Biochimica et Biophysica Acta, 508 (1978) 287—295

BBA 77966

PREFERENTIAL RELEASE OF NEW OUTER MEMBRANE FRAGMENTS BY EXPONENTIALLY GROWING *ESCHERICHIA COLI*

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(Received July 18th, 1977)

Summary

We have examined whether the outer membrane fragments released by normally growing *Escherichia coli* contain relatively old or new outer membrane.

Double-label experiments show that after incorporation of radioactive leucine into *E. coli* protein, there is a preferential release of outer membrane material which contains a high percentage of newly labeled protein. This implies that outer membrane fragments are preferentially released from those regions where newly synthesized proteins are inserted into the outer membrane. We estimate that these insertion regions cover no more than 13% of the total outer membrane, and that newly inserted proteins diffuse in the plane of the outer membrane with a diffusion constant $\leqslant 5 \cdot 10^{-13}$ cm^2/s.

Introduction

The release of lipopolysaccharide · phospholipid · protein complexes from *Escherichia coli* has been observed for growing and stationary phase cells [1—3], for cells limited in growth for a required amino acid [4—6], treated with chloramphenicol [1], saline or Tris/EDTA [7—9] and for cells exposed to phage [2]. Electron microscopy [1,10,11] and chemical composition studies [3] show that these complexes are outer membrane fragments. Other gram-negative bacteria have also been reported to release outer membrane fragments [12,13], and the relation between free endotoxin excreted by endotoxic bacteria and the outer membrane fragments released by *E. coli* has recently been reviewed [12].

It is somewhat surprising that Gram-negative cells should release any outer

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membrane at all, since the outer membrane is linked covalently to the underlying murein layer via Braun's lipoprotein at the extremely high density of 50 000—60 000 bonds per μm^2 of cell surface [14]. It is likely therefore that outer membrane fragments originate from areas where the local surface density of the bound form of Braun's lipoprotein is much lower. This might occur either if lipoprotein links are not formed at all, or if such links, after being formed, are cleaved. In the former case, the resulting outer membrane fragments will consist of newly synthesized outer membrane which has not yet been linked covalently to the murein layer. In the latter case, the released outer membrane fragments will consist of older outer membrane which is released only after already having been part of the total cell wall structure.

To discriminate between these two possible mechanisms we have compared the relative "age" of released outer membrane fragments to that of the outer membranes from which they originate.

Materials and Methods

Cell growth

E. coli JC411 (*leu*$^-$ *his*$^-$ *arg*$^-$ *met*$^-$ *lac*$^-$ *mal*$^-$ *xyl*$^-$ *mtl*$^-$ *str*^{r}) was grown in a minimal medium containing E-salts [15], 0.5% dextrose, 100 μg/ml each of L-histidine, L-arginine and L-methionine, and 50 μg/ml of L-leucine, in 20-ml vials on a waterbath shaker (200 rev./min) at 37° C. The doubling time was 60 min under these conditions. [^{14}C]- or [^{3}H]leucine were added as described below. Cell densities (mg/ml cell dry weight) were determined [16] after dilution of culture portions into medium containing 1% formaldehyde.

Radioactive labeling and sampling of cells

Cells were grown to about 0.5 mg/ml in 2.65 ml medium containing $7.7 \cdot 10^8$ dpm L-[4,5-^{3}H]leucine (spec. act. 1 mCi/μmol; Radiochemical Centre, Amersham, U.K.), resulting in 40% incorporation of label into protein. The cell density was measured during the last few hours to confirm that the culture was growing exponentially. At a time point defined as $t = 0$ min, 0.7 ml prewarmed medium containing $7.7 \cdot 10^7$ dpm L-[U-^{14}C]leucine (spec. act. 330 mCi/mmol; Radiochemical Centre) was added. Samples were taken at —0.25, +0.5, 1, 2, 4, 10, and 20 min by pipetting 0.4 ml culture into a microvial cooled in an ice bath. In the reverse experiments cells were grown in 3 ml medium containing $7.7 \cdot 10^7$ dpm [^{14}C]leucine, also resulting in 40% incorporation of label into protein, and at $t = 0$, 0.35 ml prewarmed medium containing $7.7 \cdot 10^8$ dpm [^{3}H]leucine was added.

