Biochimie 123 (2016) 103–109

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Effects of elevated growth temperature and heat shock on the lipid composition of the inner and outer membranes of *Yersinia pseudotuberculosis*

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ARTICLE INFO

Article history:
Received 10 December 2015
Accepted 2 February 2016
Available online 4 February 2016

Keywords:
Phospholipids
Lysophosphatidylethanolamine
Phosphatidylglycerol
Fatty acids
Membranes
Gram-negative bacteria

ABSTRACT

Differences in the distribution of individual phospholipids between the inner (IM) and outer membranes (OM) of gram-negative bacteria have been detected in mesophilic *Escherichia*, *Erwinia* and *Salmonella* species but have never been investigated in the psychrotrophic *Yersinia* genus. Therefore, the influence of an elevated growth temperature and heat shock on the phospholipid and fatty acid (FA) compositions of the fractionated *Yersinia pseudotuberculosis* envelope was investigated. The shift of the growth temperature from 8 °C to 37 °C to mimic the switch from saprophytic to parasitic growth of this bacteria and the exposure of the cells to heat shock, which was induced by a sharp increase in the temperature from 8 °C to 45 °C, increased the lysophosphatidylethanolamine content from zero and 1% to 6% and 10% in the IM and OM, respectively. These changes were accompanied by a decrease in the phosphatidylethanolamine (PE) content and a drastic increase (up to 3-fold higher) in the phosphatidylglycerol (PG) level in the OM of the bacteria, which increases the net negative charge of the cell envelope. The levels of the predominant saturated palmitic (16:0) and cyclopropane FAs were approximately 1.5- and 7.5-fold higher, respectively, but the content of the predominant unsaturated palmitoleic (16:1n-7) and *cis*-vaccenic (18:1n-7) FAs was approximately 10–30-fold lower in both membranes that were isolated from the cells grown at elevated temperatures. Due to these changes, reflecting the process of "homeoviscous adaptation", the ratio between the unsaturated and saturated FAs decreased but remained higher in the IM than that in the OM. Simultaneously, no significant changes were observed in the FA composition of cells subjected to heat shock, demonstrating a difference between the responses of the heat-shocked and heat-adapted *Y. pseudotuberculosis*. The unique ability of *Y. pseudotuberculosis* to reciprocally regulate the ratio of anionic PG and net neutral PE and therefore adjust the negative charge of the OM may be a common strategy used by pathogenic bacteria to promote the barrier function of the OM.

1. Introduction

The lipid matrix of bacterial membranes is the primary target for different environmental factors, which trigger adaptation mechanisms that result in changes in the polar head groups and fatty acid (FA) components of the phospholipids. In particular, alterations in the environmental temperature affect membrane fluidity by regulating the ratio of saturated to unsaturated fatty acids through a bacterial response called "homeoviscous adaptation" [1]. However, the effect of abiotic factors on the phospholipid and fatty acid compositions of bacterial species inhabiting different ecological niches has not been investigated systematically [2].

The cell envelope of gram-negative bacteria consists of inner (IM) and outer membranes (OM) separated by a periplasmic space.

Abbreviations: DPG, diphosphatidylglycerol; FA, fatty acids; IM, inner membranes; LPE, lysophosphatidylethanolamine; OM, outer membranes; PAGE, polyacrylamide gel electrophoresis; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PldA, outer-membrane phospholipase A_1; SFA, saturated fatty acids; UFA, unsaturated fatty acids.

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http://dx.doi.org/10.1016/j.biochi.2016.02.004
0300-9084/

Although data are available on the arrangement of individual phospholipids in the envelope of the mesophilic gram-negative bacteria [3–8], no such data have been reported for psychrotrophic bacteria, including the enteropathogen *Yersinia pseudotuberculosis*, which is characterized by high ecological plasticity [9]. Despite the important role of membrane lipids in the function and adaptation of bacteria, the available information about the lipid distribution between the IM and OM is mostly limited to the model organism *Escherichia coli*.

However, recent lipidomic approaches have surprisingly demonstrated that there is little overlap between the *Escherichia* model lipid profiles and those of specialized pathogens, emphasizing the need for constructing organism-specific lipidomic databases [10,11]. Moreover, the differences in both the protein and lipid compositions of the IM and OM that were isolated from different gram-negative bacteria were documented [11,12]. Therefore, data on the distribution of phospholipids between the IM and OM are required to properly interpret the conformational and functional changes of the membrane proteins as the bacteria adapt to the various growth conditions. To our knowledge, there are no reports on the effect of either high growth temperatures or heat shock on the membrane phospholipid and fatty acid compositions in the *Yersinia* genus. Heat stress can be classified either as a heat adaptation, which assumes that a cell is exposed to a temperature above its optimal growth for long periods, or heat shock, which assumes that a cell is exposed to temperatures above its normal growth maximum for a very short period. Therefore, to understand the response of *Y. pseudotuberculosis* to both types of heat stress, the changes in the membrane lipid and fatty acid compositions in the heat-adapted and heat-shocked cells were thoroughly investigated by comparing the cells that were either growing at temperatures that correspond to the saprophytic and parasitic phases of the life (at 8 °C and 37 °C, respectively) or subjected to heat shock by a sharp increase in the temperature from 8 °C to 45 °C. Quantitative differences in the phospholipid composition of the IM and OM of the heat-adapted and heat-shocked psychrotrophic *Y. pseudotuberculosis* cells were observed, which reciprocally (PE and PG) or differently (LPE) change their phospholipid compositions within the envelope compared with mesophilic *E. coli*.

