptation might be differentiated from those due to the non-sporulating phenotype by comparing wild-type and Spo$^-$ culture strains. With this in mind, we analyzed the envelope composition of YB886 wild-type strain cultures grown in LB and LBN media and the 8E isogenic *spoOA* mutant strain grown in LB medium.

Hydrophobicity index. The hydrophobic character of bacteria plays a central role in their interaction with different surfaces. Changes shown in the envelope composition during hypersaline adaptation may be reflected in the hydrophobic properties of bacterial surface. This measure would be indicative of the wall structure, being in fact the most exposed envelope at the bacterial surface. Figure 2 shows the values of the hydrophobicity index obtained by the partition of the different cultures between water and organic solvent (hexane). The decrease of absorbancy of the aqueous phase was used as a measure of cell surface hydrophobicity. The hydrophobicity index of the wild-type strain was higher when grown in LBN than in LB medium, but of the same order as 8E strain (Spo$^-$) cultured in LB medium. This result would indicate that during growth in hypertonic medium, *B. subtilis* has undergone changes in the composition of its external envelope, which behaves similarly to those of a non-sporulating 8E strain.

Phospholipid and glycolipid composition. Phosphatidylglycerol (PG), phosphatidylethanolamine (PE), cardiolipin (CL), and lysilphosphatidylglycerol (lysilPG) are the major membrane phospholipids present in *B. subtilis* [3, 24]. Phospholipid composition of the different cultures studied in this work are shown in Table 2. There is no significant difference in phospholipid composition

Table 2. Phospholipid composition of *Bacillus subtilis* cultures

Lipid[a]	LB[b] (Ave ± SD)	LBN[b] (Ave ± SD)	LB (8E)[b] (Ave ± SD)
CL	23.99 ± 3.00	46.00 ± 6.30	20.67 ± 3.59
PG	42.29 ± 1.66	31.50 ± 1.41	48.97 ± 8.97
PE	19.66 ± 0.82	17.20 ± 4.92	22.86 ± 4.16
LPG	14.05 ± 2.79	5.29 ± 3.80	19.05 ± 4.85

[a]CL, PG, PE, and LPG stand for cardiolipin, phosphatidylglycerol, phosphatidylethanolamine, and lysylphosphatidylglycerol respectively. [b]YB886 strain was cultured in LB and LBN media for 24 or 48 h respectively. The Spo⁻ mutant strain 8E was cultured in LB. Lipids were extracted as indicated in Materials and Methods. Values represent means of three determinations ± SD.

Table 3. Glycolipid/phospholipid ratio of the *Bacillus subtilis* cultures

Growth condition	(μmol glucose/μmol phosphorus)
LB	0.049 ± 0.02
LBN	0.072 ± 0.02
LB (8E)	0.048 ± 0.03

Values represent means of three determination ± SD.

between wild-type and 8E strains grown in LB medium. Nevertheless, the wild-type strain grown in LBN medium showed a net decrease in PG, and increased levels of CL. The major phospholipid in membranes of *B. subtilis* YB886 strain grown in LB medium is PG (about 40 mole/%), and the CL content is about 24 mole/% of total phospholipids. In LBN medium, CL is the major membrane phospholipid (46 mole/%), and PG falls to about 31 mole/%. A mild decrease in lysilPG was also observed in LBN medium. Similar changes have been pointed out in other laboratories for *Escherichia coli* [18] and *Staphylococcus aureus* [13] cultured in a hypersaline medium. This change in membrane phospholipid composition must be closely related to different membrane functions and/or properties (see Discussion).

Among the membrane lipids, *B. subtilis* also presented a glycolipid, diglucosyl-diglyceride [3, 5, 24] that represented about 2.5% (not shown). The contribution of this glycolipid to the membrane lipid composition of *B. subtilis* cultures was measured as the glucose/total phospholipid ratio. As shown in Table 3, the fraction of glycolipid found in YB886 and 8E strains growing in LB medium was similar. In contrast, the glucose/total phospholipid ratio of YB886 growing in LBN medium was higher and equivalent to an increase of about 46%. A similar increase in the amount of glycolipid was observed in *Saccharomyces cerevisiae* grown in hypertonic NaCl medium [30].

