The spatial distribution of phospholipids and glycolipids in the membrane of the bacterium *Micrococcus luteus* varies during the cell cycle

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Abstract Recently, we have developed a photocrosslinking approach which uses anthracene as a photoactivatable group and which allows us to determine the lateral distribution of lipids in membranes quantitatively. In synchronous cultures of the gram-positive bacterium *Micrococcus luteus*, this approach shows that the spatial distribution of phosphatidylglycerol and dimannosyldiacylglycerol, the two major lipids in the bacterial membrane, varies greatly during the cell cycle. Minimum heterogeneity was observed during cell growth while maximum heterogeneity was detected during cell division.

Key words: Lipid distribution; Anthracene; Photocrosslinking; Cell cycle; *Micrococcus luteus*

1. Introduction

Today, biological membrane cannot be described as homogeneous systems with bulk phase physical properties and regular distributions of lipids and proteins. In addition to a marked asymmetry in the transverse distribution of these molecules [1,2], membranes display lateral microcompartmentations which extend from the nanometer up to the micrometer scale [3–11]. However, there is no unique definition of these membrane domains and their structure and composition are still poorly understood. The functional significance of these microstructures and the mechanisms which contribute to their existence also remain to be elucidated.

Recently, we have developed a photocrosslinking approach which enables the lateral distribution of the various molecular species of lipids present in a membrane to be determined quantitatively [12]. This approach uses anthracene as a photoactivatable group after it has been attached to lipids via the anthracene-fatty acid 9-(2-anthryl)nonanoic acid (ANno) [13]. Lipids labeled with ANno display phase and physical properties similar to those of normal lipids [13]. As another advantage, ANno can be incorporated into the lipids of prokaryotic [14] and eukaryotic [15] cells at a high rate via regular lipid metabolic pathways, thus enabling constitutive lipids to be studied in situ. In the membrane of the gram-positive bacterium *Micrococcus luteus*, this approach coupled with a study of the transverse distribution of lipids revealed the existence of heterogeneities in the lateral distribution of the two major lipids, namely phosphatidylglycerol and dimannosyldiacylglycerol [16]. These results were obtained with non-synchronously growing cells, harvested at the end of the exponential phase of growth. It has recently been suggested that changes in the distribution of lipids might occur in bacterial membranes during the cell cycle [17]. When applied to synchronously dividing *M. luteus* cells, our photochemical approach shows that the spatial distribution of phosphatidylglycerol and dimannosyldiacylglycerol varies greatly during the cell cycle.

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Abbreviations: PG, phosphatidylglycerol; DMDG, dimannosyldiacylglyerol; ANno, 9-(2-anthryl)nonanoic acid; ^{3}H-ANno, 9-(2-anthryl) [9-^{3}H(N)]nonanoic acid.

2. Materials and methods

2.1. Chemicals

Syntheses of 9-(2-anthryl)nonanoic acid (ANno) [13] and 9-(2-anthryl)[9-^{3}H(N)]nonanoic acid (^{3}H-ANno, spec. act. 9.25 Gbq/mmol) [16] have already been described. 2-Phenylethanol was obtained from Sigma (USA).

2.2. Bacterial culture

M. luteus (ATCC 4698-4) was grown in PWYE peptone-containing culture medium at 30°C under agitation as previously described [16]. Absorbance of cell suspensions was measured with a spectrophotometer (model Lambda 16, Perkin-Elmer, UK) at a wavelength of 550 nm.