Separation of cells and cell free supernatant

The samples were centrifuged (0° C, 8 min, 8000 × *g*). The supernatant was recentrifuged (0° C, 8 min, 8000 × *g*) to remove any cells which might still be present. The second supernatant was used for the isolation of outer membrane fragments as described below. The cell pellet was washed with 0.4 ml cold medium and suspended in 0.2 ml cold medium. To determine the radioactivity incorporated into cell proteins, two 10-μl portions of washed cells were each added to 5 ml ice-cold 10% trichloroacetic acid containing 0.2% (w/v)

L-leucine. After 30 min at 0—4°C the precipitates were collected on a 0.45 μm filter (Selectron-Filter type BA85, Schleicher and Schuell, Dassel, West-Germany), washed twice with 5 ml ice-cold 10% trichloroacetic acid containing 0.2% L-leucine, dried, and counted. The remaining 0.18-ml cells were centrifuged (0°C, 8 min, 8000 × *g*), suspended in 0.2 ml 15% glycerol, frozen in liquid nitrogen and stored at −80°C until the membrane separation was performed.

Isolation of outer membrane fragments

Outer membrane fragments were isolated from the recentrifuged supernatant by applying 0.2 ml to a Sephadex G-200 column (0.7 × 20 cm), and eluting with E-salts [15] containing 0.05% (w/v) sodium azide. The void volume fraction (about 1.0 ml as determined with Blue Dextran (Pharmacia, Uppsala, Sweden) was collected and counted. The radioactivity found in the void volume was due to labeled membrane vesicle proteins [3]; it was not due to binding of radioactive leucine to medium vesicles, since elution with the same buffer, supplemented with 10 mM L-leucine, did not lower the radioactivity found in the void volume.

Preparation and isolation of membrane fractions

The labeled cells were diluted about 100-fold with 25 mg (dry weight) of unlabeled exponential phase cells harvested at a density of 0.5 mg/ml, and suspended in 5 ml 200 mM Tris · HCl (pH 8.0). Spheroplasts were prepared at 0—4°C [17]; spheroplasting was usually complete within 30 s after the mild osmotic shock [17]. Mg^{2+} was added to a concentration of 10 mM to stabilize the spheroplasts, which were lysed in a French pressure cell (Aminco, Silver Spring, Md., U.S.A.) at 640—800 atm [18]. Membranes were collected, washed [18], and resuspended in 1 ml of a buffer containing 10 mM Tris · HCl (pH 8.0), 1 mM EDTA, and 20% sucrose. The membranes were applied on a continuous sucrose gradient (25—55% sucrose/1 mM EDTA/10 mM Tris · HCl (pH 8.0)) and centrifuged in a SW 41 rotor (Beckman Instr. Inc.) at 4°C (36 h, 284 000 × *g*). Gradients were extruded [17], fractions of 0.3 ml were collected, and 0.1 ml of each was counted.

Radioactivity determinations

Aqueous solutions were counted in a Liquid Scintillation Analyzer (Philips, Eindhoven, The Netherlands) in 10 ml toluene/Triton X-100 (2 : 1, v/v) containing 0.4% (w/v) diphenyloxazole (PPO) and 0.04% *p*-bis-2-(5-*p*-biphenyloxazolyl)benzene (POPOP) [19]. Filters were counted in 6 ml toluene containing 0.4% (w/v) PPO and 0.008% (w/v) *p*-bis-(*o*-methylstyryl)benzene (bis-MSB).

Crossover and counting efficiency correction curves for ^{14}C and ^{3}H were determined for each counting system; these curves were used to determine corrected ^{3}H and ^{14}C dpm values.

Results

Experimental approach

If outer membrane fragments are released randomly, they should contain as

much newly synthesized protein as the *E. coli* outer membranes from which they derive. However, if cells release fragments predominantly from newly synthesized or older outer membrane regions, the fragments should contain more or less new protein, respectively, than cellular outer membranes.

To distinguish between these alternatives, cells were grown overnight in the presence of one isotope (old) to label all proteins, including those in the released outer membrane fragments. At time zero, the second isotope (new) was added to the exponentially growing culture and the appearance of new protein in newly released outer membrane fragments was compared to the incorporation of new protein into the outer membrane. That is, membrane fragments released from time zero up to a given time t_x were compared to cell membranes at t_x. It was necessary therefore to stop the incorporation of newly labeled protein into cell membranes at t_x, and to obtain the outer membrane fragments which had been released from the cells up to t_x.