Table 4. Fatty acid composition of membrane lipids of *Bacillus subtilis* cultures

Fatty acid	LB	LBN	LB (8E)
Σ Straight saturated	15.24 ± 2.15	34.06 ± 5.08	18.34 ± 3.43
Σ Branched saturated	80.59 ± 3.88	48.74 ± 10.08	76.26 ± 8.36
Σ Mono-unsaturated	4.84 ± 1.48	14.72 ± 4.94	4.76 ± 1.97
Σ Poly-unsatur.ω6	1.38 ± 0.72	3.52 ± 1.13	0.84 ± 0.46

From YB886 and 8E strains cultured in LB and LBN or LB respectively, fatty acids were determined as indicated in Materials and Methods. The different groups of fatty acids (straight saturated, branched saturated, monounsaturated, polyunsaturated) and determinations were summed and noted. Values represent means of three individual determinations ± SD

Table 5. Ratios among fatty acids of different chains present in themembrane lipid of *Bacillus subtilis* cultures

Ratio	LB	LBN	LB (8E)
iso/anteiso	1.58	0.99	1.26
branched/unsat.	12.76	2.64	13.24
branched/straight	5.29	1.44	4.21
unsat./straight	0.41	0.54	0.31

Fatty acids were determined as indicated in Table 4, and ratio between series calculated and reported in table; iso/anteiso stands for iso-branched chain saturated fatty acids/anteiso-branched chain saturated fatty acids; branched/unsat. stands for branched chain saturated fatty acids/unsaturated fatty acids; branched/straight stands for branched chain saturated fatty acids/straight chain fatty acids; unsat./straight stands for unsaturated/straight chain saturated fatty acids.

Fatty acid composition. In accordance with Kaneda [12], we observed that branched-chain fatty acids were the principal components esterified to membrane phospholipid in *B. subtilis*. In addition, no significant difference in fatty acid composition in membrane lipids was observed between YB886 and 8E strains grown in LB medium. In contrast, wild-type strain grown in hyperosmotic LBN medium showed a significant increase in straight-chain saturated fatty acids and a remarkable decrease in the branched chain saturated ones (Table 4), in particular in the iso-branched fatty acids (Table 5). Monounsaturated fatty acids, whose major component is 18:1 (ω-9) fatty acid, also increased in the membrane lipids of the wild-type strain grown in LBN; 18:1 (ω-9) in particular, rose from 2% to 6.2% of total fatty acids in LBN medium (not shown). The ratios between iso and anteiso-branched chain, straight chain, and monounsaturated fatty acids are shown in Table 5. The different values of the branched/straight chain fatty acids ratio between *B. subtilis* YB886 grown in LB and LBN medium in particular were noteworthy. This result pointed out that fluidity and transport properties of *B. subtilis* membranes might be altered in LBN cultures.

Discussion

The adaptation of *B. subtilis* to grow in hypertonic saline media led to important variations of its envelope. This finding was supported by morphological modifications, such as an extensive filamentation (Fig. 1) and different sensitivity towards antibiotics whose target was at the envelope (cell wall and membrane; Table 1). Concerning the wall structure, it seems unlikely that peptidoglycan composition should vary, since it is generally taken as a taxonomic criterion of species; however, the results of reduced lysozyme sensitivity (Table 1 and protoplast obtention) indicated that an important increase in the thickness of the wall had occurred. In addition, the lower sensitivity of LBN cultures to penicillin G would probably reflect the degree of peptidoglycan cross-linking. Thus, the wall of hypertonic cultures would be thicker but less compact, allowing minor or gradual osmolarity effects inside the cell and increasing the uptake of compatible osmolytes in transit across the wall. In *S. aureus*, another Gram-positive bacterium, results that agree with this conclusion have been reported [34]. Failure to undergo phage lysis presented by *B. subtilis* LBN cultures infected with either the virulent ϕ29 or the temperate ϕ105 phages corroborated that important modifications of the envelope composition had occurred. Since the lytic cycle of these phages takes place in different hypertonic conditions [28, 35] and they do not share common receptors, the most probable assumption is that receptors were masked by the important thickening of the wall.

The nature of the molecules conferring hydrophobicity on the bacterial surface has not yet been identified, but it has been suggested [19, 21] that lipoteichoic acid, together with proteins, are the most important wall components responsible for surface hydrophobicity in bacteria. The hydrophobic index of *B. subtilis* YB886 is higher in LBN than in LB cultures, but of the same order as 8E (SpoOA) grown in LB medium (Figure 2). However, the components involved in the surface property must be different in these last two conditions (wild type in LBN and Spo- in LB) since removal of surface proteins by a trypsin treatment with these cultures did not lead to the same HI value (data not shown). Thus, variations in the external envelope of LBN cultures would rather be related to the osmotic response than to the absence of sporulation.

