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A porin-type protein is the main constituent of the cell envelope of the ancestral eubacterium *Thermotoga maritima*

Reinhard Rachel[1], Alfred M. Engel, Robert Huber°, Karl-O. Stetter° and Wolfgang Baumeister

Max-Planck-Institut für Biochemie, D-8033 Martinsried b. München and °*Universität Regensburg, Institut für Biochemie, Genetik und Mikrobiologie, D-8400 Regensburg, FRG*

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Thermotoga maritima is an extremely thermophilic eubacterium and represents the deepest known branch and the most slowly evolving line within this kingdom. This bacterium possesses a sheath-like envelope which forms characteristic balloons (the 'toga') around the cell poles. We have shown by electron microscopy in conjunction with image processing and by biochemical analyses that this sheath is essentially an outer membrane. Its predominant 42 kDa protein, which is regularly arrayed in vivo, has all the characteristics of the trimeric porins known from the phylogenetically very distant proteobacteria. This was an unexpected finding, which has some interesting evolutionary implications.

Porin; Bacterial outer membrane; Evolution; Electron microscopy; Two-dimensional protein crystal; (*Thermotoga maritima*)

1. INTRODUCTION

Thermotoga maritima [1], and likewise *Thermotoga thermarum* [2], *Thermotoga neapolitana* [3], and *Thermosipho africanus* [4], represent a group of organisms (the 'Thermotogales'), which according to various molecular criteria, clearly belong to the eubacterial kingdom. Amongst the eubacteria they are the most extreme thermophiles, which led to the hypothesis that the 'original' eubacteria were, in fact, thermophiles [5]. *Thermotoga* also possesses some very unusual biochemical attributes, such as the occurrence of novel types of lipids [6] or an uncommon peptidoglycan structure [1]. They are only distantly related to all the major lineages in the eubacterial phylogenetic tree, and, representing the deepest known branch within the eubacteria [7], they can be expected to have retained some ancestral characteristics.

The rod-shaped cells are surrounded by a 'sheath' which is firmly attached to the cylindrical part of the cell body, but 'balloons' around the cell poles [1]. An earlier electron microscopy study has already shown that the 'sheath' is composed of a regular array of particles [1]. We have further investigated this 'sheath' and show that it contains as a major component a trimer of a 42 kDa protein. The 3-dimensional structure of this protein, revealed by electron microscopy and image processing, is highly reminiscent of the 'classical' porins of the outer membrane of *E. coli*, e.g. OmpF [21] or PhoE [17].

Correspondence address: W. Baumeister, Max-Planck-Institut für Biochemie, D-8033 Martinsried b. München, FRG

[1] *Present address:* MRC, Laboratory of Molecular Biology, Structural Studies, Hills Road, Cambridge CB2 2QH, England

2. MATERIALS AND METHODS

Thermotoga maritima MSB 8 was available either as a suspension of cells or as frozen cell paste. Cells were suspended in 25 mM triethanolamine-HCl, pH 7.5, 5 mM EDTA, 1 mM PMSF, and broken by pulsed sonification (5 min at 0°C, using a Branson Sonifier B15 at 40 W) in the presence of 0.5 M $NaClO_4$. Undisrupted cells were removed by centrifugation at 6000 × *g* (10 min, 4°C); the cell wall fragments were collected at 40000 × *g* (15 min, 4°C) and washed twice in the same buffer. Further purification of sheaths was achieved on a Percoll (LKB-Pharmacia) gradient (starting concentration: 30% v/v) in the same buffer for 60 min (20000 × *g*). The uppermost band in this gradient at a density of 1.02 g/cm^3 contained almost pure sheaths. For purification of the molecular constituents, the material was solubilized by addition of octyl-polyoxyethylene (octyl-POE; Bachem) to a final concentration of 1%. After ultracentrifugation (60 min at 100000 × *g*, 4°C) the supernatant was fractionated by column chromatography on Sephacryl S-300 HR (LKB-Pharmacia) in 100 mM Na-phosphate, pH 7.6, 2 mM DTE, 1% octyl-POE. The fractions containing the purified trimer of the 42 kDa protein were concentrated and the detergent removed by methanol-chloroform precipitation [8]. SDS-PAGE was performed essentially according to Laemmli [9] in gradient slab gels (7–15% acrylamide).