2.3. Cell synchronization

Cells in the exponential phase of growth ($A_{550} \sim 7$) were incubated for 45 min at 30°C under agitation in the presence of 2-phenylethanol (0.75%, v/v) and then submitted to low-speed centrifugation at 4°C ($1000 \times g$, 5 min). Stopping a synchronous culture to remove cell aliquots was found to disturb cell synchronization. For that reason, the pelleted synchronous cells were resuspended in the normal growth medium at moderate concentration ($A_{550} \sim 4$) and distributed over 10 culture flasks (10 ml cell suspension in a 100 ml flask). Except for one (sample 1 at time 0), these flasks were incubated simultaneously in a shaker incubator (model G24, New Brunswick, USA) at a temperature of 30°C. They were withdrawn one by one from the incubator, the first after 45 min (sample 2) and the others at 30-min intervals (samples 3–10) whilst maintaining agitation and their cell content was processed for the desired assay. To count cells, 1 ml of cell suspension was diluted 10^7 fold and 100 µl of this diluted suspension were sprayed onto PWYE agar plates with glass beads. After 48 h incubation at 30°C, cell colonies were counted.

2.4. Metabolic incorporation of the anthracene-fatty acid into synchronously dividing cells

For photodimerization experiments, cells were grown in the presence of radioactive anthracene fatty acid from the beginning to the end of the culture. The culture medium was supplemented with fatty acid-free bovine serum albumin (1 mg/ml) to favor dispersion of the anthracene fatty acid [15]. ^{3}H-ANno (spec. act. 4.6 GBq/mmol) dissolved in dimethylformamide (10 mg/ml) was slowly added to the culture medium with a syringe, under gentle stirring, up to a final concentration of 40 µg/ml. Stable microemulsions were obtained by two brief sonications (30 s each) of the suspension in a water bath type sonicator. Cells were synchronized and cultured as described above. For each flask removed from the incubator, the 10 ml cell suspension was divided into two identical batches.

(i) One batch, for the counting of lipid monomers, was immediately poured into 15 ml of a chloroform/methanol mixture (1:2, v/v) chilled to a temperature of −20°C and maintained at this temperature overnight. The various samples were warmed to 20°C, agitated, further

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maintained 1 h at room temperature and then centrifuged. 5 ml water and 5 ml chloroform were added to the monophasic supernatant and lipids were recovered from the chloroform phase. This slightly modified Bligh and Dyer [18] extraction procedure yielded the lipids while preventing excessive conversion of phosphatidylglycerol into cardiolipin [19].

(ii) The other batch, for photodimerization experiments, was submitted to the following procedure.

2.5. Photoirradiation of bacteria

As previously described [16], 2-ml samples of the bacterial suspension (A_{550} = 5) were illuminated for 3 min at a temperature of ~30°C in a fluorescence cuvette disposed on an optical bench transmitting light (above 340 nm) from a 200-W mercury arc lamp. The illuminated cells were washed to remove residual anthracene fatty acid present in the cell wall and the lipids were extracted as described above.

2.6. Lipid separation and radioactivity counting

Total lipid extracts from illuminated or nonilluminated bacteria were dissolved in a certain volume of chloroform/methanol/water (90:10:0.5 v/v) in proportion to the absorbance of the original bacterial suspension, at a rate of 100 μl for an absorbance of 10. 10 μl of these solutions (each containing about 40 μg lipids) were deposited on channelled 60CF254 silica gel thin-layer plates (Merck, Germany) composed of a series of 1 cm wide by 20 cm long migration lanes which forced the lipids to move linearly and which were well suited in size to the 1.5 cm wide by 25 cm long counting window of the radioscanner used for direct counting of the radioactive anthracene-labeled derivatives on the plates (Automatic TLC-Linear Analyzer, Berthold, Germany). After migration of the lipids with chloroform/methanol/acetic acid/water (65:25:10:4 v/v), the plates were dried overnight and scanned. As control experiments, increasing amounts of purified radioactive anthracene-labeled dimannosyldiacylglycerol were deposited on channelled plates, chromatographed and scanned as described above. Radioactivity counting was found to be strictly proportional to the amount of lipid deposited. With this procedure of constant weight lipid deposit coupled with careful drying of the plates after lipid migration, the reproducibility of radioactivity counting was better than 5%. The data presented for each sample of the growth curve are issued from three thin-layer chromatograms, each scanned five times. Because metabolic incorporation of ANno occurs nearly exclusively at the *sn*-1 position on the glycerol backbone (which means one anthracene fatty acid per labeled lipid molecule) [14], the relative radioactivity countings attached to lipid monomers and dimers could be converted directly into relative weights of lipids expressed in mol%.

