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Biochemical and biophysical studies of *Bacillus subtilis* envelopes under hyperosmotic stress

C.S. López[a,c], H. Heras[b], H. Garda[b], S. Ruzal[c], C. Sánchez-Rivas[c], E. Rivas[a,*]

[a]*Instituto de Biología Celular, Facultad de Medicina, Paraguay 2155, 1121 Buenos Aires, Argentina*
[b]*INIBIOLP, Instituto de Investigaciones Bioquímicas de La Plata, UNLP, La Plata, Argentina*
[c]*Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, UBA, Buenos Aires, Argentina*

Abstract

The behaviour and state of the envelopes from *B. subtilis* cultures grown in Luria Bertani (LB) medium with and without 1.5 M NaCl are compared. Under hypertonic conditions, the hydrophobicity of the cultures increases. The phospholipid and fatty acid (FA) compositions show important differences: a higher cardiolipin (CL) content [at the expense of phosphatidylglycerol (PG)], and a higher unsaturated and straight chain FA content. The fluidity of the membranes, determined with fluorescent probes, indicates an increase in viscosity of the cytoplasmic membrane. The consequences of these variations in membrane permeability and osmotolerance are discussed. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Bacillus subtilis; Stress; Membranes; Phospholipids; Fatty acids

1. Introduction

The osmotic response of bacteria is of current and increasing interest due to the important aspects of its applications. Most of the studies on prokaryotes have centred on genetic regulation and physiological aspects of their adaptation to salt (Csonka and Hanson, 1991). The behaviour of the envelopes under different osmotic conditions has practically been unexplored. Nevertheless, several lines of evidence suggest that cell envelope composition also plays an important role (Kanemasa et al., 1972; Sharma et al., 1996). *Bacillus subtilis* is a Gram-positive, sporulating bacteria able to adapt to large variations of osmotic strength. This bacterium develops different strategies to grow in hypersaline media, among which is the uptake and accumulation of compatible solutes (Kempf and Bremer, 1998), and also important modifications of its envelope, essentially composed of a membrane and a thick wall. These hypertonic cultures resist lysis by the virulent ϕ29 or the temperate ϕ105 phages, and show different sensitivity to antibiotics or enzymes acting at the wall or membrane level, such as phosphonomycin, lysozyme, penicillin G, or polymyxin B (López et al., 1998). Reduced lysozyme and penicillin sensitivities indicate that the walls of hypertonic cultures are thicker but less compact than

*Corresponding author. Tel.: + 54-11-4508-3675, extn. 21; fax: + 54-11-4962-5457.
E-mail address: erivas@fmed.uba.ar (E. Rivas)

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those of isotonic cultures, a property that allows increasing uptake of compatible osmolites inside the cell. In addition, under high osmotic strength, *B. subtilis* cultures develop extensive filamentation with asymmetric septa, and fail to sporulate, behaviour attributed to the different membrane composition (Ruzal and Sanchez-Rivas, 1994; Ruzal et al., 1998)

In order to pursue the characterisation of the envelope modifications that take place during growth in hyperosmotic media, biochemical and biophysical parameters of the structure and composition of the membrane and wall were analysed.

2. Materials and methods

2.1. Microorganism

Bacillus subtilis strain YB886 (*metB5*, *trpC2*, *xin-1*, SPβ^s) was grown with vigorous agitation at 37°C in rich Luria Bertani (LB) medium (normal conditions) or hyperosmotic LBN medium by adding 1.5 M NaCl to the basal LB.

2.2. Hydrophobicity determination

Hydrophobicity was determined by partition of a bacterial suspension between 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 litre of distilled water, pH 7.1) and hexane, introducing a small modification to the rapid and simple assay method described by Op den Camp (1985). The results are expressed as the proportion of cells excluded from the aqueous phase.

