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In vivo study of the state of order of the membranes of Gram-negative bacteria by Fourier-transform infrared spectroscopy (FT-IR)

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Temperature-induced order/disorder transition profiles were obtained from the membranes of intact Gram-negative bacterial cells by FT-IR analysis of the frequency shifts of the acyl chain methylene symmetric stretching band as a monitor. Cells grown at different temperatures yielded distinct transition profiles. At the individual growth temperatures, however, the nearly alike frequency values indicated a very similar 'state of order' of the bacterial membranes. The FT-IR data were complemented by GC analysis of whole cell fatty acid composition. The FT-IR data obtained in vivo gave direct evidence of the adaptation of the 'state of order' and 'fluidity' of bacterial membranes to varying growth temperatures.

Fourier transform infrared spectroscopy (FT-IR); Gas chromatography; Cytoplasmic membrane; Outer membrane; Bacteria; In vivo spectroscopy

1. INTRODUCTION

In contrast to Gram-positive procaryotes, Gram-negative bacteria possess — besides to the cytoplasmic membrane — a second, asymmetric membrane barrier, the so-called 'outer membrane' (OM). The outer leaflet of this asymmetric membrane is generally composed of only one type of amphiphatic molecules, the lipopolysaccharides (LPS) [1-4]. The LPS are negatively charged amphiphiles which play an essential role in the stabilization of the asymmetric configuration of this particular membrane [2–4,7–11]. Apart from acting as an effective permeation barrier against hydrophilic, high-molecular solutes, the OM also excludes a variety of important low-molecular hydrophobic drugs [3–6]. In contrast to the cytoplasmic membrane and related phospholipid model membranes, little is known about how the OM and reconstituted LPS-membranes are organized at the molecular level and in which way the outer membrane architecture is influenced by genetic, microbiological, and chemical variations [1–11]. Certain types of 'deep-rough' LPS-mutants, as an example, (the LPS of which are devoid of O-specific side chains and which possess a highly defective oligosaccharide core-structure [1,3]), express a remarkably higher permeability towards hydrophobic molecules and fuse more readily with externally added phospholipid vesicles than do wild-type strains [3–6,12–14]. Due to the inherent complexity of Gram-negative organisms, reports on in vivo experiments testing the 'state of order' and/or 'fluidity' of either of the two membranes directly are rare [15,16]. Here, we present an FT-IR spectroscopic technique for testing membrane related order/disorder phenomena of viable Gram-negative cells which does not require any probe molecules (spin lables, deuterated fatty acids, etc.).

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2. EXPERIMENTAL

Cells were streaked onto solid agar plates using a four-quadrant streak pattern and were grown at 37, 22 and 15°C for 24, 48 and 72 h, respectively. Solid agar plates were prepared from solutions of 50 g peptone from meat (Merck 7214), 70 g lab-lemco powder (Oxoid L29), 75 g agar (Merck), 15 g NaCl and 15 g $Na_2HPO_4 \cdot 2H_2O$ dissolved in 5 l water. The solutions were autoclaved at 120°C, filtered and adjusted to pH 7.3. Cells were harvested with platinum loops from areas of confluently growing colonies in the third quadrant of the agar surface; they were suspended in 1% NaCl/D_2O solutions and pelleted at 4°C by centrifugation. For FT-IR measurements, the pellets were prepared as gel films between CaF_2-windows (equipped with 25 μM Teflonspacers) and were sealed in a gas-tight, temperature controlled IR-cuvette system which has been designed in our laboratory. Temperature was controlled by a temperature bath purchased from Haake, Germany. For temperature profile measurements, the temperature was linearly increased (0.2°C·min^{-1}) while effectively one averaged and ratioed FT-IR spectrum was collected and stored per 1°C temperature increase. FT-IR measurements and data evaluations were performed as already described [11,17]. GC-analysis of whole cell fatty acid composition was performed on an HP 5890A gas chromatograph (Hewlett Packard). Cell harvesting, saponification, methylation, extraction procedures, instrumental parameters and data evaluation were similar as described elsewhere [18–20].

