Phospholipid Metabolism and Membrane Synthesis During Sporulation in Bacillus megaterium

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In view of previously published reports of localized membrane growth in exponentially growing Bacillus megaterium and in sporulating Bacillus cereus, an attempt was made to describe phospholipid metabolism and the topology of membrane synthesis during sporulation in B. megaterium. The cells were pulsed with radioactive glycerol or acetate at the time of septum formation, and the specific activity of the lipid fraction was measured at various times through the free spore stage. The bulk of the material labeled during septation could not be recovered in the spore. Rather, it was found that the labeled lipid fraction underwent considerable turnover during spore development. Additionally, other experiments revealed that the lipid made before the initiation of sporulation was also subject to extensive turnover. In order to minimize both the confounding effects of lipid turnover and the possible presence of lateral diffusion of labeled lipid in the membrane, autoradiography of cells pulse labeled with radioactive glycerol at the time of septation was performed; a symmetrical grain distribution resulted. Thus, despite previously published suggestions to the contrary, the current experimental techniques could not demonstrate the existence of localized membrane synthesis in B. megaterium during sporulation.

As an early step in bacterial endospore formation, the cell is divided into two compartments of unequal size by a septum, thereby defining one end of the cell as the presumptive spore or forespore. Subsequently, the forespore is surrounded by a second membrane in a process referred to as engulfment. Although these phenomena are very definitely a unique feature of a differentiating system, they have also been viewed as representing a modification of normal cell division (14). As a consequence, a study of the nature of membrane synthesis during sporulation may have relevance not only for the elucidation of bacterial differentiation, but also for an understanding of membrane synthesis during cell division and growth in general.

Recently, considerable effort using a variety of approaches has gone into establishing the topology of bacterial membrane synthesis (9, 16). The majority of the data from rod-shaped organisms has suggested that the bacterial peripheral membrane grows all over in a symmetrical fashion (13, 19). On the other hand, there has been a report that polar membrane growth occurs in Bacillus megaterium (20) and that there is localized membrane synthesis at the site of forespore septum formation during sporulation in Bacillus cereus (8). This paper reports the results of experiments designed to investigate membrane synthesis during septation and engulfment in sporulating cells of B. megaterium. Particular emphasis is placed on differentiating between symmetrical and localized growth. The difficulties encountered in detecting the mode of membrane synthesis are discussed.

MATERIALS AND METHODS

Bacterial strain and growth conditions. All experiments were conducted with a strain of B. megaterium kindly provided by Charles Gilvarg. The minimal medium and growth conditions used and the sporulating properties of the organism have been described (11). The cessation of vegetative growth and initiation of sporulation, as reflected by a drop in turbidity, were monitored with a Klett-Summerson colorimeter.

Purification of spores. Sporulating cells were incubated until the majority of the refractile bodies were released from their sporangia. The cells were then harvested, resuspended in a small volume of water, and subjected to sonic oscillation for 1 min at 3 A (Branson Sonifier, Branson Instruments, Inc., Stamford, Conn.). The volume was then adjusted to 20 ml, and the cells were centrifuged through 62% Renografin (E. R. Squibb & Sons, New York, N.Y.) (12). The spore pellet appeared, by microscopy, to be essentially 100% clean spores.
Cell breakage. Reports in the literature (1, 7), as well as data from this laboratory (unpublished data), point to the desirability of cell breakage in order to ensure extraction of all phospholipid classes uniformly. Therefore, the following procedure was used prior to all lipid extractions. Harvested cells or clean spores (10⁸ to 10¹⁰) resuspended in 1 ml of water were added to a Toothmaster Amalgamator Capsule (The Toothmaster Co., Racine, Wis.) which was half-filled with glass beads. The capsule was sealed with melted paraffin to prevent leakage and agitated in a Hi-Speed Amalgamator at a setting of high for either 30 s (vegetative cells) or for two 30-s bursts separated by a 30-s rest (spores). The broken cells were decanted into a conical tube, and the glass beads were allowed to settle. The supernatant fraction was removed, and the empty capsule and glass beads were washed with the 0.05-M solutions of water to recover any remaining cellular material. The washes were combined with the original supernatant fraction, and the lipid was extracted.