2.3. Lipid extraction

Lipids were extracted from cellular pellets according to Rivas and Luzzati (1969) with some modifications as described by Kent et al. (1973)

2.4. Lipid analysis

Lipid classes were analysed by TLC, in chloroform/methanol/acetic acid (65:25:6, v/v/v). Phospholipids and glycolipids were detected with iodine vapour and identified by comparison with authentic purified standards and by treatment with specific coloration reagents. Zones of the plates corresponding to each lipid fraction were scraped off and quantified. Lipid phosphorous was determined according to Bartlett (1959), and total carbohydrates were quantified as indicated by Hanson and Phillips (1981).

2.5. Fatty acid analysis

Total lipid extract was hydrolysed and methylated as described previously (López et al., 1998). Fatty acid methyl esters were analysed by gas chromatography using a Shimadzu RC8 with an Omegawax 250 column (30 m $\times$ 0.25 mm, 0.25 μm film) (Supelco).

2.6. Steady state fluorescence measurements with DPH and TMA-DPH; emission and excitation spectra of Laurdan

1,6-Diphenyl-1,3,5-hexatriene (DPH) and 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene *p*-toluenesulfonate (TMA-DPH) were obtained from Sigma, and stock solutions were prepared in tetrahydrofuran. Multilamellar lipid vesicles were prepared by mixing the appropriate amounts of total lipids in chloroform–methanol (2:1, v/v) with the probes. Samples were dried under nitrogen, resuspended in phosphate buffer 20 mM pH 7, and vortexed vigorously. The final probe/phosphorous ratio for DPH and TMA-DPH was 1/200 and 1/800, respectively. In all experiments performed, the sample absorbency never exceed an OD of 0.1 at 360 nm. Excitation and emission wavelengths for both probes were measured at 355 and 425 nm, respectively, using a SLM 4800 spectrofluorometer equipped with a standard polarisation accessory. Experimental data were corrected for the G factor and for the intrinsic fluorescence of unlabelled multilamellar vesicles, which never exceeded 1% of the labelled samples.

6-Dodecanoyl-2-dimethylaminonaphthalene (Laurdan) was purchased from Molecular Probes. A stock solution was prepared in methanol. The final Laurdan probe/lipid phosphorous ratio was 1/400. The emission spectrum in lipid vesicles was obtained using a fixed excitation wavelength of 340 nm, and the excitation spectrum at a fixed emission wavelength of 440 nm, using the same equipment. All spectra were corrected for background by subtracting the signal of unlabelled samples.

3. Results and discussion

The hydrophobic character of bacteria plays a central role in their interaction with different cell surfaces, and its measurement is indicative of the structure and properties of the most exposed envelope of these bacteria. When cells from both cultures were submitted to the partition test described in Materials and methods, the proportion of cells excluded from the aqueous phase was 47.8±8.8% for the LBN hypertonic cultures compared with 30.9±3.9% observed for the LB control. This increase in hydrophobicity should enable this strain to endure the stress imposed by the hypertonicity of the medium. It is also indicative that, in LBN medium, *B. subtilis* have undergone important changes in the composition of their external envelopes. The nature of the molecules conferring hydrophobicity to the bacterial surface has not yet been identified. Nevertheless, it has been suggested (Miörner et al., 1983; Ofek et al., 1983) that, in Gram-positive bacteria, lipoteichoic acid, together with proteins, constitutes the most important wall component responsible for surface hydrophobicity. Diglucosyl-diglyceride is a glycolipid that represents about 2.5% of the total membrane lipids in *B. subtilis* (Bishop et al., 1967; Op den Kamp et al., 1969). It is an intermediate in the biosynthesis of bacterial envelope lipoteichoic acids. The lipid moiety of lipoteichoic acids is usually a glycolipid or phosphoglycolipid, generally anchored in the membrane (Button and Hemmings, 1976). The interrelation between lipoteichoic acid biosynthesis and membrane lipid metabolism is common to several Gram-positive bacteria and was described by Koch et al. (1984) in *S. aureus*. The presence of diglucosyl-diglyceride was measured as glucose/total phospholipid ratio. In hyperosmotic LBN medium, this ratio increased to 40%. Sharma et al. (1996) observed a similar increase in the amount of glycolipid in *S. cerevisiae* growing in a hypertonic NaCl medium. This result, together with the increase of the hydrophobicity index, allows us to postulate that lipoteichoic acid would play an important role in the adaptation of *B. subtilis* cells to high ionic strength