Concerning the variations in lipid composition, our results are similar to those observed in the Gram-negative *Escherichia coli* [18] or *Staphylococcus aureus* [13] grown in hypertonic media: a fraction of PG is converted to CL plus glycerol (Table 2). It is interesting to note that during protoplast fusion experiments, in which bacteria support high osmotic strength, similar variations have been observed (in agreement with Sánchez-Rivas & Bohin [27]). Hoch [10] argues that the conversion of two PG molecules to CL plus glycerol increases the order on the membrane surface; in addition, the increased CL may be engaged in the regulation of ionic lateral conduction by the plasma membrane, because it is known that the glycerol moiety of the CL molecule takes part in the lateral conduction of protons through H-bonded networks [10]. Moreover, Kanemasa, Yoshioka, and Hayashi [13] have suggested that changes in the concentration of CL might behave like a barrier against the high ionic level, or to contribute to the active transport. Skorko-Glonek et al. [31] have recently shown that the PG content influenced the activity of a membrane protease involved in heat stress. Glycerol has also been described as a compatible osmolyte accumulated during the adaptation of different microorganisms to osmotic stress [1, 4]. In our culture conditions, glycerol, whose cytoplasm concentration was not found to increase (data not shown), might be metabolized in part by glycerokinase to produce glycerol-3-phosphate, whose accumulation has been shown to produce abnormal septation and to inhibit sporulation [22]. It is known that PG serves as biosynthetic precursor in at least three reactions [23]: i) formation of aminoacyl-phosphatidyl-glycerol (lysilPG); ii) synthesis of CL, PE and phosphoglycolipids; iii) transfer of an unacylated sn-glycerol-1-phosphate group to non-lipid substances [14] in the lipoteichoic acid synthesis. The interrelationship between lipoteichoic acid biosynthesis and membrane lipid metabolism is common for several Gram-positive bacteria [17, 33] and was described by Koch, Haas and Fischer [16] in *Staphylococcus aureus*. Glycolipid is another intermediate in the biosynthesis of bacterial envelope lipoteichoic acid. The lipid moiety of lipoteichoic acid is usually a glycolipid or phosphoglycolipid normally present in the membrane [6, 36]. Diglucosyldiglyceride is also present among the *B. subtilis* membrane lipids, and its concentration strongly increases in YB886 strain grown in hyperosmotic medium. Table 3 shows that in LBN medium, there is a remarkable increase in the glucose/total phospholipid ratio. These results, together with the possible production of glycerol-1-phosphate [14] and a phosphoglycolipid intermediary by PG discussed above, plus the increase of hydrophobicity index of the *B. subtilis* grown in hyperosmotic conditions, allow us to think that lipoteichoic acid may be engaged in the response of *B. subtilis* to osmotic stress.

Branched-chain fatty acids are the main components sterified to membrane phospholipid in Bacillus [12]. There is no significant difference in fatty acid composition of membrane lipids between wild-type and 8E (Spo0A) strains grown in LB medium. Nevertheless,

wild-type strain grown in hyperosmotic medium shows a clear increase in straight-chain saturated fatty acids and a remarkable decrease in the branched-chain saturated ones compared with the cultures grown in LB medium (Table 4). In particular, there is a decrease in the iso-branched fatty acids. The different values of the branched/straight chain saturated fatty acids ratio found between *B. subtilis* YB886 grown in LB or LBN medium is remarkable. The lipid composition of several Gram-positive bacteria was found to be influenced by the culture conditions during growth [11]. In the growth media employed, *B. subtilis* biosynthesized a relatively high proportion of monounsaturated fatty acids compared with that described in the literature [8, 12]. In addition, a remarkable increase of monounsaturated fatty acids was observed, in particular in LBN medium (Table 4). Since several reports have pointed out the role of 18:1 (ω-9) fatty acid as an inhibitor of KinA, in turn a regulator of the sporulation process [32], we believe that the increase in monounsaturated fatty acids [in particular, 18:1 (ω-9) increases 3-fold in LBN medium] is more related to the inhibition of sporulation than to changes in membrane fluidity.

Nevertheless, during osmotic stress, the decrease of the branched/straight chain saturated fatty acids would indicate a decrease of the fluidity of membrane lipids partially balanced by the increase of monounsaturated fatty acids. Sharma et al. [30] have observed that fluidity of *Saccharomyces cerevisiae* membrane lipids is increased in hypersaline culture conditions. The fluorescence anisotropy technique could give us more clear information about the actual change in membrane lipid fluidity during hyperosmotic growth and will be the aim of our ongoing investigation.

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