Lipid extraction. Sporangia (5 to 40 ml) were added to an equal volume of cold 10% trichloroacetic acid and harvested immediately. The harvested cells were broken as described above and then extracted in chloroform-methanol (1:2, vol/vol) according to the procedure of Bligh and Dyer (2). The extracted lipids were taken to dryness at 40°C under a stream of nitrogen. Clean spores were treated in essentially the same manner except that the trichloroacetic acid precipitation was omitted.

Thin-layer chromatography. Extracted lipids were resuspended in methanol-chloroform (1:1, vol/vol) and spotted on silica gel plates. The phospholipids were moved off the origin by ascending chromatography using a solvent system consisting of chloroform-methanol-water (1:1:0.04, vol/vol/vol). After the solvent front had proceeded approximately 10 cm from the origin, the plate was air-dried and developed in a second solvent consisting of diethyl ether-benzene-acetic acid (40:50:2:0.02, vol/vol/vol) as described previously (10). It was established in a representative experiment that the phospholipid spots traveled with the first solvent front by autoradiographic localization of material labeled with [2-¹⁴C]glycerol (16.1 mCi/mmole) by using Kodak No-Screen X-ray film. The phospholipid area was scraped with a toothpick and collected with aspiration into the large end of a Pasteur pipette containing a glass wool trap. Three successive 0.5-ml portions of chloroform-methanol (1:1, vol/vol) were dried through the pipette, and the eluant was collected directly into scintillation vials. The solvent was evaporated, and 10 ml of Liquifluor-toluene counting solution (New England Nuclear Corp., Boston, Mass.) was added. All radioactivity was assayed in a Beckman L-233 liquid scintillation spectrometer.

Saponification of lipids and extraction of fatty acids. The following procedure was used (G. Hege- man, personal communication). Cells were harvested, and total lipids were extracted as described above. A 5-ml mixture of 50% aqueous KOH-methanol-water (1:1:1, vol/vol/vol) was added to the lipids, and the tubes were flushed with nitrogen and capped. After saponification for 1 h at 65°C in a water bath, the nonsaponifiable lipids were extracted and removed by using two 5-ml portions of petroleum ether (bp 30 to 60). The remaining material was acidified to pH 2, and the fatty acids were extracted with three 5-ml portions of diethyl ether.

Phosphorus analysis. The following procedure is a modification of the procedure of Chen et al. (4). The lipid extracts and phosphorus standards were evaporated in 3-ml test tubes. Thirty to 50 uliters of 72% perchloric acid were added, and ashing was carried out at 210°C for 3 h in a metal block under reflux. The samples were cooled to room temperature and spun briefly in a table-top centrifuge to retrieve any drop-lets remaining on the walls of the tubes. A 1-ml amount of phosphate reagent diluted 1:1 with water was added, and the samples were heated for 30 min at 50°C. The absorbance was read at 820 nm against a distilled water blank. The pH in this assay is very critical. Therefore, the amount of perchloric acid used for ashing must be carefully controlled, and 50 uliters must not be exceeded.

Radioactive isotope incorporation. For the determination of the total amount of isotope incorporated, cells were exposed to the radioactive compound for the time indicated, and a sample was then precipitated with an equal volume of cold 10% trichloroacetic acid. After 30 min at 4°C, the samples were filtered through membrane filters (Millipore Corp., Bedford, Mass.; pore size, 0.45 μm), washed three times with cold 5% trichloroacetic acid and three times with water, transferred to scintillation vials, and dried. Radioactivity was assayed as described above.

Double label analysis. The counting efficiency for each sample was determined by using both the external standard of the spectrometer and a tritiated toluene internal standard. No direct heating effects were detected. The extent of spillover between the ¹⁴C and ³H channels was calculated, and all the samples were corrected accordingly.