Extrapolation of the incorporation data to zero time indicated that incorporation of new label into total cell protein ceased within 10 s after a portion of cells was cooled to 0°C at t_x. Since this time is short compared to the time scale of our experiments, rapid cooling appeared to be a satisfactory method to stop incorporation of new label into cells at t_x. The outer membrane fragments released by cells were separated from the cooled cells by centrifugation. It was determined in a separate experiment that rapid cooling and standing at 0°C for 45 min did not cause the release of outer membrane material beyond that which was present in the medium prior to cooling.

Incorporation of old and new leucine into E. coli JC411

Exponentially growing cells were labeled with [^{3}H]leucine as the long term label, and at time zero, [^{14}C]leucine was added as the new label. New and old label disappeared coordinately from the supernatant after time zero (data not shown), and were incorporated into total cell protein (Fig. 1).

To follow the incorporation of labeled protein into cell membranes, membrane separations were performed on samples obtained at 1, 4, 10, and 20 min after the addition of new label. Fig. 2 shows a typical sucrose gradient profile for such membrane separations. The cytoplasmic membrane sedimented to a density of 1.16–1.18 g/cm^3, the outer membrane to a density of 1.22–1.24 g/cm^3. The ratio of outer membrane protein to cytoplasmic membrane protein was higher when calculated based on ^{14}C content than on ^{3}H content. This phenomenon may introduce errors in comparisons of the new label content of various membrane fractions. To eliminate these errors, the use of [^{3}H]- and [^{14}C]leucine as old and new label, respectively (Fig. 1) was reversed and the results of these two different types of experiments were averaged, as shown below.

Release of newly labeled outer membrane fragments from E. coli JC411

For each sample taken in the experiments of Fig. 1, outer membrane fragments were isolated from the culture medium by gel filtration. The void volume fractions contained about 0.2% of the total long term label incorporated into the cells, in agreement with the results of Hoekstra et al. [3]. By subtracting the amount of old label found in the void volume fractions of the 0.4

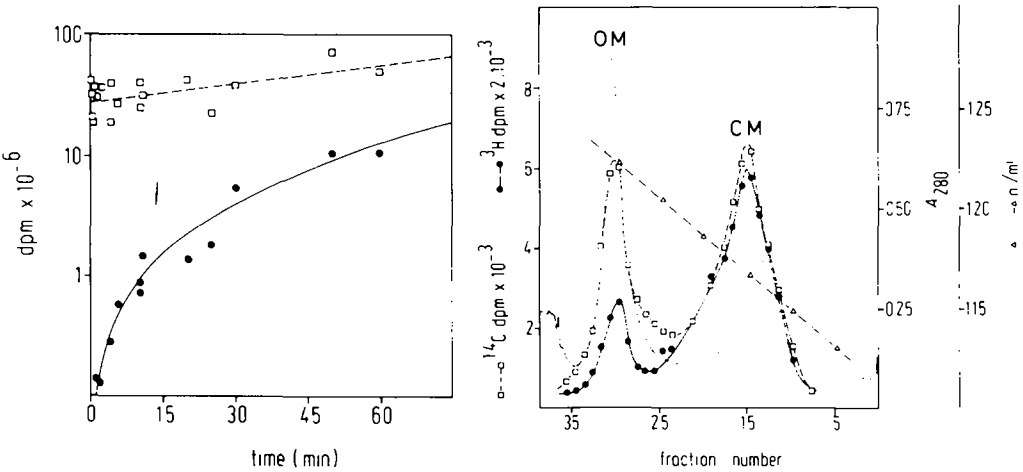

Fig. 1. Incorporation of old and new leucine into total cell protein of *E. coli* JC411. Cells were grown overnight on medium containing [^{3}H]leucine. At time zero [^{14}C]leucine was added and samples were taken during the following hour. Total radioactivity incorporated per mg cells was determined. The values given are the combined data from three experiments. □- - - - -□, ^{3}H dpm; ●———●, ^{14}C dpm.

Fig. 2. Typical separation of outer and cytoplasmic membranes of radioactively labeled *E. coli* JC411. The membrane separation shown was performed on a culture which had been labeled overnight with [^{14}C]leucine and for 20 min with [^{3}H]leucine. CM, cytoplasmic membrane; OM, outer membrane.

ml culture sample taken just before the addition of new label, from that found in the void volume fractions of each sample taken after the addition of new label, we calculated the amounts of old and new label which appeared in the outer membrane fragments released after time zero; these are the outer membrane fragments which were released after the incorporation of newly labeled proteins into the cytoplasmic and outer membranes had started.