Infrared (IR) spectroscopy of the isolated 'sheaths' was performed with the ATR technique [10] in an IR-spectrometer Perkin-Elmer 325. The sample was applied on a Germanium plate, and the IR spectrum was recorded between 4000 cm^{-1} and 1000 cm^{-1}. A bandshape analysis of the amide I and amide II region was used for estimation of the content of α-helix/random coil vs β-sheet [10].

For electron microscopy, isolated 'sheaths' were applied on carbon-coated grids and negatively stained with Na-phosphotungstate (1%; pH 7.2) or uranyl acetate (2%). Micrographs were recorded on Philips electron microscopes (EM 420, CM12) at primary magnifications between 30000 and 49000 (nominally). Im-

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age processing of selected areas was done with the correlation averaging procedure [11]. Tilt series were recorded between 0 and 78°, and the 3-dimensional structure was determined by a hybrid real space/Fourier space approach [12].

For the determination of the surface relief of the 'sheath', cells were frozen in a Cryojet QFD101 (Fa. Balzers) at −170°C in propane/liquid nitrogen slush. Subsequently, they were cleaved and then freeze-etched at −95°C for 2 min in a Balzers BA 360 freeze-etching apparatus and shadowed undirectionally with Pt/C (2 nm) under an angle of 45° and the replica stabilized with carbon (25 nm). The reconstruction of the surface relief was done as described in [13].

3. RESULTS AND DISCUSSION

Freeze-etching shows that the entire 'sheath' is covered by a patchwork of regular arrays (fig.1), i.e. the 2-dimensional crystalline domains which make up the 'sheaths' have only poor long-range order. Image analysis of such images using correlation averaging techniques in combination with surface relief reconstruction [11,13] revealed a regular array of orifices triangular in shape, with indications of 3 separate holes some distance below the outer surface. The spacing of the p3 lattice is 11 nm. This particular motif as well as the dimensions of the lattice are highly reminiscent of some well-characterized pore-forming proteins ('porins') from the outer membranes of 'classical' Gram-negative eubacteria such as OmpF, OmpC, or PhoE from *Escherichia coli* [14–17]. Whereas these bona fide porins from *E. coli* naturally exist in a non-crystalline form (or form only crystal patches of a minimal size) and therefore have to be reconstituted in vitro for structural studies, outer membrane proteins ('rOMPs') regularly arrayed in vivo are not uncommon in some subdivisions of the proteobacteria (see e.g. [18,19]).