2. Materials and methods

2.1. Strain and growth conditions

Strain 488 of *Y. pseudotuberculosis* was cultivated in Lysogeny Broth (LB) (Becton, Dickinson and Company). The bacterial cells were grown to log phase ($OD_{600} = 1$) at 8 °C and 37 °C under aerobic conditions with shaking at 180 rpm. One part of bacterial cells grown at 8 °C were heat-shocked at 45 °C for 30 min by transferring flask to a water bath shaker (RSB - 12, Remi Elektrotechnik Limited). The bacteria were separated from culture medium by centrifugation at 1200 g for 20 min and then washed twice with physiological saline (0.85% NaCl).

2.2. Preparation of spheroplasts and total membranes and isolation of the inner and outer membranes

The procedure for separating the IM and OM was based on equilibrium sucrose density gradient centrifugation of the total membrane fraction, which was obtained by lysing the spheroplasts prepared by lysozyme-EDTA treatment, in the accordance with Osborn's method [4] that was modified by Park et al. [13]. This method allows reproducible separation of the IM and OM fractions of considerably high purity. The cells were resuspended in ice-cold 10 mM Tris–HCl, pH 7.8, containing 0.75 M sucrose (1 g of cells *per* 30 ml of solution). Lysozyme was added to a final concentration of 100 μg/ml, and the cells were then incubated on ice for 2 min. The suspension was slowly diluted with 2 vol of 1.5 mM EDTA-Na_2, pH 7.5, on ice. The spheroplasts were lysed by sonication for 4 times for 30 s each, with a 1 min interval *per* 25 ml of suspension. The unbroken cells and membrane aggregates were removed by two centrifugations at 1200*g* for 20 min. The supernatant was centrifuged at 200,000*g* and 10 °C for 60 min in the TLA 110 rotor (Optima MAX-XP, Beckman Coulter). The membrane pellet from 1 g of cells was diluted with 9 ml of an ice-cold solution containing 0.25 mM sucrose, 3.3 mM Tris–HCl and 1 mM EDTA, pH 7.8, and centrifuged at 200,000 g for 90 min.

The washed membrane pellet was dissolved in 2 ml of ice-cold 25% sucrose (w/w) in 5 mM EDTA, pH 7.5. The pellet was thoroughly homogenizated by vigorous vortexing, and sample of 0.8 ml of the crude membrane fraction was layered on the top of 3.2 ml of a 35–50% linear sucrose gradient (w/w) containing 5 mM EDTA, pH 7.5. After centrifugation at 310,000*g* for 6.5 h at 4 °C in the SW 60 Ti Rotor (Optima L-90 K, Beckman coulter), membrane fractions of 0.1–0.15 ml were collected from the bottom of tubes.

The presence of proteins in the membrane fractions was established spectrophotometrically (UV-2550, Shimadzu) at 280 nm. The OM membrane fractions were identified by the presence of OM-specific markers – OmpF-like porin and outer-membrane phospholipase A_1 (PldA), whereas the IM membrane fractions were identified by their corresponding buoyant densities and the complete absence of PldA.

Polyacrylamide gel electrophoresis (PAGE) or SDS-PAGE was performed according to the method of Laemmli [14] using a 12% acrylamide gel. A set of colored proteins (Thermo Scientific, Lithuania) with molecular weights of 10, 15, 25, 35, 40, 55, 70, 100, 130 and 170 kDa was used. The separated proteins were stained with Coomassie R-250 in 10% acetic acid and 30% methanol. Pure OmpF-like porin, which was isolated from *Y. pseudotuberculosis* (Laboratory of Molecular Basis of Antibacterial Immunity, G.B. Elyakov Pacific Institute of Bioorganic Chemistry, FEB RAS), in 50% sucrose was utilized to identify membrane fractions that were analyzed by SDS-PAGE. The localization of PldA in the membrane fractions corresponding to peaks I, II and III was assessed by western blotting. After SDS-PAGE, the proteins were transferred from the non-stained gel to a nitrocellulose membrane (0.2 μm, Millipore, USA) using semi-dry transfer equipment at a current of 0.8 mA/cm^2 overnight at 4 °C, according to a standard procedure [15]. Immunodetection was performed by the protein detection system SNAP i.d., according to the manufacturer's instructions (Millipore, USA). To prepare a murine serum against PldA, adult BALB/c mice were immunized thrice by subcutaneous injection of recombinant PldA of *Y. pseudotuberculosis* [16], emulsified with complete Freund's adjuvant (Sigma-Aldrich). It was applied a dose of 10 μg of PldA per mouse at an interval of seven days. The serum was obtained 30 days after the first immunization.

HRP-Goat Anti-Mouse antibodies (Invitrogen, USA) were used according to the manufacturer's instructions. The antigen-antibody complexes were detected on the nitrocellulose membrane using hydrogen peroxide detection with 3,3′-diaminobenzidine for 20 min at room temperature.

2.3. Phospholipid analysis of the inner and outer membranes and whole cells of Y. pseudotuberculosis

The total lipids were extracted from the membrane fractions or whole cells of *Y. pseudotuberculosis* using the method of Folch et al. [17]. The combined sucrose gradient fractions containing the OM or IM were diluted 5-fold with water to more completely extract the lipids. The phospholipids were separated by thin-layer