2.7. Calculation of lipid distribution

The theoretical background for calculating the lipid distribution has been exposed in detail elsewhere [16]. Briefly, the lipid phase in a membrane can be considered as a collection of n molecules which distribute between $x_1,...x_j,...x_n$ molecules ($x_1 + ...x_j + ...x_n = n$) of $M_1,...M_j,...M_n$ different molecular species, respectively. However, it is not necessary to count all the lipid homo-and heterodimers which can form in a biological membrane. Analysis of the system can be restricted to only two molecular species M_i and M_j and the probabilities of forming homo-and heterodimers in case of random distribution of these molecules are:

$$p(M_i{-}M_i) = x_i^2/(x_i + x_j)^2$$

$$p(M_i{-}M_j) = 2x_ix_j/(x_i + x_j)^2$$

$$p(M_j{-}M_j) = x_j^2/(x_i + x_j)^2$$

Interestingly, (i) it is not the absolute value of these probabilities which is the important parameter but their relative values and (ii) from the relative weight of the homo-and heterodimers formed between two molecular species, it is possible to go back to the relative weight of the two native monomers in the original mixture [16].

In this study, as in the previous one [16], we have restricted our analysis to phosphatidylglycerol and dimannosyldiacylglycerol and the following procedure was applied: (1) The relative weights of the homo-and heterodimers originating from the two lipids after photoirradiation of the bacteria were determined. (2) From these values, the relative weights of the two native lipid monomers were calculated back and compared to those found when counting the lipid monomers extracted from the membrane of non illuminated bacteria. (3) These relative weights of monomers so computed were used to recalculate the relative weights of the dimers with the assumption of random distribution of the two lipids within the membrane. (4) Finally, the experimental and calculated relative weights of dimers were compared.

As a measure of the difference between experimental and calculated values, we introduce the heterogeneity index δ as:

$$\delta_{ij} = |x_{ii}^{exp} - x_{ii}^{calc}| + |x_{ij}^{exp} - x_{ij}^{calc}| + |x_{jj}\text{exp} - x_{jj}^{calc}|$$

in which x_{ii}, x_{jj} and x_{ij} are the relative weights of the homo-and heterodimers, respectively, expressed in mol% ($x_{ii}+x_{jj}+x_{ij}=100$). δ is nil in the case of random mixing of lipids.

3. Results

Good cell culture synchronization was achieved by combining two procedures as described by Lacave et al. for *Mycobacterium aurum* [20]: (i) cell incubation in the presence of 2-phenylethanol which stops DNA synthesis in a reversible way and (ii) low-speed cell ccentrifugation which leads to the selection of certain cells in size. This procedure gave the stepwise absorbance versus time growth curve shown in Fig. 1, which accounts for synchronously dividing cells with a doubling time of ~90 min and which can be compared to the continuous normal growth curve. In parallel and as a control experiment, the number of cells present in each sample of the growth curve was inferred from the number of colonies