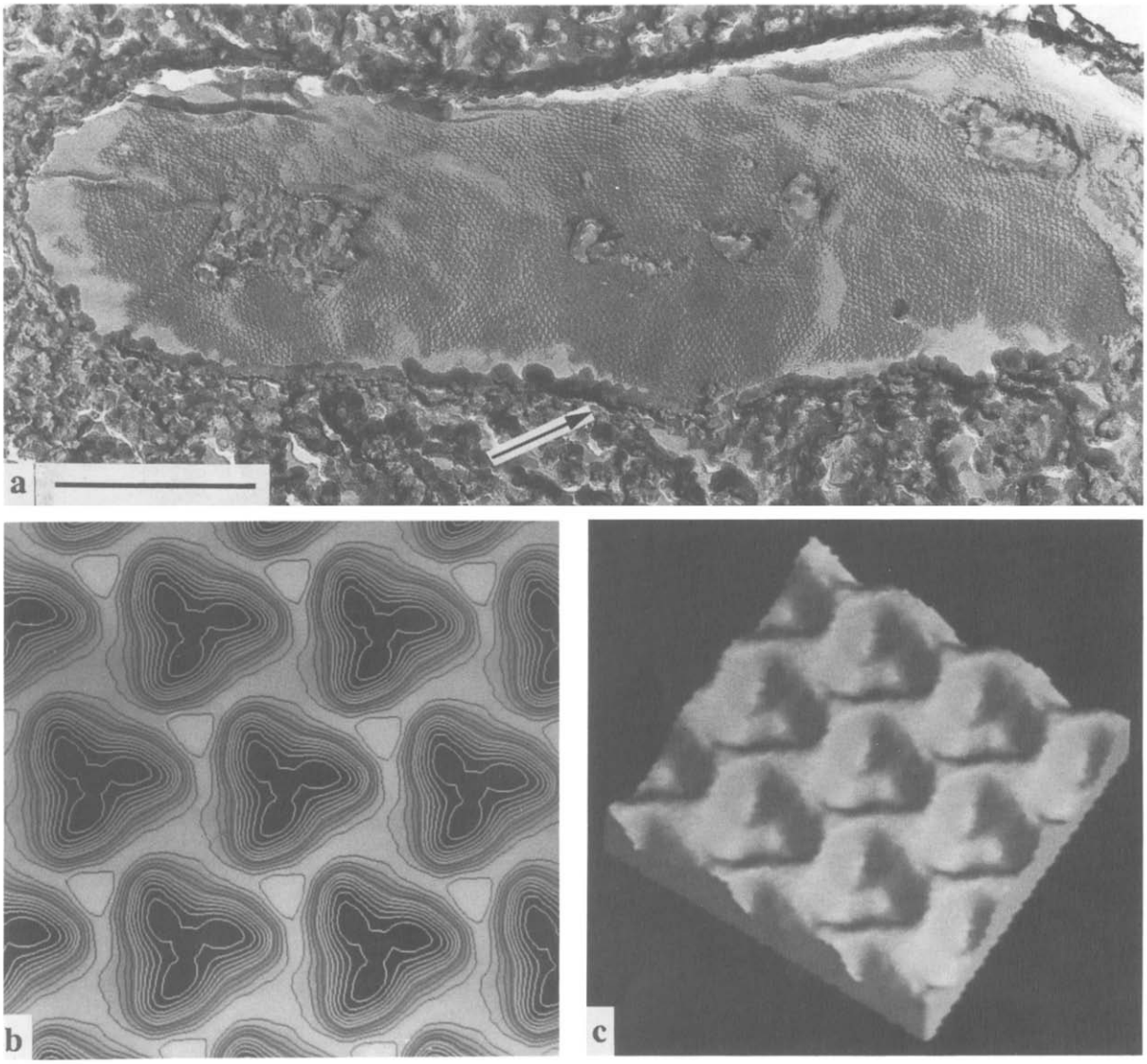

Fig.1. (a) Micrograph of a freeze-etched cell of *Thermotoga maritima*, unidirectionally shadowed with Pt/C. Scale bar = 300 nm. The arrow indicates the direction of the shadowing. (b) Surface relief reconstruction of a small area of (a) in contour representation (contour levels representing height). (c) Same as (b) in a 3-dimensional surface shading representation.

For further characterizations we have isolated *Thermotoga maritima* cell envelopes by sonification and Percoll density gradient centrifugation yielding a single opaque band at a density of 1.02 g/cm^3. This fraction was highly enriched in 'sheath' material, although it still contained remnants of plasma membrane, as seen by electron microscopy or SDS-PAGE. Attempts to remove these by exposure to Triton X-100, as routinely done with Gram-negatives [20] failed, since the 'sheath' turned out to be labile in this detergent, and no regular pattern could be detected any longer by electron microscopy. Envelopes obtained after differential centrifugation or from the Percoll gradient centrifugation were negatively stained with Na-phosphotungstate and micrographs were subjected to image processing. Correlation averages of sheath fragments clearly show triplets of stain-filled holes surrounded by a network of stain-excluding material (fig.2). We have further used such preparations to perform a 3-dimensional reconstruction via tilt series. The data obtained by tilt series of electron micrographs extended to a resolution of 2–2.5 nm. Horizontal sections through the structure