Autoradiography. Cells (1 ml) were added to a flask containing 0.2 mCi of [2-³H]glycerol (5 Ci/ mmole; New England Nuclear Corp.). After 3 min of incubation, 1 ml of 4% OsO₄ (ice cold) was added, and the flask was plunged into an ice water bath. The cells were fixed in the cold for 3 h, washed with water, and resuspended in 1 ml of water. Microscope slides were precleaned with ethanol and water and, immediately before use, each slide was pretreated by spreading a drop of Ullricht’s adhesive to yield a thin film (3). As soon as the film had dried, a drop of cells was placed on the slide and was spread by using the side of a Pasteur pipette. The slides were air-dried overnight and then washed in three changes of cold 5% trichloroacetic acid followed by three changes of water. The slides were air-dried for 3 h and then dipped in G5 emulsion (Ilford Ltd., Essex, England) at 40°C that had been diluted 1:1 (vol/vol) with water. The slides were arranged vertically in a test tube rack to dry overnight, transferred to black bakelite boxes containing vials of silica gel, and stored in the refrigerator. At various intervals, slides were developed in D-19 (East- man Kodak Co., Rochester, N.Y.) for 2 min at 20°C. The slides were examined, and grains were counted at
RESULTS

Timing of forespore septum formation and engulfment. Because the majority of the lipids of B. megaterium can be expected to be limited to the bacterial membrane (23), experiments were conducted to establish the time of maximal lipid synthesis. At various times after the onset of sporulation (T₀), cells were exposed to a 2-min pulse of [2-¹⁴C]glycerol and assayed for cold-acid-insoluble radioactivity. The maximal rate of glycerol incorporation for the 2-min pulses was achieved between 2 and 3 h after the initiation of sporulation (i.e., between T₂ and T₃). Cells were then examined with an electron microscope at various times between T₂ and T₃. It was apparent that at T₂, a majority of the cells were in the process of septation and engulfment. All further experiments designed to label the forespore membranes were therefore conducted between T₂ and T₃.

Distribution of glycerol incorporated during sporulation. Reports in the literature have suggested that the incorporation of [2-¹⁴C]glycerol can be expected to directly reflect lipid synthesis (5). However, this suggestion has not been shown to have universal application (6). Experiments conducted with the present system revealed that only for pulses of a few minutes duration was greater than 85% of the cold trichloroacetic acid-precipitable radioactivity recovered in the lipid extract. Therefore, in all further experiments, lipid was routinely extracted prior to radioactivity determination.

Fate of lipid synthesized during engulfment and turnover of lipids during sporulation. If the membrane made during engulfment were synthesized at the site of septum formation, one might expect that this new membrane would be found in the spore. In order to establish whether, in fact, the lipid made at T₂,₇₅ is preferentially incorporated into and conserved in the resultant spore, the following experiment was conducted. At T₂,₇₅, cells were exposed to [¹⁴C]glycerol or [¹³C]acetate for 20 min and then chased with a 30-fold excess of the appropriate compound. Samples were removed at the appropriate times, phosphorus analyses were performed on the total extracted lipids, and radioactivity was measured in the phospholipids isolated by thin-layer chromatography; (it was initially determined that almost all of the extracted lipid counts were in the phospholipid fraction). The same experiments were conducted where the labeling period took place 2 h before the onset of sporulation (T₀). From the results presented in Table 1, one can see that (i) the material labeled during septation (T₂,₇₅) is not to be found preferentially in the spore, (ii) the specific activity of the lipid fraction has dropped substantially while the spore is still encased within the mother cell (T₀), suggesting progressive lipid turnover, (iii) material labeled before the onset of sporulation (T₀) is turned over to an extent similar to material labeled at T₂,₇₅, and (iv) the turnover of glycerol is more extensive than the turnover of acetate. Additional experiments were performed varying the period of lipid labeling from 5 min to indefinite periods of time (i.e., no nonradioactive chase). Essentially the same loss in specific activity was detected as described above.

<table>
<thead>
<tr>
<th>Time of label</th>
<th>Time of sample</th>
<th>Specific activity of phospholipid *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>[¹³C]acetate</td>
</tr>
<tr>
<td>T₀</td>
<td>T₀</td>
<td></td>
</tr>
<tr>
<td>T₂</td>
<td>T₂</td>
<td></td>
</tr>
<tr>
<td>T₂</td>
<td>Free spores</td>
<td>2,322</td>
</tr>
<tr>
<td>T₂,₇₅</td>
<td>T₂,₇₅</td>
<td>2,202</td>
</tr>
<tr>
<td>T₂,₇₅</td>
<td>Free spores</td>
<td>1,420</td>
</tr>
</tbody>
</table>

* Cells were exposed to [¹³C]acetate (0.4 μCi/ml) or [¹⁴C]glycerol (0.5 μCi/ml) at the times indicated. After 20 min, the radioactive label was chased with the appropriate nonlabeled compound. Phosphorus analyses were performed on total lipid extracts, and radioactivity was assayed in the phospholipid fractions.