In accordance with Bishop et al. (1967) and Op den Kamp et al. (1969) we found that the major phospholipids present in the membrane of *B. subtilis* YB886 are phosphatidylglycerol (PG), phosphatidylethanolamine (PE), cardiolipin (CL) and lysilphosphatidylglycerol (lysilPG). In normal LB growth, the PG content is about 40%, while the CL content is about 24% of total phospholipids. In contrast, in hypertonic LBN medium, CL is the major phospholipid (46%), and PG decreases to about 31% (Fig. 1). Similar changes have been reported in other bacteria such as *E. coli* (Lusk and Kennedy, 1972) and *S. aureus* (Kanemasa et al., 1972) cultured in hypersaline medium, or *M. loti* growing at low pH (Correa et al., 1999). These variations in lipid composition could be either the result of adaptation of *B. subtilis* to hyperosmotic growth or a more general modification of the membrane in response to different stresses. Hoch (1992) argues that the conversion of two PG molecules to CL plus glycerol increases the order on the membrane surface, and that CL may be engaged in the regulation of ionic lateral conduction by the plasma membrane, because it is well known that the glycerol moiety of the CL molecule takes part in the lateral conduction of protons through H-bonded networks. The increase in CL concentration might behave as a barrier against the high ionic level.

Branched-chain fatty acids (FA) are the principal components esterified to membrane lipids in *Bacillus* (Kaneda, 1977). Cultures grown in hyperosmotic LBN medium show a significant increase in straight-chain saturated FA, and a remarkable decrease in branched-chain saturated FA, in particular the iso-branched FA. Table 1 shows the ratios between iso- and anteiso-branched chain, straight-chain and mono-unsaturated FA in lipid membranes in both

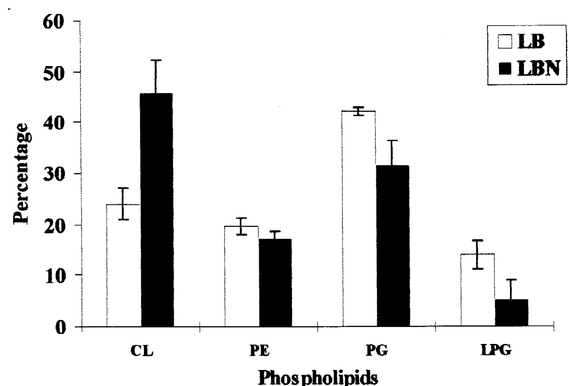

Fig. 1. Phospholipid composition. Values represent the mean of three determinations ±S.D.

Table 1
Fatty acid composition[a]

	LB	LBN
Fatty acid		
Straight saturated	15.2±2.2	34.1±5.1
Branched saturated	80.6±3.9	48.7±10.1
Mono-unsaturated	4.8±1.5	14.7±4.9
Poly-unsaturated ω-6	1.4±0.7	3.5±1.1
Ratio		
Iso/anteiso	1.58	0.99
Branched/unsaturated	12.76	2.64
Branched/straight	5.29	1.44
Unsaturated/straight	0.41	0.54

[a] Values represent the mean of three determinations ±S.D. Iso, iso-branched-chain saturated FA; anteiso, anteiso-branched-chain saturated FA; unsaturated, unsaturated FA; straight, straight-chain saturated FA.

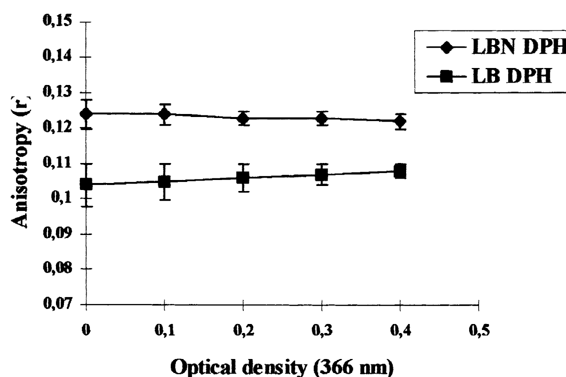

Fig. 2. DPH anisotropy versus optical density of the multilamellar suspensions. Values represent the mean of four determinations ±S.D.

