Vol. 85, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 14, 1978 Pages 326-334

TRANSLOCATION OF LIPOPOLYSACCHARIDE IN THE CELL WALL OF A GRAM-NEGATIVE MARINE BACTERIUM

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Received September 25,1978

SUMMARY: Suspensions of *Alteromonas haloplanktis* 214 were pulsed with [^{14}C] galactose and chased with unlabelled galactose for various periods. The three outermost layers of the cell wall were separated and the lipopolysaccharide from each was isolated. Depending upon the composition of the suspending medium and the extent of aeration, [^{14}C] lipopolysaccharide appeared primarily either in the periplasmic space or in the loosely bound outermost layer of the cells after the shortest pulse time tested (1 min). ^{14}C-lipopolysaccharide appeared in appreciable amounts in the outer membrane only after longer periods of pulsing and chasing. The observations reported are consistent with the conclusion that newly synthesized lipopolysaccharide moves through the periplasmic space, through the outer membrane, and spreads over the outer surface of the outer membrane of this organism. Lipopolysaccharide in the outer membrane may either be synthesized independently of this more mobile form or gradually infiltrated by it.

INTRODUCTION: Osborn and co-workers (10) have concluded from pulse chase studies that LPS (lipopolysaccharide) is synthesized in or at the cytoplasmic membrane of *Salmonella typhimurium* and is subsequently translocated irreversibly to the outer or cell wall membrane. Mühlradt *et al* (7, 8) using ferritin-conjugated antibodies showed with the freeze etch technique that new LPS appeared first in patches on the surface of *S. typhimurium* and subsequently spread evenly over the entire cell surface. Since thin sections of plasmolyzed cells showed the newly synthesized LPS to be associated largely with points of adhesion between the inner and the outer membrane it was proposed that LPS translocation occurred at these adhesion sites. Subsequent lateral movement of LPS in the outer membrane was believed to be responsible for the even distribution of LPS on the bacterial surface.

Kulpa and Leive (4) concluded that LPS enters the outer membrane of *Escherichia coli* non-randomly at various insertion points and subsequently spreads throughout the membrane. Subsequent studies by Leive (6) were

ABBREVIATIONS: LPS, lipopolysaccharide; CST, buffered complete salts

0006-291X/78/0851-0326$01.00/0

interpreted as indicating that LPS synthesized early during growth was organized into regions that are not freely interpenetrated with more recently synthesized LPS.

When cells of the Gram-negative marine bacterium *Alteromonas haloplanktis* 214 are washed with 0.5 M NaCl a loosely-bound outer layer is removed from the surface of the cells. Subsequent suspension of the cells in 0.5 M sucrose causes the outer membrane to break into large fragments releasing the contents of the periplasmic space (2). Analysis of the fractions recovered revealed that only 17 percent of the LPS in the cell wall was actually present in the outer membrane. The remainder was divided almost equally between the loosely bound outer layer and the periplasmic space (1). The results of pulse chase experiments designed to follow the progress of newly synthesized LPS through the three outermost layers of the cell wall are recorded.

MATERIALS AND METHODS: Cells of *Alteromonas haloplanktis* 214 variant 3 (ATCC 19855) were grown to early stationary phase in a medium and under conditions described previously (1) with the medium modified to contain 0.13% galactose. Cells from 250 ml of medium were harvested by centrifugation (unless indicated otherwise, all centrifugations were conducted at 16,000 x g at 4° for 10 min) and resuspended in 40 ml (to approximately 7 mg dry wt of cells per ml) in either sterile growth medium without galactose (complex medium) or buffered complete salts (CST) solution containing 0.22 M NaCl, 0.01 M KCl, 0.026 M $MgCl_2$ and 0.001 M Tris hydroxymethylamino methane HCl pH 7.2. The suspension at 25° was either shaken on a rotary water bath shaker or sparged with sterile air. These conditions provided 10 percent and 70 to 80% saturation with air, respectively, as measured using a YSI Model 53 biological oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio). At 0 time 25 μCi D-[^{14}C(U)]-galactose (200-300 mCi/mmol) in 1 ml of H_2O was added.