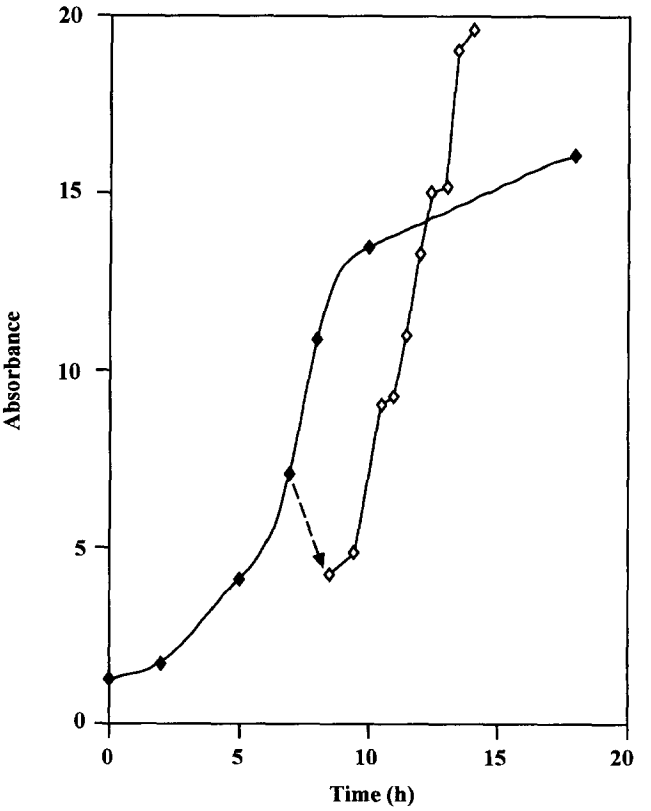

Fig. 1. *Micrococcus luteus* ATCC 4698-4 non-synchronous (◆) and synchronous (◇) growth curves. Absorbance of cell suspensions was measured at the wavelength of 550 nm. The dotted line indicates the cell density at which non-synchronous cells were processed (incubation in the presence of phenylethanol and then centrifugation) to promote cell synchronization.

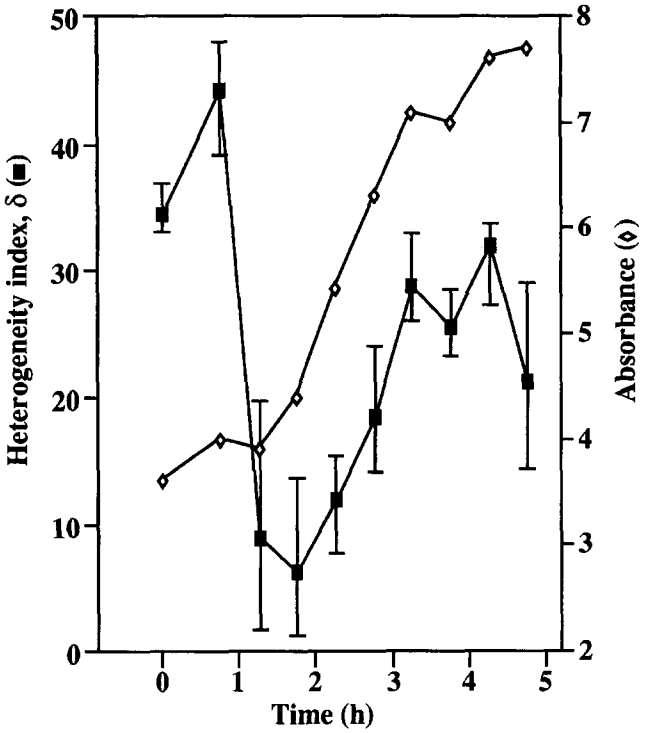

Fig. 2. Growth curve (◇) and heterogeneity index δ (■) for synchronous cultures of *Micrococcus luteus* in the presence of 9-(2-anthryl)nonanoic acid. Absorbance of cell suspensions was measured at the wavelength of 550 nm. δ relates to the spatial distribution of phosphatidylglycerol and dimannosyldiacylglycerol, the two major lipids within the membrane. It is defined as the sum of the absolute differences between experimental and calculated dimer relative weights (see text). Bars indicate dispersion of the data.

counted after subculture of cell aliquots on agar plates. The number of cells was found to be proportional to the absorbance of the original cell suspension and near doubling of the cell population was observed from one plateau to the other (data not shown). Cell synchronization was also assessed by the fact that growth curves were constructed from 10 independent culture flasks, thus indicating that the various bacterial batches were synchronous in themselves and with respect to each other.