growth conditions. A surprising result is the significant increase of unsaturated FA, which represents at best 6% and reaches 18% during LBN growth. Its importance can be directly related to the hyperosmotic tolerance response, as shown in photosynthetic *Synechocystis* bacteria (Allakhverdiev et al., 1999). In addition, among the mono-unsaturated FA, the major component is 18:1 (ω-9) and its contribution increases from 2% of total fatty acids to 6.2% in LBN medium. Several reports have pointed out its role as a KinA inhibitor, an important regulator of the phosphorylation pathway switching on the sporulation process (Strauch et al., 1992). We believe that its increase should contribute to the inhibition of the sporulation process (Ruzal et al., 1998). Furthermore, the modifications in lipid composition observed here suggest that variations of membrane fluidity and transport properties have occurred.

For the purpose of monitoring the lipid fluidity at different zones of the membrane bilayer of these cultures, the steady state fluorescence anisotropy (r_s) was determined with DPH and TMA-DPH fluorescent probes. A gradual decrease in the r_s of DPH between 10 and 60°C was observed for vesicles prepared with lipids extracted from both cultures. There were no significant differences in r_s values within the whole temperature range under normal and hyperosmotic conditions (not shown).

Nevertheless, when the fluorescence anisotropy of DPH in multilamellar suspensions was plotted against the optical density (Fig. 2) and corrected according to light scattering, the measurements showed significant differences in anisotropy for data extrapolation at OD = 0. These data were plotted in a form consistent with Teale's basic finding (Lentz et al., 1979) that fractional error introduced into the anisotropy by light scattering should be proportional to the optical density of the turbid solution. The steady state anisotropy experiments indicate that, during the osmoadaptation process, *B. subtilis* has a tendency to decrease the fluidity of lipid paraffinic chains in order to retain intracellular osmocompatible compounds. The fluorescence anisotropy of TMA-DPH also decreases gradually with temperature and was higher than that of DPH at all temperatures studied, indicating that the cationic probe is located in a more rigid environment (not shown).

Laurdan is a more sensitive probe having the advantage of displaying spectral sensitivity to the phospholipid phase state (Parasassi et al., 1994). Fig. 3 shows the fluorescence excitation and emission spectra at 37°C of vesicles with Laurdan. The plot shows a blue shift in the emission spectra of vesicles prepared with cell lipids grown in LBN medium. This indicates that, during the process of osmoadaptation, an increase in lipid membrane microviscosity has occurred in the glycerol backbone of the phospholipids.

During the adaptation of *B. subtilis* to hyperosmolarity the envelopes undergo significant biochemical and structural changes. The increase in membrane lipid microviscosity may well indicate its role as a permeability barrier that prevents the entrance of NaCl and the escape of compatible osmolites. How-

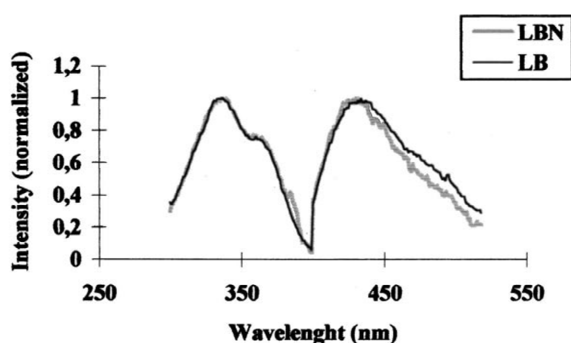

Fig. 3. Emission and excitation spectra of Laurdan. Spectra were obtained as described in Materials and methods.

ever, it would be important to identify variations due to general stress responses from those specific for osmotolerance. It is well known that several proteins, not particularly involved in osmotic stress, are also induced and some prepare the bacteria for supporting additional hits. In this sense, it should be asked if some of the envelope variations observed are involved in a more general 'pre-adaptive' state of the cells.

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