Relative age of medium vesicles compared to cell membranes

To compare the relative age of outer membrane fragments and the cells from which they were derived, the ratio of new to old label (denoted by the symbols Q_F, Q_{OM}, Q_C, for the released outer membrane fragments, the outer membrane, the cytoplasmic membrane and the total cell protein, respectively) was determined for each fraction. Fig. 3 shows the resulting curves of Q versus time, for the experiments described in Fig. 1. A comparison of Q_F and Q_{OM} indicates that outer membrane fragments initially contained more new label than the cellular outer membrane.

Fig. 3 also shows however that new label appeared faster in the outer than in the cytoplasmic membrane (i.e. $Q_{OM} > Q_{CM}$), which is explained at least in part by the fact that [^{3}H]leucine (old label) is incorporated or detected relatively less effectively in the outer membrane than [^{14}C]leucine (new label), as shown in Fig. 2. Thus, the ratio $^{14}C/^3H$ can be expected to be greater for the outer membrane than for the cytoplasmic membrane. Since such considerations may also apply to the differences between Q_F and Q_{OM}, the results of Fig. 3 cannot be interpreted unequivocally. Accordingly, the experiment of Fig. 3 was performed with [^{14}C]leucine as the long term label, while [^{3}H]leucine was added

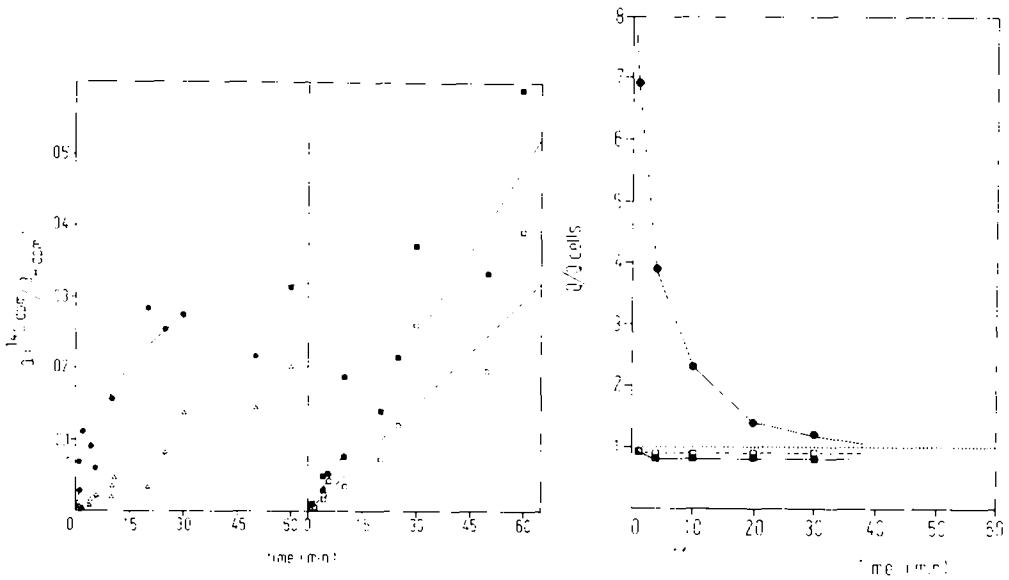

Fig. 3. The incorporation of new label into whole cells, released outer membrane fragments, and cytoplasmic and outer membranes. Cells were labeled overnight with [^{3}H]leucine as shown in Fig. 1, and culture samples were removed at various times after the addition of new label ([^{14}C]leucine). The ratio Q (dpm in new label/dpm in old label) was determined as a function of time for each of these fractions. ○- - - - -○, Whole cells; ●———●, outer membrane fragments; □- - - - -□, cytoplasmic membrane; ■———■, outer membrane. The values given are the combined data from three experiments.