As can be seen in Fig. 2, the presence of ANno in the culture medium did not modify cell growth and synchronization. From the absorption spectra of known amounts of purified lipids, about 15 mol% ANno were incorporated with respect to the lipids.

Fig. 3 shows typical thin-layer chromatograms of total lipid extracts from non-illuminated and illuminated bacteria. As previously described, cardiolipin, phosphatidylglycerol and dimannosyldiacylglycerol constituted the major lipids in the bacterial membrane, with only traces (~3%) of phosphatidylinositol [16]. PG and DMDG homo-and heterodimers formed the major dimer products. They could be easily separated from each other and from the lipid monomers. Cardiolipin gave only small amounts of homodimers and the dimers of PI with the other lipids could be ignored [16].

Consequently and as previously described [16], analysis of dimer products could be restricted to those formed between PG and DMDG. The values of the heterogeneity index δ obtained for the various samples of a synchronous culture have been plotted in Fig. 2, together with the corresponding growth curve. Table 1 shows the experimental and calculated relative weights of monomers and dimers and the corresponding values of δ for samples 2 and 4 of the growth curve. As a first remark, the relative weights of monomers determined experimentally or by back-calculation were similar. As can be seen in Fig. 4, they remained practically unchanged during the cell cycle. It is more than likely that the systematic and slight differences in lipid composition found between illuminated and non-illuminated bacteria originate from differences in the conversion rate of phosphatidylglycerol into cardiolipin. Consistently, the calculated relative weights of monomers were used to recalculate the relative weights of dimers. As a second observation, it is remarkable to observe that the experimental and calculated relative weights of dimers differed greatly (δ~45) in sample 2 which, at the beginning of the first plateau of the growth curve, corresponds to the end of the first period of cell division. By contrast, they tend to be similar (δ~6) in sample 4 which, like sample 3, corresponds to the period of cell growth which begins the next cell cycle. Then, δ was observed to increase again reaching a value of ~30 for sample 7, at the end of the second period of cell division. At the end of the culture (sample 10, A_{550} ~8), and probably due to partial loss of cell synchronization, δ stabilized around an intermediate value of 25. The same trend, i.e. low δ value during cell growth and high δ values during cell division, was observed when duplicating this experiment (data not shown).

4. Discussion

The above data clearly shows the existence of large devia-

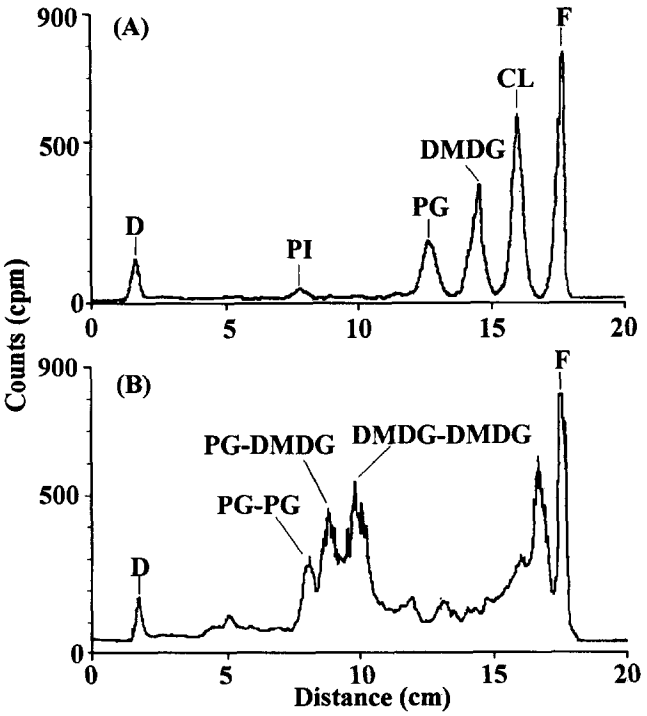

Fig. 3. Thin-layer chromatograms of lipid monomers (A) and dimers (B) from *Micrococcus luteus* cells cultured in the presence of radioactive 9-(2-anthryl)nonanoic acid. Lipid monomers and dimers migrated as indicated. D and F refer to lipid deposit and solvent front, respectively. Radioactivity was counted directly on the chromatograms using a radioscanner.

