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Topography of Outer Membrane Assembly in Salmonella

P. F. Mühlradt

Gesellschaft für Molekularbiologische Forschung mbH, Mascheroder Weg 1, 3300 Braunschweig-Stöckheim, West Germany

The topography of lipopolysaccharide insertion into the outer membrane of Salmonella is discussed in context with a review of recent findings pertaining to general properties of the outer membrane, such as asymmetry and lateral mobility of surface components.

Key words: outer membrane, lipopolysaccharide, bacteriophage

INTRODUCTION

The specific question of outer membrane assembly is closely linked to more general problems of membranology such as permeability, fluidity, asymmetry and, last but not least, composition of this particular membrane. I shall have to review the available findings pertaining to the general properties of the outer membrane before focussing attention to the actual question of how and where this membrane is assembled. The answer will be necessarily incomplete, and as far as possible mechanisms are involved, highly speculative.

COMPOSITION OF THE OUTER MEMBRANE

Figure 1 shows a schematic presentation of the gram-negative cell wall. This figure implies that the membrane is highly asymmetric, protein and lipopolysaccharide (LPS) being on the outer face, phospholipids on the inner face. What is the experimental evidence for such a model (1), and how stable is this asymmetry? Van Gool and Nanninga (2) and also Smit et al. (1) have shown by freeze fracture techniques that protein particles are seen mainly in the outer fracture face. Henning and coworkers used an entirely different approach, namely crosslinking of outer membrane proteins, and reached similar conclusions (3). In our laboratory it was shown, using ferritin antibodies directed against the polysaccharide moiety of the LPS (see Fig. 2), that the wild type LPS is positioned at the outer face of the outer membrane. This result was obtained only when lysozyme treatment to remove the mucopeptide layer and labeling were carried out at ice-bath temperature. Incubation of the preparation for only one minute at 37°C preceding the antibody labeling already led to extensive redistribution of the ferritin label to both sides of the membrane, indicating a partial rearrangement of the LPS (4). A quantitation of this process is not possible with this method. The experiment shows, however, that asymmetry of the outer membrane, as far as the LPS distribution is concerned, is not a stable state once the mucopeptide has been removed. We also know from V. Braun's work (5) that the

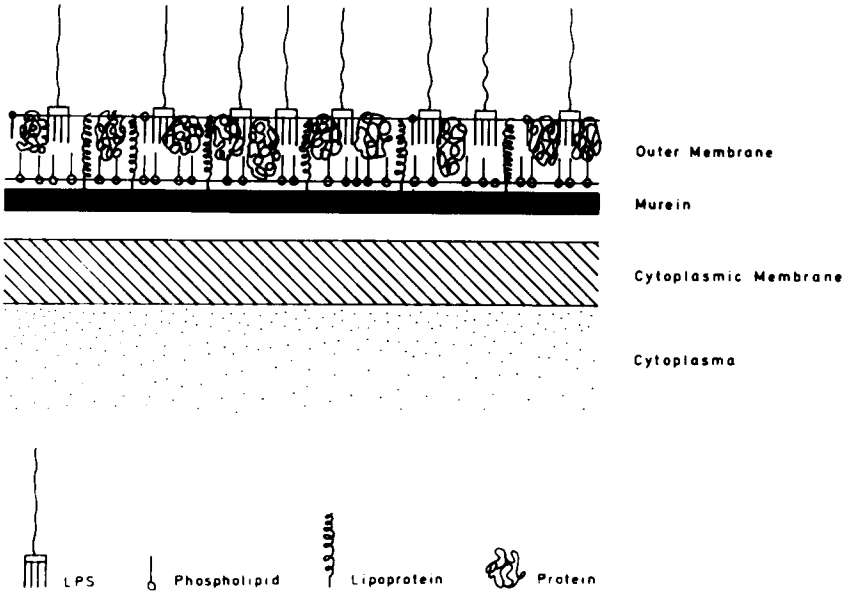

Fig. 1. Schematic structure of gram-negative cell envelope.