3. RESULTS AND DISCUSSION

3.1. *The spectroscopic parameter used for probing order/disorder transitions*

A mid-infrared spectrum of living bacterial cells suspended in D_2O is shown in Fig. 1. IR-features are com-

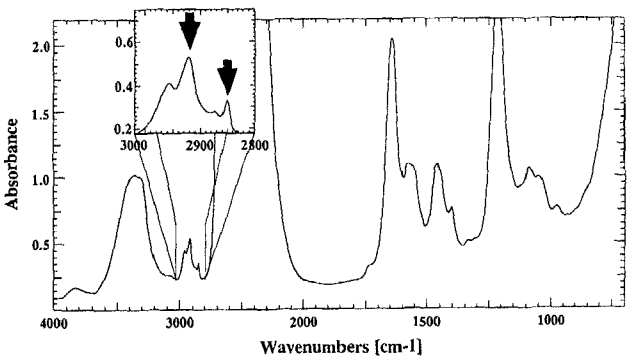

Fig. 1. Survey FT-IR spectrum of intact bacterial cells suspended in 1% NaCl/D_2O solution. The arrows of the inset point to the $>CH_2$ asymmetric and symmetric stretching bands, respectively. The $>CH_2$ symmetric stretching mode, in particular, is a valuable probe for testing order/disorder transition phenomena.

plex because all constituents of the cells (lipids, proteins, carbohydrates and nucleic acids) are contributing to the spectral contour observed. Symmetric and asymmetric stretching vibrations of acyl chain methylene ($>CH_2$) segments of the lipids are observed in a narrow spectral range between 3000 and 2800 cm^{-1} (see inset to Fig. 1). The symmetric $>CH_2$ stretching band near 2852 cm^{-1} is particularly valuable as a monitor for probing the 'state or order' of biological membranes, since its frequency and band-width parameters respond sensitively to order/disorder transitions and to changes of translational and rotational mobility of the fatty acid chains, respectively [21–26], and since contributions from other vibrational modes are minimal. Fig. 2 displays the frequency of this absorption band as a function of temperature. Three traces are plotted from measurements of repetitively cultured samples demonstrating the reproducibility level of the technique. Increasing frequency values imply introduction of disorder (i.e. gauche rotamers) to lipid acyl chains [21]. As expected, complex membraneous mixtures of lipids and proteins

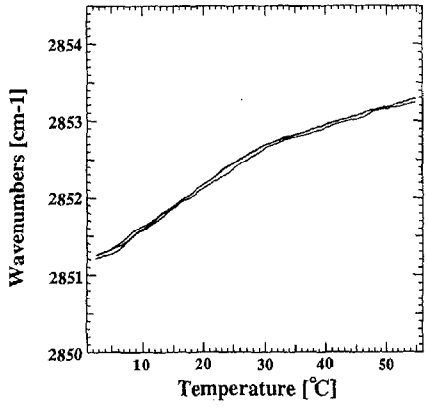

Fig. 2. Reproducibility test: frequency/temperature profiles of $>CH_2$ symmetric stretching band of bacterial cell/suspensions in D_2O. The curves 1 through 3 depict the melting profiles as detected for three different samples of repetetively cultured cells of wild-type *Salmonella minnesota*, SF 1114S.

do not reveal any pronounced transition features typical for pure model membrane systems. The profiles range from frequency values near 2851,2 at 2°C to around 2853,4 at 54°C. Two different slopes with a breakpoint near 30°C are evident. Although only small remnants of cooperative acyl chain melting are observed, the temperature induced increase in disorder between 4 and 54°C is considerable and corresponds to an approximate frequency increase of two wavenumbers. Protein-free bovine brain phosphatidylserine bilayer vesicles, as an example, exhibit frequency shifts of three wavenumbers within the same temperature range [26], while DMPC and DPPC bilayer vesicles yield a shift of approximately four and five wavenumbers, respectively, within the temperature range of gel to liquid crystalline phase transition [21].

3.2. *The growth temperature deeply influences the membrane melting profiles of bacterial cells*

It is well known that culture conditions may alter phospholipid and fatty acid composition of bacterial cell membranes [27–31]. Fig. 3A displays the frequency/temperature profiles of $>CH_2$ symmetric stretching of 'wild-type' *Salmonella minnesota*, strain SF 1114S grown at 37, 22 and 15°C, respectively. The three profiles are markedly different: The lower the temperature of growth, the higher the frequency values at any given temperature. Apparently, acyl chain order/disorder transitions of natural membranes strongly depend on the growing conditions (e.g. temperature of growth). There is another striking result: the characteristic frequency values detected at the respective growth temperatures are confined to a very narrow interval of only 0.15 cm^{-1} (2852.80 and 2852.95 cm^{-1}, see Fig. 3A). From the latter finding we conclude that acyl chain order/disorder is kept constant by adaptation of membrane structure and fluidity to the individual temperatures of growth. This is most probably predominantly accomplished by active regulation of acyl chain composition of the membrane lipids, which trigger the 'state of order' and the 'fluidity' necessary for cell-growth, cell-division and activity of membrane proteins and enzymes.