For cells pulsed for different periods, 5 ml of cell suspension were withdrawn after each pulse time and added to 5 ml of either chilled complex medium or CST solution (depending upon the experiment) each maintained in an ice bath and containing sufficient NaCN to give a final concentration of 10 mM. At the same times, 5 ml of cell suspension were withdrawn for the chase and added to 20 ml of either complex medium or CST solution containing sufficient unlabelled galactose to give a final concentration of 200 mM. The chase suspensions were shaken on a rotary shaker at 25°. After the appropriate chase period each suspension was added to 1 ml of chilled complex medium maintained in an ice bath and containing sufficient NaCN to give a final concentration of 10 mM.

For cells pulsed for 1 min and chased for various times, 5 ml of the cell suspension containing [^{14}C]-galactose were withdrawn and added to 5 ml of complex medium containing NaCN (final concentration 10 mM) maintained in an ice bath. For the chase, sufficient galactose was added to the remainder of the cell suspension to give a final unlabelled galac-

tose concentration of 200 mM. The suspension was incubated at 25° with sparging. At the end of each chase period 5 ml of the suspension were withdrawn and added to 5 ml of complex medium containing NaCN maintained in an ice bath.

At the end of the pulse and chase times all suspensions were centrifuged and the supernatant solutions were discarded. The cells were resuspended in 10 ml of CST containing 10 mM NaCN and again sedimented by centrifugation.

The three outer layers of the cell wall, the loosely bound outer layer, the outer membrane and the periplasmic fraction were separated and isolated as described previously (2, 9). The NaCl solution used to remove the loosely bound outer layer and the sucrose solution employed to separate the outer membrane and release the contents of the periplasmic space were each modified to contain 10 mM NaCN.

LPS was extracted from each of the fractions by phenol extraction and recovered by centrifugation at 144,000 x g using methods which have been reported (1). Treatment of the isolated LPS with RNase and DNase was omitted since the enzymes did not change the radioactivity profiles of the LPS fractions in the polyacrylamide gels.

The isolated LPS was subjected to polyacrylamide gel electrophoresis and the gels were fractionated and counted as described previously (1).

RESULTS: Cells suspended in complex medium. When cells were suspended in fresh growth medium (minus galactose) for pulsing with [^{14}C] galactose and chasing with unlabelled galactose the results in Fig. 1 were obtained. Previous studies have shown that the LPS of this organism is heterogeneous and depending on the age of the culture can be separated by polyacrylamide gel electrophoresis into as many as 3 fractions referred to as LPS I, II and III (1). After a 1 min pulse with [^{14}C] galactose a fraction corresponding to LPS II became radioactive in the periplasm, no [^{14}C]LPS was detected in the outer membrane while a small but distinct peak appeared in the loosely bound outer layer. After a 2 min chase, some [^{14}C]LPS appeared in the outer membrane. On pulsing for 2 min [^{14}C]LPS was found in both the periplasmic space and the outer layer and none was present in the outer membrane. Only after additional pulsing and chasing did any appreciable build-up of radioactivity occur in the LPS of the outer membrane. After a 10 min pulse followed by a 10 min chase the 3 peaks corresponding to LPS I, II and III were discernible in the LPS of the outer membrane.

Cells suspended in salt solution. When cells were suspended in a buffered salt solution similar to that used to prepare the growth medium the rate of transfer of newly synthesized LPS to the loosely bound outer layer was