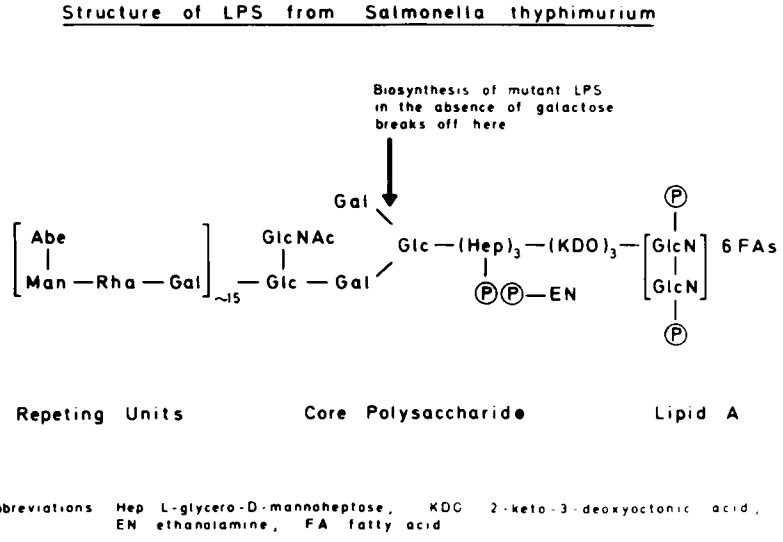

Fig. 2. Structure of LPS from Salmonella typhimurium.

TABLE I. Surface Density of LPS in Salmonella Outer Membrane

Strain	Growth phase	Molecules LPS per μm^2 (a)	% Surface covered by fatty acids of LPS (b)	Intermol. distance Å
R 1195	l. exp.	2.9×10^5	40	20
S 1195	l. exp.	2.6×10^5	35	21
S 1195	l. exp.	3.2×10^5	43	19
S 1135	m. exp.	3.0×10^5	40	20
S 1135	l. exp.	2.0×10^5	27	24
S 1908	m. exp.	3.0×10^5	41	20
S 1908	l. exp.	2.3×10^5	32	22
S 1908	l. stat.	2.4×10^5	33	22

(a) Data taken from (6). It was assumed that the smallest subunit of LPS contains 2 glucosamines in the lipid A, and that no covalent crosslinks exist between these subunits.

(b) Data taken from (6). It was assumed that one diglucosamine subunit is substituted by 6 fatty acid residues, each of a surface area of 20–25 $Å^2$.

mucopeptide and the outer membrane are physically closely linked via the lipoprotein, which is at least in part covalently bound to the mucopeptide and at the same time is integrated into the outer membrane. Consequently one should not regard the outer membrane as a separate structural and functional entity.

Interesting insights into the structure of the outer membrane can be gained by quantitative determinations of the outer membrane components. Such determinations were carried out in our laboratory for the number of LPS molecules per μm^2 surface, the intermolecular distances of the molecules, and the percentage of the surface covered by the lipid A portion (6). These published data have been reevaluated because they were based on the assumption that the LPS is covalently crosslinked via phosphate ester bonds in the lipid A moiety. However, phosphate nuclear magnetic resonance data gave no compelling evidence for the existence of such crosslinks (V. Lehmann and P. Mühlradt, unpublished). The revised data are presented in Table I. The cell surface, although densely populated by LPS, is by no means covered by the lipid A (1). Interestingly, if one compares the number of negative charges from the carboxyl and phosphate groups of the LPS on the cell surface with those negative charges from the carboxyl groups of the diaminopimelic acid on the mucopeptide as determined by Braun et al. (7), one finds that approximately equal numbers of negative charges oppose each other on both faces of the cell wall. When the mucopeptide is removed, as in the above described ferritin-labeling experiment, an outer membrane results which is crowded with negative charges on the outer face. It is perhaps for this reason that the LPS undergoes the observed rearrangement.

It is with these general properties of the outer membrane in mind that we now turn to the actual problem of cell wall and outer membrane assembly.

CELL WALL ASSEMBLY

When bacterial cells are enlarging, they are faced with a problem not encountered by animal cells: They have to build their walls in an environment of low osmotic pressure, and they have to assemble them on the other side of a severe permeability barrier, outside the cytoplasmic membrane.