Table 1
Experimental and calculated relative weights of phosphatidylglycerol (A) and dimannosyldiacylglycerol (B) monomers and dimers in the membrane of *M. luteus* for samples 2 and 4 of the synchronous growth curve in Fig. 2 (also shown is the corresponding heterogeneity index δ)

	Experimental relative weights (mol%)					Calculated relative weights (mol%) for a homogeneous distribution of the two lipids					
	Monomers		Dimers			Monomers		Dimers			
	A (I)	B (II)	A-A (III)	A-B (IV)	B-B (V)	A (VI)	B (VII)	A-A (VIII)	A-B (IX)	B-B (X)	δ
Sample 2	46	54	23	23	54	35	65	12	45	43	45
Sample 4	32	68	17	45	38	40	60	16	48	36	6

Columns I–V show the experimental relative weights of the two monomers and the three dimers obtained from radioactivity counting on thin-layer chromatograms. Columns VI and VII correspond to the relative weights of monomers calculated from the experimental relative weights of dimers (columns III–V). Columns VIII–X show the relative weights of dimers which are computed using the calculated relative weights of monomers (columns VI and VII) and assuming a homogeneous distribution of lipids. δ is the sum of the absolute differences between the experimental and calculated relative weights of dimers.

tions between the experimental and calculated distributions of anthracene-labeled PG and DMDG species in synchronously dividing *M. luteus* cells. As previously shown [16], these deviations are not due to any artifact or to any peculiar properties of these lipids since in mixed liposomes of known composition, the photodimerization technique revealed random distribution of anthracene-labeled PG and DMDG molecules [16].