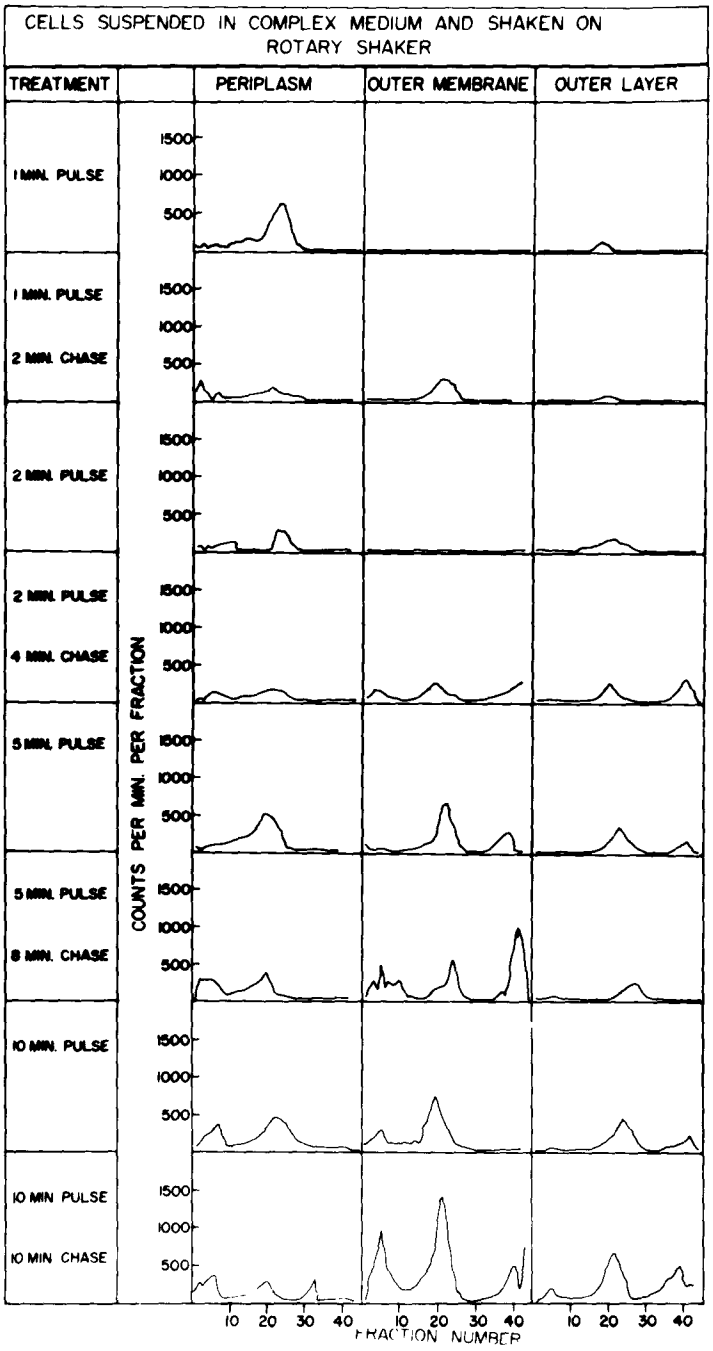

Fig. 1. Radioactivity profiles obtained when LPS isolated from the 3 outermost layers of the cell wall of *A. haloplanktis*, the loosely bound outer layer, the outer membrane and the periplasmic fraction was subjected to polyacrylamide gel electrophoresis. In this experiment cells suspended in complex medium and shaken on a rotary shaker were pulsed with [^{14}C]galactose and chased with unlabelled galactose for the periods shown.