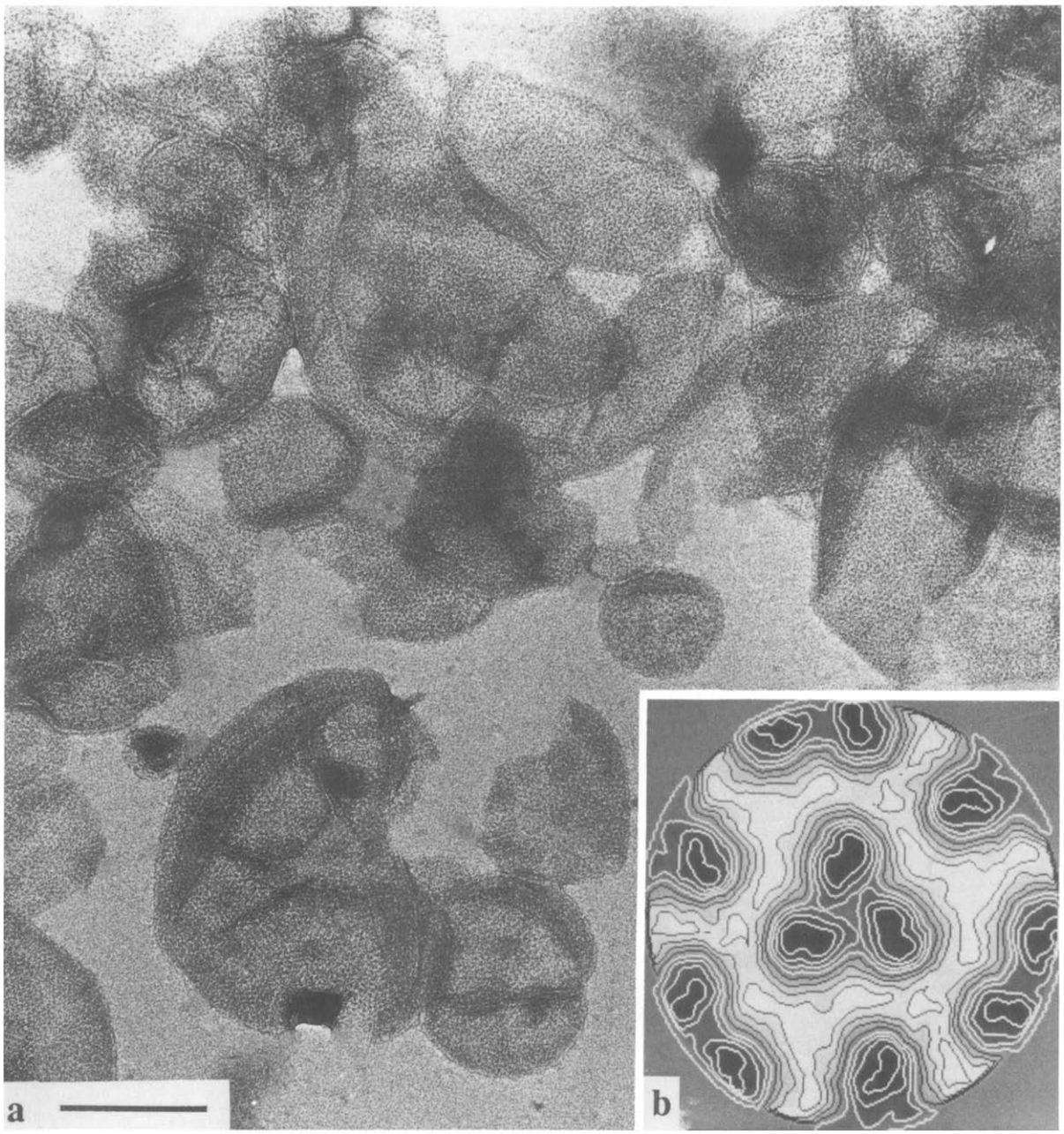

Fig.2. (a) Micrograph of purified 'sheaths' after Percoll gradient centrifugation, negatively stained with Na-phosphotungstate. Scale bar = 300 nm. (b) Result after correlation averaging of a small patch. The resolution is approx. 2 nm. Dark is negative stain, white is stain-excluding material.