Fig. 4. Comparison of the incorporation of newly labeled proteins into released outer membrane fragments and cell membranes. The data of Fig. 3 and the reverse experiments were averaged (see text) to yield the ratio Q/Q_C, which shows the content Q of new label in a given fraction as compared to the content Q_C of new label in whole cells. . . . . Q_C/Q_C; ●———●, Q_F/Q_C; ■———■, Q_{OM}/Q_C; □- - - - -□, Q_{CM}/Q_C.

at time zero. If differences in the content of new label in two fractions (e.g. $Q_{OM}/Q_{CM} = 1.5$) are due to artifacts, then the reversal of labels should also reverse the relative content of new label in these fractions (e.g. Q_{OM}/Q_{CM} should be $1/1.5 = 0.67$). The corrected relative content of new label in these two fractions is the geometric mean of the results obtained in the two experiments (e.g. $Q_{OM}/Q_{CM} = \sqrt{1.5 \times 0.67} = 1$).

The experiment with labels reversed was performed twice (data not shown). In these experiments $Q_{OM} < Q_{CM} < Q_C$, indicating that the differences between Q_{OM}, Q_{CM}, and Q_C were in fact due to differences in incorporation or counting efficiency of label in different cell fractions. Differences between Q_F and the other Q values remained however, indicating that new label appeared sooner in outer membrane fragments than in the other cell fractions.

The results of Fig. 3 and those obtained with the labels reversed were avereaged after expressing all results in terms of Q_C (i.e. by calculating Q/Q_C for all Q values at 1, 4, 10, 20, and 30 min). The resulting plots of Q/Q_C versus time (Fig. 4) show that the released outer membrane fragments contained a relatively high amount of newly synthesized protein shortly after the addition of new label, compared to either the cellular outer membrane, the cytoplasmic membrane or the total cell protein. In time, the relative amount of newly syn-

thesized protein in the released outer membrane fragments approached that found in the outer and cytoplasmic membranes and in whole cells.

Discussion

The results of Fig. 4 imply that outer membrane fragments must be released from insertion regions where most of the newly synthesized outer membrane proteins are inserted. It is unlikely therefore that there is random insertion of proteins into the outer membrane; if this did occur there would be no difference in the new protein content of released fragments and the outer membrane they originate from.

Since shortly after the addition of new label the new protein content of the released outer membrane fragments exceeds that of the cellular outer membrane by more than 7.5-fold (Fig. 4), it can be calculated * that protein insertion regions cover less than 13% of the total outer membrane. This value is comparable to that estimated for lipopolysaccharide insertion regions, which cover 15—30% of the outer membrane of *E. coli* J5 [20].

In recent years much research has been done on the topology of membrane growth (for reviews see refs. 21 and 22), resulting in data for the outer membrane varying from a single equatorial growth zone [23] and polar growth zones [24] to many, evenly spread insertion regions [25]. A comparative study of the protein composition of the released fragments studied here and the outer membrane they originate from ref. 3 shows that the released outer membrane fragments lack protein G, an outer membrane protein with a molecular weight of 15 000. A positive relationship between protein G and inhibition of septation has been suggested [26,27]; its absence from outer membrane fragments may indicate therefore that these fragments are released from the outer membrane regions where septation occurs at the time of cell division. This conclusion is also favoured by electron microscopic studies of septum formation in *E. coli* B/r, where vesicles have been observed on and near the septum [10], and by studies of the growth pattern and cell division in *Neisseria gonorrhoeae* [13].

Lateral diffusion of outer membrane proteins

The new protein content of the released outer membrane fragments decreases with time (Fig. 4). This may be explained as follows. As time goes on after the addition of new label, newly labeled proteins will continue to be

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* When cells are exposed to a steady state label (O for old), it accumulates to the extent of O_{ir} and O_{OM} in the insertion regions and the total outer membrane, respectively. When cells are exposed to new label (N) at time zero, it will appear in insertion regions (N_{ir}) and in the total outer membrane (N_{OM}). The ratios Q_{ir} and Q_{OM} of new to old label will be, respectively: $Q_{ir} = N_{ir}/O_{ir}$ (Eqn. 1); $Q_{OM} = N_{OM}/O_{OM}$ (Eqn. 2). If outer membrane fragments originate from insertion regions, the content of new label in released outer membrane fragments reflects the content of new label in insertion regions; thus, $Q_{ir} = Q_F$ (Eqn. 3). Fig. 4 shows that as time increases, the ratio O_F/Q_{OM} decreases. Conversely, as t approaches zero, Q_F/Q_{OM} exceeds 7.5, while N_{OM} must approach N_{ir} because there will have been little or no diffusion of new label from insertion regions to the remainder of the outer membrane at very small t. Thus, at very small t, from Eqns. 1, 2 and 3, $Q_F/Q_{OM} = Q_{ir}/Q_{OM} = (N_{ir}/O_{ir})/(N_{ir}/O_{OM}) > 7.5$ or $O_{ir}/O_{OM} < 0.13$, and it appears, therefore, that on the average, insertion regions cover no more than 13% of the total outer membrane surface.