There are two possible ways the walls could be assembled: a) Low molecular weight precursors are exported through the cytoplasmic membrane and are joined outside to form high molecular weight cell wall constituents. B) Biosynthesis of cell wall constituents takes place at the inside of the cytoplasmic membrane, and the high molecular weight components are then exported to their ultimate destination.

Both possibilities pose essential permeability problems, in the one case for the precursors, in the other for the products. Precursors for one principal and typical outer membrane and thus cell wall component, the LPS, are nucleotidediphospho-sugars and ATP. The cytoplasmic membrane is impermeable to both these compounds, which makes possibility A unlikely. On the other hand, wild type lipopolysaccharide carries polysaccharide chains containing about 60 carbohydrates (see Fig. 2), which means that, if the products were exported (possibility B), this huge carbohydrate portion would have to permeate both membranes and the murein layer in order to reach the cell surface. However, Nikaido and others have shown that any oligosaccharide bigger than a tetrasaccharide will not permeate the outer membrane (8) or the cell wall (9).

From theoretical considerations alone one might thus reach the conclusion that bacterial cell walls cannot possibly enlarge. But specific sites must exist at which the insertion of new cell wall material can take place.

None of the constituents of the outer membrane are synthesised externally, but either in the cytoplasm or at the inner side of the cytoplasmic membrane. For phospholipids this was shown by D. White et al. (10); for LPS it was shown by M. J. Osborn, et al. (11). Since nobody suspects ribosomes in the outer membrane, we can safely assume that proteins are also translocated to the outer membrane. Thus, apparently possibility B is realized, i.e., components of the outer membrane are synthesized inside and assembly then takes place outside.

When we pose the question where new cell wall material is inserted, we have to be aware of the fact that the answer may be a different one for each cell wall component (12, 13); although temptation is great to postulate universal growth points.

We have focussed our attention in our laboratory on the insertion of "new" LPS into the outer membrane of Salmonella typhimurium. The distinction of newly made LPS from already existent "old" LPS was made possible by a type of mutant which lacks UDP-galactose-4-epimerase and produces in the absence of external galactose only incomplete LPS (see Fig. 2) because it cannot form UDP-gal, the precursor for the transfer of galactose. However, when galactose is externally added to the growth medium, UDP-gal is formed via gal-1-P and complete LPS is synthesized. We raised antibodies to the complete LPS, absorbed with the incomplete LPS, and coupled with ferritin. These labeled antibodies are only directed against "new" LPS, formed after addition of galactose to the medium. In other words the ferritin-antibodies will show where the new LPS is located on the surface.

Cells were exposed for various times to galactose, rapidly chilled, and labeled at 0°C with ferritin-conjugate. After 30 sec exposure to galactose, new LPS could be demonstrated on the cell surface in freeze-etch preparations. Thus, translocation is very rapid. This finding agrees with the data of Osborn (11), who showed with her membrane separa-

tion technique that radioactively labeled LPS very rapidly appeared in the outer membrane fraction. Both groups, Osborn's and ours, have also shown that translocation is irreversible, so that what we are seeing here is not the completion of already existing LPS, but the appearance of new LPS (11,14).

The ferritin indicating the new LPS appeared in well defined patches. The initial number of patches were counted to be 70-100/μm^2 surface (14) i.e. 350–500 per cell of 5 μm^2. With longer times of exposure to galactose the patches enlarged, merged, and after exposure to galactose for longer times than 3 min the cells were completely covered by ferritin-antibody. We conclude that a limited number of sites exist where newly-made LPS emerges, and that it then spreads by lateral diffusion (14). Early independent ultracentrifugation experiments of L. Leive were also indicative of the lateral mobility of LPS (15). Such lateral mobility could directly be shown in our following experiment (6). The idea was to first create patches of new LPS on cells briefly exposed to galactose. Then the LPS synthesis, as well as lateral mobility, was stopped by rapidly chilling the cells. The cells were washed, then divided in two portions: one was kept at 0°, the other warmed to 37° for 10 min, then again cooled. Both portions were labeled as usual with ferritin antibodies at 0°. The experiment is illustrated in Fig. 3. The outcome of this experiment was that the cells warmed for 10 min in the absence of galactose show a thinly dispersed ferritin pattern, whereas the cells kept at 0° have their ferritin indicative of the new LPS still in relatively few, well defined areas.