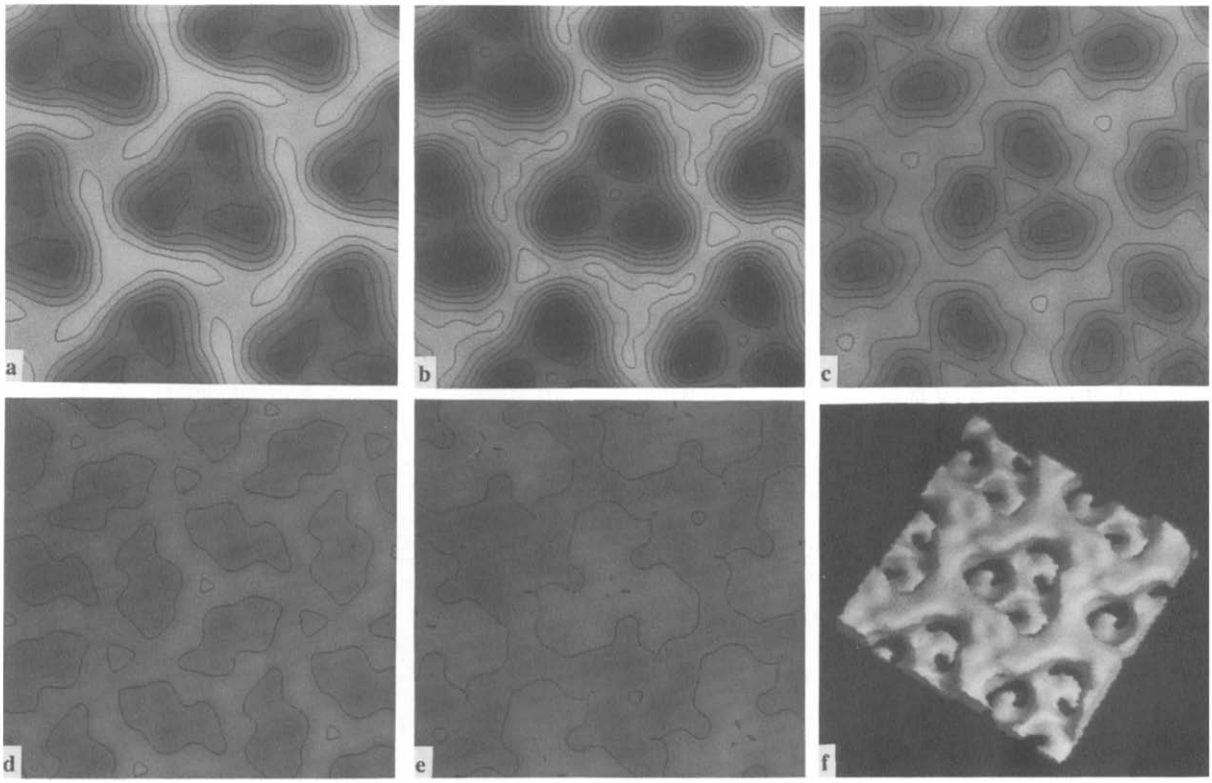

Fig.3. 3-dimensional reconstruction of the porin of *Thermotoga maritima*, negatively stained with Na-phosphotungstate. (a–e) Horizontal sections through the 3-dimensional data set parallel to the membrane plane, at −1.5 nm, +0.3 nm, +1.5 nm, +2.4 nm, and +3.0 nm, relative to the center of the stack. The section thickness is 0.3 nm. (f) A computer-generated view of the thresholded 3-dimensional data set.