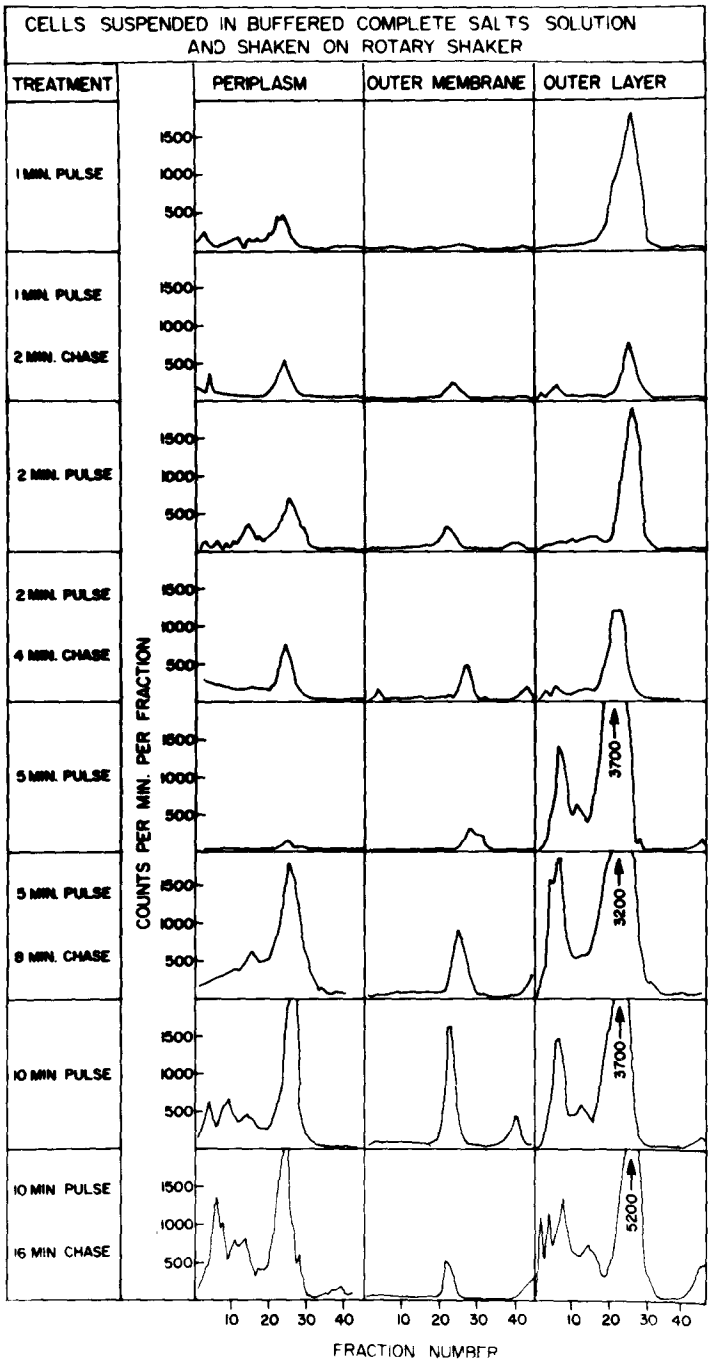

Fig. 2. As in Fig. 1 except that the cells were suspended in buffered complete salts (CST) solution.

very rapid, Fig. 2. After a 1 min pulse most of the [^{14}C]LPS was found in this layer with smaller amounts in the periplasm while none was detectable in the outer membrane. The outer membrane LPS became appreciably labelled only after longer periods of pulsing and chasing.

Cell suspension sparged. When cells suspended in fresh growth medium (minus galactose) were provided with additional aeration by sparging, most of the [^{14}C]LPS isolated after a 1 min pulse was found in the loosely bound outer layer, Fig. 3. Appreciable radioactivity appeared in the underlying layers only after chases of relatively long duration.

DISCUSSION: Separate experiments have shown that other compounds besides LPS become radioactive when the cells are exposed to [^{14}C]galactose for even the shortest pulse time tested. The complete disappearance of radioactivity in one layer and its appearance in another when the cells were chased with unlabelled galactose was seldom observed, presumably because LPS can be synthesized continuously from a pool of radioactive precursors arising from the metabolism of the [^{14}C]galactose. This is different from the situation in cells where [^{14}C]galactose can be used to label LPS specifically (10). The time of appearance of radioactive LPS in the various layers, however, can be used to indicate the route that newly synthesized LPS took through the cell envelope.

It is clear from the results with cells suspended in complex medium under conditions of limited aeration that [^{14}C]LPS appeared first in the periplasmic space. When the cells were further aerated or suspended in CST most of the [^{14}C]LPS appeared in the loosely bound outer layer after the shortest pulse time tested. Only later was [^{14}C]LPS found in appreciable quantities in the outer membrane. This indicates that newly synthesized LPS must have moved from the periplasmic space directly to the loosely bound outer layer without equilibrating with the LPS in the outer membrane. The rate and extent of this movement was affected however by the conditions under which the experiments were run with