Values are expressed as counts per minute per microgram of phosphorus.
In order to confirm the differential turnover of the glycerol- and acetate-labeled material, cells were exposed simultaneously to both \(^{14}C\)acetate and \(^{3}H\)glycerol for a 20-min pulse at \(T_{2.15}\). At appropriate times, samples were removed, and lipids were extracted. The results presented as the ratio of \(^3H\) to \(^{14}C\) (Table 2) confirm the earlier suggestion that the glycerol label is lost at a faster rate than the acetate label.

**Distribution of glycerol and acetate labels in the lipid fraction.** Although it can be reasonably assumed that the \(^{14}C\)acetate label would largely be confined to the fatty acids and the \(^{3}H\)glycerol label to the glycerol backbone of the lipid molecules, an experiment was conducted to verify this assumption. A lipid extract was made from cells that had been labeled at \(T_{2.15}\) for 20 min with both \(^{3}H\)glycerol and \(^{14}C\)acetate. The extract was subjected to alkaline methanolysis, and the \(^3H\) and \(^{14}C\) in the aqueous and ether phases were measured. As expected, virtually all of the \(^{14}C\) was associated with the fatty acid fraction, and the \(^3H\) was associated with the aqueous phase.

**Autoradiographic localization of the glycerol label.** Cells at \(T_{2.15}\) were exposed to a 3-min pulse of \(^{3}H\)glycerol and immediately prepared for autoradiography as described. The grain distribution was determined for both whole cells and half-cells (Fig. 1). Theoretical distributions for the cases in which the label is projected to be 80% localized in one-half of the cell or randomly distributed are also presented (20). A computer program was written to predict the grain distributions if less than 80% of the grains were localized in one-half of the cell. The program would not have been able to differentiate between asymmetry and symmetry if less than 15% of the grains were nonrandomly distributed. The theoretical curves generated, when matched to the data, suggested that the grains were randomly distributed. Therefore, we conclude that at least 85% of the tritium incorporated during septum formation and engulfment was randomly distributed over the cell. It was possible, however, that asynchrony of sporulation led to two populations of cells, one in which membrane synthesis was symmetrical and another where localized synthesis at the site of septum formation took place. Therefore, theoretical grain distributions were generated assuming different degrees of synchrony in the populations as well as different degrees of random labeling. It was concluded that the observed data could have been obtained in a heterogeneous population only if at least 50% of the population were synthesizing membranes symmetrically while, in the remaining septating cells, 40% or less of the grains were asymmetrically distributed. Because both light and electron microscopy observation of the sporulating cells indicated synchrony of at least 80%, we conclude that the grain distribution is essentially random in all cells, even those in the process of septum formation and engulfment.

**DISCUSSION**

**Localization of label incorporated during a pulse.** If the membrane were growing at the site of septum formation and engulfment, then a lipid precursor pulse at the appropriate time would be preferentially incorporated into the

**TABLE 2. Relative turnover of \(^{14}C\)acetate and \(^{3}H\)glycerol during sporulation**

<table>
<thead>
<tr>
<th>Time of sample</th>
<th>(^{3}H)glycerol/(^{14}C)acetate (count/min)</th>
<th>Total lipid</th>
<th>Phospholipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>(T_{2.15})</td>
<td>30.10</td>
<td>23.5</td>
<td></td>
</tr>
<tr>
<td>(T_{*})</td>
<td>8.40</td>
<td>6.4</td>
<td></td>
</tr>
<tr>
<td>Free spores</td>
<td>1.97</td>
<td>1.5</td>
<td></td>
</tr>
</tbody>
</table>