(fig.3) reveal a striking resemblance to the 3-dimensional organization of OmpF trimers of *E. coli* [21] or of the porin present in the stalks of a Hyphomicrobium-like bacterium [18]. The triangular orifice evident in the reconstruction of the outer surface from freeze-etched cells appears to develop into 3 separate channels towards the inner surface. Whether or not this merging of channels is a real feature which will also hold at high resolution remains to be seen; it depends strongly on the thresholding which is notoriously arbitrary at low resolution. This, however, does not affect the validity of our statement that the 3-dimensional organization of the regularly arrayed sheath protein of *Thermotoga maritima* is indeed almost congruent with the 3-dimensional structure of the bona fide porin OmpF. It should be mentioned, however, that the 'sidedness' of the channel system as we infer it by comparison with the freeze-etching data is opposite to the sidedness suggested for OmpF [21] or PhoE [17].

Since a high content of β-sheets is a further structural characteristic of porins [10,22], we have recorded ATR-infrared spectra of *Thermotoga maritima* cell envelopes and we have deconvoluted the amide I and II region of

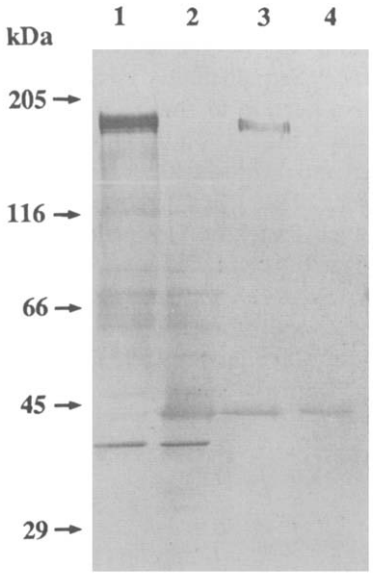

Fig.4. SDS-PAGE of the outer membrane ('sheath') and the isolated porin of *Thermotoga maritima*. Preparation was as described in section 2. Lanes 1,2: Whole 'sheaths', solubilized in 2% SDS at 35°C (1) and 100°C (2). Lanes 3,4: Isolated porin trimers (after octyl-POE extraction and column chromatography of 'sheaths'), solubilized in 2% SDS at 35°C (3) and 100°C (4).

the spectra [10]. We determined a β-sheet content of 40–50% which is somewhat less than the β-sheet content found with reconstituted pure OmpF. Taking into account that the envelope preparation was still contaminated with plasma membrane fragments, this measurement can be taken as further support of our notion that the main constituent of the sheath is a porin-type molecule.

SDS-PAGE gels of envelope preparations as well as isolated trimers (fig.4) show a dominant band with an apparent molecular mass of approx. 150–180 kDa (after treatment with 2% SDS at 35°C). The appearance and migration of this band varies somewhat from preparation to preparation, which might be due to varying amounts of LPS molecules bound to the trimer, as noticed e.g. with the OmpF porin of *E. coli* [23]. The same samples heated to 100°C in the presence of 2% SDS showed only one major band with an apparent molecular mass of 42 kDa. This is very close to the typical values found for the porins of many proteobacteria [24].

The finding that the *Thermotoga maritima* sheath is essentially a paracrystalline array of a porin-type molecule embedded in a lipid matrix and hence has the attributes of an outer membrane has some interesting evolutionary implications. It had been suggested previously [25], that the 'primordial' cell possessed two membranes. Since transport facilitating proteins are an indispensable feature in such a system, porin-type molecules can be expected to have evolved very early, which immediately raises the issue of their topogenesis. It is perhaps surprising to what extent porins appear to have conserved structural characteristics such as the trimeric organization or β-sheets as the structural elements forming the channels over an enormous evolutionary distance. Sequence analysis will show whether similarities also pertain to the primary structure level. Provided that the evolution of eubacteria is monophyletic and relationships are not distorted by horizontal gene flow, it suggests that the Gram-positives evolved from the Gram-negatives. This is in full agreement with the phylogenetic tree for eubacteria derived from 16s rRNA sequencing data, as discussed in [7], and also in accordance with a hypothesis put forward recently by Cavalier-Smith [25,26] proposing that 'Negibacteria' were the predecessors of 'Posibacteria' which lost the outer membrane as a consequence of peptidoglycan hypertrophy.

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