inserted into the insertion regions, but in the meantime newly labeled proteins also diffuse to the remainder of the outer membrane, raising the percentage of newly synthesized proteins there. After some time (depending, among other things, on the lateral diffusion rate of proteins in the outer membrane) the content of newly synthesized proteins in the rest of the outer membrane will approach that in the insertion regions.

From Fig. 4 it can be calculated * that the diffusion constant of proteins in the plane of the outer membrane has a maximum value of about $5 \cdot 10^{-13}$ cm^2/s. As yet no other quantitative data on the lateral diffusion constant of outer membrane proteins of gram-negative bacteria are available, but Ryter et al. [23] reported slow randomization of the bacteriophage λ receptor in the outer membrane of *E. coli*. In contrast, lateral diffusion constants varying from around 10^{-11} cm^2/s [28] to $2 \cdot 10^{-9}$ cm^2/s [29] have been reported for proteins in plasma membranes. However, it is not unreasonable that the diffusion of proteins in the outer membrane *E. coli* should be 20—400 times slower than that of proteins in plasma membranes. First, it is generally accepted that the fluidity of the outer membrane is relatively low compared to that of other biological membranes as a consequence of the low phospholipid content of the outer membrane [30], only part of which is actually present in the form of a bilayer [31]. Although the outer membrane phospholipid content of different *E. coli* strains may vary considerably [32], these arguments probably also apply to *E. coli* JC411 since its outer membrane contains only 0.20 mg phospholipid/mg protein [3]. Second, Braun's lipoprotein [33] is linked covalently to the underlying murein layer at a frequency of about 50 000 links/μm^2 of cell surface [14]; on the average, therefore, the distance between adjacent bound lipoprotein molecules is only 4—5 nm. Third, another major outer membrane protein (the matrix protein described by Rosenbusch [34]) or set of major proteins [35] is bound very tightly to the murein layer. Thus, interaction between the murein layer and the outer membrane may well restrict the free diffusion of most or all of the outer membrane proteins to a considerable extent. These arguments may also explain the low value for the lateral diffusion constant for lipopolysaccharides in *Salmonella*, which is about $2.9 \cdot 10^{-13}$ cm^2/s [36].

Given the above considerations, there may in fact be little or no free diffusion of proteins in the outer membrane; instead, newly inserted proteins may simply push away older proteins already present in the outer membrane. Older proteins may resist these pushing forces because of the covalent and non-covalent interactions between these proteins and the murein layer. As a result, newly synthesized outer membrane may bulge outward a little as new material is inserted. Normally, small bulges may disappear as covalent and non-covalent interactions are established between newly inserted outer membrane proteins

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* The diffusion constant D was estimated with the formula $D = x^2/4t$, where x is the average distance traveled by a diffusing molecule in a time t [28]. Extrapolation of Fig. 4 indicates that it takes about 50 min before randomization of newly inserted proteins is complete. The maximum distance which proteins need to travel for complete randomization to occur would be about 0.8 μm (a half cell length) if the insertion region(s) occupy an equatorial band around the cell. In that case $D = 5 \cdot 10^{-13}$ cm^2/s. If the insertion regions are spread out over the cell surface, the average distance traveled for complete randomization decreases, as does D. Thus, the value calculated above is an upper limit for the average diffusion constant of proteins in the outer membrane.

and the murein layer. Once an outer membrane bulge reaches a certain size however, it may no longer be possible to link the newly synthesized outer membrane to the murein layer, in which case the bulge may continue to grow until it is released as an outer membrane vesicle.

Regardless of how outer membrane vesicles originate, however, the fact that they are derived from insertion regions suggests that they will be useful in obtaining more information on the synthesis and structure of the outer membrane, especially with respect to the question of the coupled insertion of lipopolysaccharide, phospholipid and protein into the outer membrane.

Acknowledgements

We thank M. Gruber, D. Hoekstra, and L. de Leij for useful discussions, N. Panman for drawing the figures and D. Ellens for typing the manuscript.

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