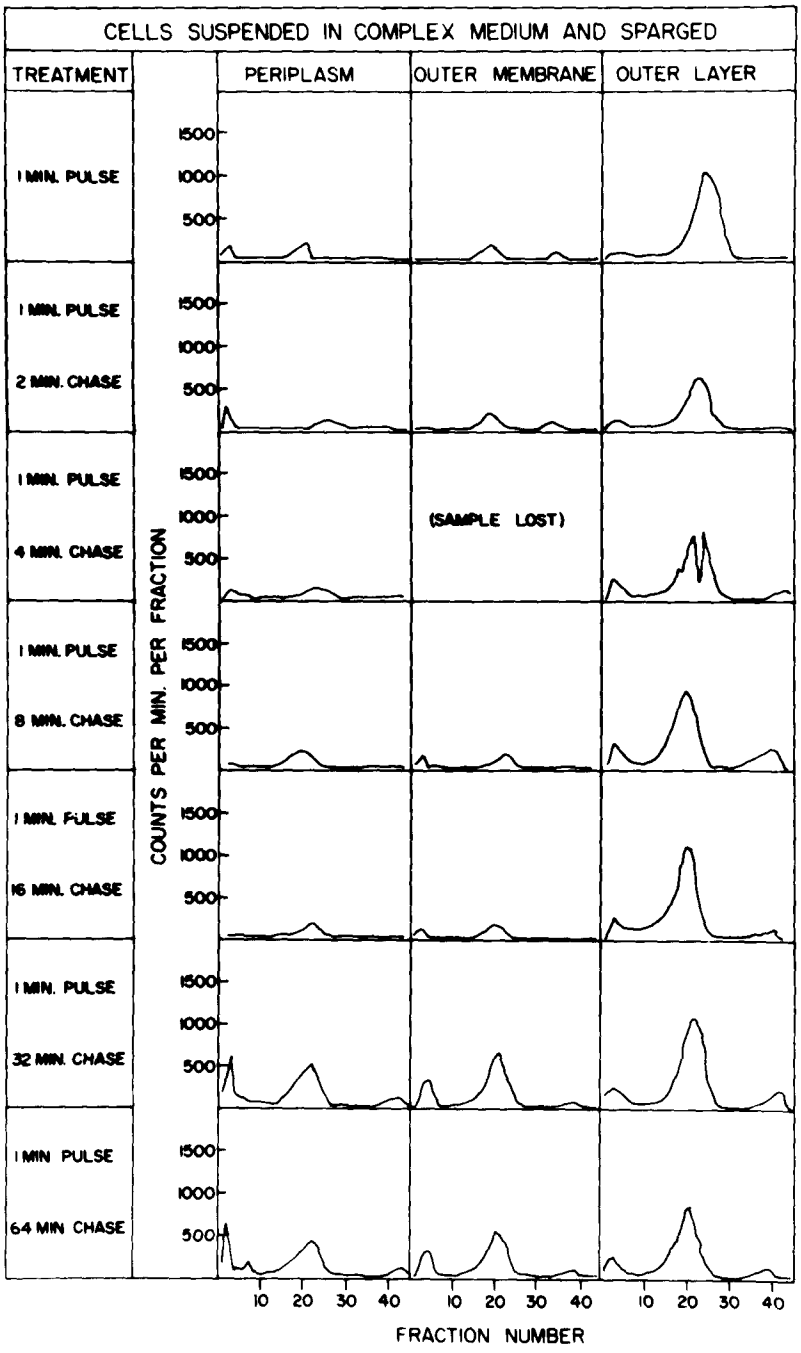

Fig. 3. As in Fig. 1 except that the cells were subjected to additional aeration by sparging.

aeration and a simplified suspending medium favoring the early appearance of newly synthesized LPS in the outermost layer. The observations reported are consistent with the conclusion that a mobile form of LPS presumably synthesized in or on the cytoplasmic membrane is transferred to the periplasmic space from whence it passes through the outer membrane, perhaps through pores (12), to a loosely bound outer layer on the surface of the cells. LPS in the outer membrane may either be synthesized independently of the more mobile form or gradually infiltrated by it.

The early appearance of radioactive LPS on the outer surface of the outer membrane of *A. haloplanktis* parallels the early appearance of newly synthesized LPS on the surface of cells of *Salmonella* species (7) and *E. coli* (4). The failure of this LPS in *A. haloplanktis* to equilibrate quickly with outer membrane LPS is consistent with Leive's demonstration of heterogeneity of LPS composition in the outer membrane fraction of *E. coli* (6). Since the rapidly labelled fraction on the outer surface of *A. haloplanktis* is associated with a layer readily removed by washing with 0.5 M NaCl it would appear that in this organism the newly synthesized LPS, rather than moving laterally within the outer membrane, spreads over its outer surface. Previous studies of the composition of the loosely bound outer layer of *A. haloplanktis* have shown it to contain lipopolysaccharide, protein and lipid including phospholipid (1, 3, 9) and to bind Mg^{++} (11). Since washing the layer with a NaCl solution displaces the bound Mg^{++} (11) and releases the layer from the cells it has been proposed that Mg^{++} cross-links bind the loosely-bound outer layer to the outer surface of the outer membrane (11). Since some 50 percent of the LPS of *E. coli*, in association with protein and phospholipid is readily released by treatment of the cells with ethylenediaminetetracetic acid (5) it is not unlikely that the EDTA-releasable fraction in *E. coli* corresponds to the loosely bound outer layer of *A. haloplanktis*. If it does, the mechanism of translocation of LPS in the 2 organisms may well be quite similar.

ACKNOWLEDGEMENT: This work supported by a grant from the National Research Council of Canada.

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