However, we have to realise that the situation at hand is a somewhat unnatural one. We have new LPS, with long carbohydrate chains, moving in an environment of old, short chain LPS. From the data in Table I, and assuming 15 repeating units for the carbohydrate chain, one can calculate that a layer of 300 Å thickness containing 6.5% wt/vol polysaccharide surrounds the cell. Remembering that much less polysaccharide can form gels, one would expect new LPS in its natural polysaccharide environment to be less mobile than in the described experiment.

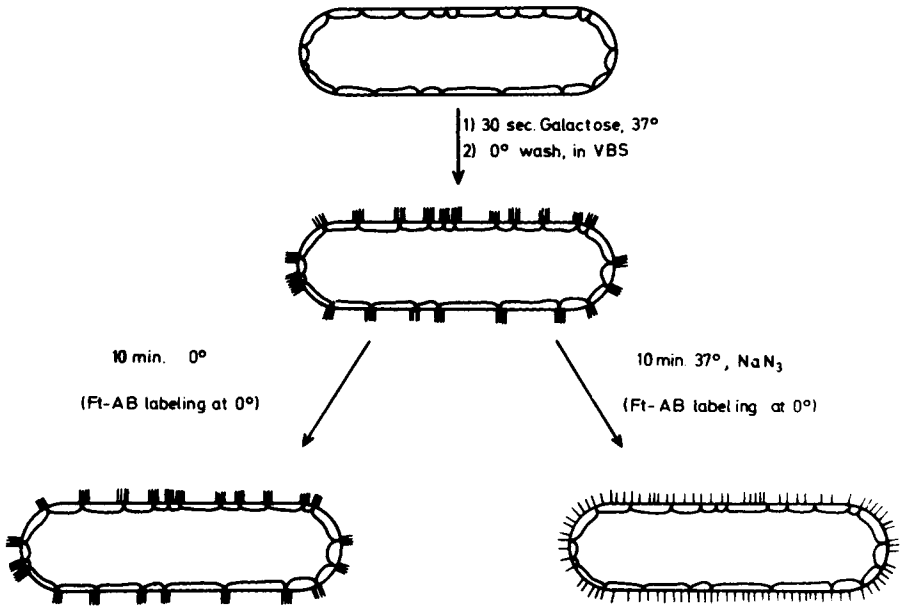

Fig. 3. Lateral surface mobility of LPS.

CONCLUSION

It was stated above that theoretically bacterial cells should not be able to grow, because the wall is impermeable to large, let alone charged, molecules such as LPS. We have now seen that at least at certain sites the wall seems perfectly permeable, namely where the LPS comes out. There is another example for such permeability, in this case in the reverse direction: namely the uptake of nucleic acid in mating and also by phage infection. M. Bayer has shown that phages such as T1-T7 and ϕX174 preferentially adsorb to specific sites of the cell surface, where adhesions of cytoplasmic membrane to the wall are visible upon plasmolysis (16). The number of these sites is roughly the same as those that we had counted as LPS translocation sites in the freeze-etch experiments, i.e. 400 per cell. We were curious to see whether these adhesion sites were identical with our LPS translocation sites. Bacteria were induced to produce LPS for a short time, were ferritin-labeled in the cold, and thin-sectioned in the plasmolyzed state. Indeed 86% of the ferritin patches were located over adhesion areas (14). These data do not strictly prove the identity of phage adsorption and LPS export sites. However, since both sites are identical with the membrane adhesion areas visible after plasmolysis (17), such identity is very likely. It thus appears that sites in the wall, needed by the cell for the export of new cell wall material, are "misused" by phages to inject their nucleic acid, very probably because these sites have special characteristic properties which make them permeable for linear charged polymers.

What is the nature of these permeable sites? It is tempting to speculate that the mucopeptide exhibits localized discontinuities at these sites, which make possible a local fusion of cytoplasmic and outer membrane. Indeed the phospholipids of both membranes are very similar in composition (18,19). It is possible that the LPS is translocated at these mucopeptideless fusion sites by a mechanism similar to that which might be involved in the rearrangement of LPS in the mucopeptideless outer membrane, described above.

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