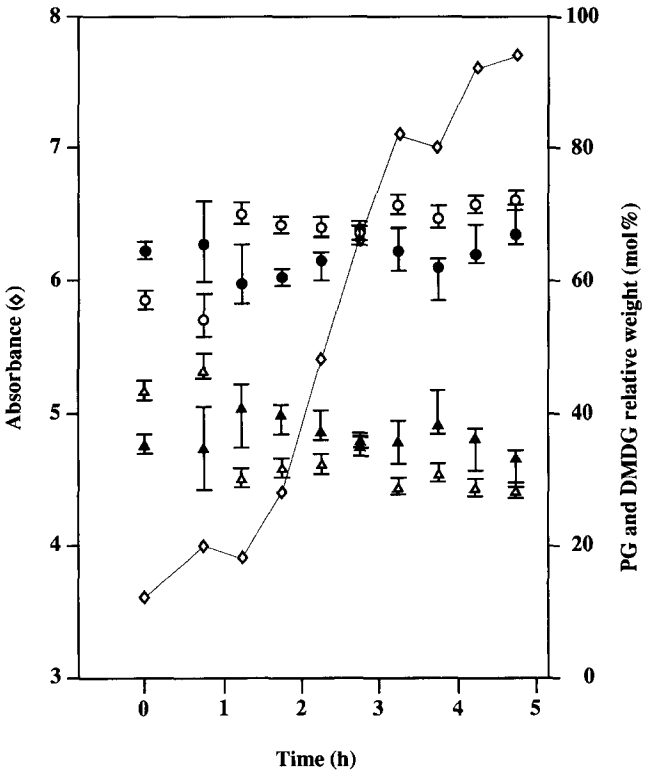

Fig. 4. Relative weights of monomeric anthracene-labeled phosphatidylglycerol (△,▲) and dimannosyldiacylglycerol (○,●) in the membrane of synchronously dividing *Micrococcus luteus* cells. Open symbols (△,○): relative weights found experimentally by counting, on thin-layer chromatograms, the radioactivity attached to lipid monomers extracted from non-illuminated bacteria. Closed symbols (▲,●): relative weights calculated from the relative weights of PG/DMDG dimers obtained experimentally by counting, on thin-layer chromatograms, the radioactivity attached to lipid dimers extracted from illuminated bacteria. Also shown is the corresponding absorbance versus time growth curve. Bars indicate dispersion of the data.

PG and DMDG from *M. luteus* were also shown to display good miscibility properties [21]. In addition, lipids in this bacterium are more than 80% substituted by *iso*-and *ante-iso* methyl-branched myristic and palmitic acids, a class of fatty acids which confers great fluidity to the lipids over a wide temperature range [14].

Because of the well defined localization of the anthracene group within the hydrocarbon chain region of the lipid bilayer [13], dimerization can be considered to occur almost exclusively within each leaflet of the lipid bilayer [12,16]. This means that non nil δ values can account for both transverse and lateral heterogeneities in lipid distribution. In our previous study with non-synchronous cell cultures [16], this indetermination was removed by following the kinetics of oxidation of PG and DMDG by periodic acid in the membrane of protoplasts. The two lipids were shown to be distributed evenly between the two leaflets of the membrane from which it was concluded that the observed deviations between experimental and calculated lipid distributions reflected heterogeneities in the lateral distribution of the two lipids. In the present study, and for obvious practical reasons, it was not possible to determine the transverse distribution of PG and DMDG for each sample of the growth curve. However, simple calculation shows that to be accounted for in terms of transverse asymmetry, a δ value of ~45 would require about 85% of one of the two lipids on one side and 85% of the other lipid on the other side. This is in contradiction with our previous results [16] and even though a certain extent of transverse asymmetry cannot be ruled out, it seems reasonable to assume that the high δ values found with synchronous cultures also reflect the existence of heterogeneities in the lateral distribution of the anthracene-labeled PG and DMDG molecules in the membrane. The high incorporation rate of the anthracene fatty acid into the bacterial lipids, the regular phase behaviour of anthracene-labeled lipids and their good miscibility with other lipids [13] mean this conclusion can be extended to the host lipids [16].

These heterogeneities in lipid distribution suggest the existence of PG-rich and DMDG-rich domains within the membrane of *M. luteus*, the structure and functional significance of which remain to be elucidated. However, and on account of the regular phase behaviour of both the anthracene-labeled and bacterial lipids, it can be concluded that proteins play a key role in the spatial organization of the membrane. Our results are also relevant to the interesting hypothesis, devel-

oped for the bacterium *Escherichia coli*, that phospholipid domains within the cytoplasmic membrane determine the spatial organization of the cell cycle [17] and may be involved in differentiation in bacteria [22]. In this model, it is postulated that the formation of phospholipid domains positions division sites. The particular phospholipid composition of a domain attracts particular proteins and determines their activity. Principally via such proteins, the interaction of the chromosome with the membrane creates a chromosomal domain. The development of chromosomal domains during replication and nucleoid formation contributes to the formation and positioning of a septal domain between them. In addition, and during the division step, drastic changes in membrane curvature in the septal zone may cause local alteration in the lipid composition [23]. Implicitly, in this dynamic view of membrane biogenesis and cell division, the postulated phospholipid domains are supposed to change in size and composition during the cell cycle. Although we cannot discriminate between these two possibilities, our results clearly indicate that the spatial distribution of lipids in the membrane of the bacterium *M. luteus* varies during the cell cycle, with maximum heterogeneity during the step of cell division. These observations strongly support the concept that changes in membrane organization are involved in the important processes of membrane biogenesis and cell division and provide experimental support to the above theoretical model.

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