The GC-analysis of whole cell fatty acid composition provides a first interpretation of the different melting profiles. Fig. 3B shows the relative peak areas in percentage of total peak area of the 26 peaks detected in the three different GC-traces from preparations of cells grown at 15, 22 and 37°C, respectively. Obviously, cell growth induces the synthesis of increased amounts of unsaturated fatty acid chains (i.e. C16:1 and C18:1), decreased amounts of fatty acid chains containing cyclopropane rings (i.e. C17:0 cyclo and C19:0 cyclo) and diminished amounts of saturated fatty acids (i.e. C16:0 C17:0 and C18:0). In a first approximation, the amounts of C12:0, C14:0 and C14:0 (β-hydroxy)acyl chains (mainly present in LPS of the outer membrane)

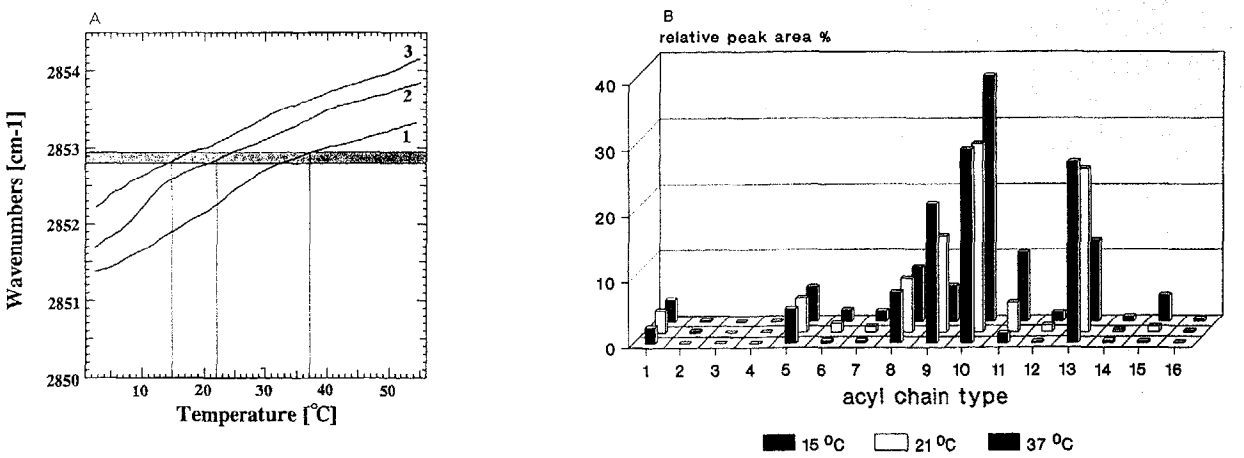

Fig. 3. FT-IR melting profiles and GC-analysis of whole cell fatty acid composition of preparations from intact cells of wild-type *Salmonella minnesota*, strain SF 1114S grown at different temperatures. (A) Frequency/temperature profiles of the $>CH_2$ symmetric stretching band as detected for cells grown at 37 (1), 22 (2) and 15°C (3), respectively. The horizontal lines define the frequency interval of $>CH_2$ symmetric stretching band maximum detected for the three different preparations at 37, 22 and 15°C, respectively, i.e. for the various different growth temperatures (see vertical lines). (B) Gas-chromatographic analysis of whole cell fatty acid composition as measured from cells grown at 37, 22 and 15°C, respectively. Black bars, relative fatty acid composition of cells grown at 15°C; open bars, relative fatty acid composition of cells grown at 22°C; hatched bars, relative fatty acid composition of cells grown 37°C. The total area of the 26 detectable GC-peaks of the chromatogram obtained from each cell preparation was set to 100%. The individual GC-peaks were then calculated in relative percent of the total area. Only the 16 most prominent GC peaks are shown.
(1) C12:0; (2) C13:0; (3) C12:0 (α OH); (4) C12:0 (β OH); (5) C14:0; (6) C15;0; (7) C14:0 (α OH); (8) C14:0 (β OH); (9) C16:1; (10) C16:0; (11) C17:0 cyclo; (12) C17:0; (13) C18:1; (14) C18:0; (15) C19:0 cyclo; (16) C19:0.

are constant. Apparently, the 'fluidity' and 'state of order' of the cytoplasmic membrane is very effectively adapted to the growing conditions by the regulation of acyl chain composition of the bacteria lipids, while the β- hydroxy-acyl chains of the LPS lipid anchor, are only slightly influenced by the growth temperature [32–35]. The herein described FT-IR technique provides, for the first time, a direct physical insight into the state of order of the membranes of intact and viable cells without using probe molecules or deuterated fatty acids. The well-defined $>CH_2$ symmetric stretching band is a valuable monitor for testing order/disorder phenomena of the membranes of intact Gram-negative cells, which either express different LPS-types [36] or have been grown under varying conditions (temperature, time, medium), and which have been chemically or physically treated, e.g. by divalent cations, polycationic peptides or by heatshock procedures.

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