Phospholipid Alterations During Growth of *Escherichia coli*

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As cultures of *Escherichia coli* progressed from the exponential growth phase to the stationary growth phase, the phospholipid composition of the cell was altered. Unsaturated fatty acids were converted to cyclopropane fatty acids, and phosphatidyl glycerol appears to have been converted to cardiolipin. With dual isotope label experiments, the kinetics of synthesis of cyclopropane fatty acid for each of the phospholipids was examined in vivo. The amount of cyclopropane fatty acid per phospholipid molecule began to increase in phosphatidyl ethanolamine at a cell density below the density at which this increase was observed in phosphatidyl glycerol or cardiolipin. The rate of this increase in phosphatidyl glycerol or in cardiolipin was faster than the rate of increase in phosphatidyl ethanolamine. After a few hours of stationary-phase growth, all the phospholipids were equally rich in cyclopropane fatty acids. It is suggested that the phospholipid alterations observed are a mechanism to protect against phospholipid degradation during stationary phase growth. Cyclopropane fatty acid synthetase activity was assayed in cultures at various stages of growth. Cultures from all growth stages examined had the same specific activity in crude extracts.

Several changes in bacterial lipid composition have been reported to occur during the transition from the exponential growth phase to the stationary growth phase. The accumulation of cyclopropane fatty acids during this time has been reported in *Serratia marcescens*, *Lactobacillus* sp., and *Escherichia coli* (for reviews, see 14, 24). Cardiolipin (diphosphatidyl glycerol) has been observed to accumulate during the stationary phase in *Clostridium butyricum* (1), *Staphylococcus aureus* (9), and *Thiobacillus thiooxidans* (28). The disappearance of phosphatidyl glycerol has been noted in *E. coli* (11). The accumulation of O-amino acid esters of phosphatidyl glycerol has been reported in *S. aureus* (9) and in *C. welchii* (18).

The kinetics of several such changes during growth of *E. coli* are reported in this paper. The amounts of the various phospholipids and the amounts of cyclopropane fatty acids in each of these phospholipids have been measured as a function of growth. Cyclopropane fatty acid synthetase activity has also been measured in cells at various stages of growth.

**Materials and Methods**

*Cultivation of bacteria.* *E. coli* K-12 strain AB301 (met') was grown in medium 56 with glucose (0.4%) as previously described (4). When cells were labeled with 32P O4, medium 56-LP was used. In medium 56-LP, the phosphate buffer was replaced by a tris-(hydroxymethyl)aminomethane (Tris)-chloride buffer (0.05 M; pH 7.3), and 10 ml of medium 56 was added per liter of medium 56-LP (final phosphate concentration, 10^-2 M). The growth rate was the same in both media. Growth was measured by turbidity (4) and was standardized to dry cell weight by a standard curve.

*Extraction and analysis of phospholipids.* Samples of cell cultures were treated with an equal volume of 10% (w/v) cold trichloroacetic acid. The precipitate was collected by centrifugation and was extracted by the method of Kanfer and Kennedy (11). The resulting washed chloroform extract was used for determination of radioisotope incorporation into phospholipids and for analysis by thin-layer chromatography. The phospholipids were separated on activated Silica Gel G thin-layer plates (layer thickness, 250μ) with the following solvent systems: (i) chloroform-methanol-water (65:30:4); (ii) chloroform-methanol-water (65:35:4); and (iii) chloroform-methanol-acetic acid (glacial)-water (50:25:7:3) (22). The phospholipids were identified by their behavior, as compared with authentic standards in the above solvent systems, and by the behavior of their decyalted (11) products on Whatman no. 1 paper chromatograms. The paper chromatograms were developed in phenol saturated with 0.1% ammonia (11) or in 2-propanol-water-concentrated ammonia (7:2:1) (19).

Areas of the thin-layer plates localized by auto-
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radiography or by Rhodamine 6G spray (30) were scraped into scintillation vials. Either a dioxane-water (5:1) scintillation solution (30) or a Cab-O-Sil in toluene scintillation solution (30) was added, and the samples were counted in a Beckman CPM-100 instrument. Cross-contamination of the 32P and 14C channels was corrected by use of appropriate standards. The same results were obtained with either counting system upon conversion to molar units.

Assay of cyclopropane fatty acid synthetase activity. Two different reaction mixtures were used for the assay. Assay mixture 1 was modified from that of Borek (2). The assay mixture consisted of phosphoenol-pyruvic acid, 15 μmoles; pyruvate kinase, 100 μg; adenosine triphosphate (ATP), 2 μmoles; MgCl₂, 100 μmoles; 2-mercaptoethanol, 100 μmoles; 14C-H₃-methionine, 0.1 mole (1.5 μCi); Tris (pH 7.0), 100 μmoles; and 0.1 to 0.5 ml of crude enzyme preparation (final volume, 1.0 to 1.5 ml).

Assay mixture 2 was that of Zalkin et al. (34), and consisted of only S-adenosyl methionine-methyl-14C, 2 μmoles, with mercaptoethanol, Tris, and crude enzyme preparation as above.

Phospholipid dispersions were prepared by dialysis (7) or by sonic oscillation from phospholipids obtained from either log phase E. coli B or from Azotobacter agilis. Lipid was added to a concentration of 0.2 to 0.3 mg per ml of either assay mixture.