*For sporulating cells (\(T_{2.15}\)) were exposed simultaneously to \(^{14}C\)acetate (0.6 \(\mu\)Ci/ml) and \(^{3}H\)glycerol (1.0 \(\mu\)Ci/ml) for a 20-min pulse followed by a nonradioactive chase.
developing spore. Were this the case, one would expect the specific activity of the free spore lipid to be greater than the specific activity of the sporulating cell. This, in fact, was reported to be the case for B. cereus (8). However, we were not able to demonstrate this for B. megaterium. The specific activity of the spore phospholipid as reflected by the specific activity of the \(^{[3}H\)glycerol label is only about 14% of that of the septating cells at the time of the pulse (Table 1). This result can be interpreted in three ways, reflecting the occurrence of different processes. First, there is the possibility that the membrane is growing at a point remote from the area of septum formation and, therefore, the material labeled at \(T_{2,74}\) would be preferentially excluded from the developing spore. In fact, Morrison and Morowitz have reported polar growth in B. megaterium by using autoradiographic analysis of radioactive palmitate incorporated during vegetative growth (20). A second possibility to explain the data would suggest that the lipid molecules are undergoing extensive turnover during development and that the labeled material, localized at the site of septum formation, is being replaced by nonradioactive precursors. If this turnover were sufficiently extensive, the specific activity of the spore would be low. It has been estimated that the spore contains approximately 30 to 50% of the phospholipid of the sporulating cell (1, 21). Therefore, only a turnover rate which exceeds 50 to 70% of the labeled material per developmental cycle would be sufficient to obscure detection of localized growth if it were present. Finally, it is possible that the membrane is either made symmetrically or that a localized label becomes symmetrically distributed during sporulation (to be discussed later) and that turnover again reduces the specific activity during development.

It is apparent (Table 1) that the specific activity of the phospholipid labeled at \(T_{2,74}\) has already dropped extensively by \(T_s\), a time at which refractile forms are first beginning to appear in the cells and preceding the time when a net loss of phospholipid begins (1). This extensive rate of turnover might be sufficient to reduce the specific activity of the spore to well below the specific activity of the sporangium, even if the radioactive label were confined primarily to the developing forespore. The data also suggest that there are no large conserved classes of phospholipid. The losses in specific activity observed when the cells were exposed to the radioactive precursor for a 5-min pulse or for an extended period of time were not very different (unpublished data). Additionally, material made before the onset of sporulation appeared to undergo turnover at a rate similar to that for material made during septation (Table 1).

**Autoradiography.** It is becoming apparent that the most likely structure for most natural membranes is a fluid-mosaic structure (24). This theoretical construct, as well as several lines of empirical evidence, suggests that components in the membrane may enjoy a large degree of freedom of motion (22). As a result of this membrane fluidity, one cannot rigorously eliminate the possibility that the absence of localized lipid labeling is not the result of randomization of molecules after their incorporation. By subjecting the cells to a short exposure (3 min) to radioactive glycerol and by immediately fixing the cells with osmium tetroxide in the cold (15), it was hoped to minimize the randomization phenomenon. Localized incorporation was not detected (Fig. 1). However, the possibility of lateral migration even with this preparative technique cannot be excluded. The effect of OsO\(_4\) as a membrane fixative is known to occur at the site of double bonds in unsaturated fatty acids (17). The work by Jost et al. (15) demonstrating inhibition of migration by osmium was done with membranes containing a high degree of unsaturation, whereas B. megaterium contains mostly saturated fatty acids. If, in fact, migration of the radioactive label did take place in this system, then it would suggest that during sporulation there is no physical barrier to lateral diffusion between the cell compartments.

Morrison and Morowitz have reported the existence of polar membrane growth in vegetative cells of B. megaterium pulsed with radioactive palmitate and analyzed autoradiographically (20). In the current work, attempts to use their labeling and washing procedures consistently failed to remove substantial amounts of adsorbed but not incorporated radioactivity (unpublished data). Therefore, autoradiography using palmitate could not be attempted.

Fitz-James and Young, in a study using B. cereus (an organism closely related to B. megaterium), have reported that an acetate pulse administered during sporulation can be preferentially recovered in the lipids of the resultant spore (8). In the current studies with B. megaterium, this result has not been duplicated. It would appear that the existence of extensive phospholipid turnover and the possibility of rapid randomization of the radioactive label immediately following its incorporation (18) make it impossible to detect asymmetric membrane growth.
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LITERATURE CITED