The assays were halted by the addition of methanol and concentrated KOH to achieve a solution of 5 to 10% KOH in 50% aqueous methanol. The samples were then saponified and extracted by the method of Zalkin et al. (34). The resulting washed ether extract was evaporated and then counted in toluene scintillation solution (4). One unit of enzyme activity is defined as one micromole of cyclopropane fatty acid formed per minute, under the above conditions. The specific activity is defined as enzyme units per milligram of protein.

Preparation of enzyme extracts. Samples of growing AB301 cultures were chilled (all further steps were done at 0 to 5 °C), centrifuged, washed with 0.1 M Tris-chloride (pH 7.0), and resuspended in the same buffer plus mercaptoethanol (0.01 M). This suspension was then treated five times for 1 min at full power in a Branson 20-kc sonifier. The sonically treated extract was then centrifuged at 50,000 × g for 30 min, and the resulting supernatant fluid was used as the crude enzyme preparation. In some experiments, a particulate fraction was prepared in the following manner. The sonically treated extract was centrifuged at 5,000 × g for 10 min to remove unbroken cells and large bacterial debris; the resulting supernatant fluid was centrifuged at 50,000 × g, and the pellet was resuspended in Tris-mercaptoethanol as above. This resuspended pellet contained less than 5% of the supernatant activity and gave no increase in activity when added back to the supernatant fluid. This was true for cells at all stages of growth. The yield of supernatant protein, determined by the method of Lowry et al. (21) with bovine serum albumin as standard, was relatively constant for cells at all stages of growth [0.21 to 0.30 mg of protein per g (dry weight) of cells].

Isolation and analysis of fatty acids. Fatty acids were isolated and esterified as previously described (5). A Barber column 3000 instrument with a column (6 ft by 2 mm) of 10% Apiazon L on 60/80 mesh Chromosorb W was used for gas chromatography. A temperature program of 2°C rise per minute from 190 to 260°C was used to separate the fatty acid methyl esters. Identification of the fatty acids was accomplished by comparison of retention times with the retention times of authentic standards. 14C-labeled fatty acid methyl esters were fractioned as previously described (5).

Materials. L-14C-H₃-methionine and S-adenosyl-L-methionine-methyl-14C were purchased from New England Nuclear Corp. (Boston, Mass.). Carrier-free 32P was obtained from Nuclear Supplies (Encino, Calif.). Phosphoenolpyruvic acid, pyruvate kinase, and ATP were from Calbiochem (Los Angeles, Calif.). Fatty acid methyl ester standards and the chromatography supplies were from Applied Sciences (State College, Pa.). “Chromatographically pure” phospholipids, bacterial phosphatidyl ethanolamine and bovine cardiolipin and bovine phosphatidic acid, were obtained from the Pierce Chemical Co. (Rockford, Ill.) and General Biochemicals (Chagrin Falls, Ohio), respectively.

RESULTS

The cyclopropane fatty acids of E. coli are cis-9,10-methylene hexadecanoic acid and lactobacillic acid (cis-11,12 methylene octadecanoic acid) (3, 14). It has been shown that all of the counts incorporated into E. coli fatty acid from 14C-H₃-methionine are found in the cyclopropane fatty acids (3, 17), and that the labeled carbon of these fatty acids is the ethylene carbon of the cyclopropane ring (20). Law and co-workers (17, 32) have demonstrated in vitro that the substrate for cyclopropane fatty acid synthesis is an unsaturated fatty acid present in an intact phospholipid.

Kinetics of cyclopropane fatty acid synthesis. Figure 1 shows the kinetics of incorporation of 32P and of 14C-H₃-methionine into the unFractionated phospholipids of AB301. Control experiments have shown that 97% of the 14C-H₃-methionine incorporated into phospholipids is found in fatty acids. Cyclopropane fatty acid synthesis (14C incorporation) shows the characteristic lag (compared with the growth curve) of AB301 (4) and other E. coli strains (14-18), whereas 32P incorporation is a direct function of growth.

Thin-layer chromatograms of lipid extracts of E. coli AB301 yielded five radioactive spots (Fig. 2). The spots were identified by their behavior in all three thin-layer solvent systems and by the behavior of their deacylation products upon paper
chromatography. Spot 1 is cardiolipin (diphosphatidyl glycerol), which has recently been reported in E. coli (6, 10, 22). Spot 2 is phosphatidyl glycerol and spot 3 is phosphatidyl ethanolamine, both of which have been previously reported in E. coli (10, 11, 22). Spots 4 and 5 migrated as though they could be lyso-phospholipids, phosphatidyl serine, or O-amino acid esters of phosphatidyl glycerol. They were not further characterized because they comprise only about 0.1% of the total phospholipids. The amount of $^{32}$P incorporated into phospholipids in this experiment indicated that 7.85% of the dry weight of the cell was phospholipid. This is consistent with established values (10, 14, 22). The enzyme reactions responsible for the biosynthesis of cardiolipin, phosphatidyl glycerol, and phosphatidyl ethanolamine in E. coli have been demonstrated by Kennedy and co-workers (12, 29).

This separation scheme led to the following experiment. AB301 was grown from an original density of $10^7$ cells per ml (from an overnight culture) in the presence of $^{32}$P and $^{14}$C-H$_2$-methionine. Samples were taken at various intervals and treated with trichloroacetic acid. The phospholipids were extracted and chromatographed. Appropriate areas of the developed plate were scraped into scintillation vials and counted. The synthesis of cyclopropane fatty acids in vivo began sooner and proceeded at a slower rate with phosphatidyl ethanolamine as the substrate than with phosphatidyl glycerol or cardiolipin as the substrate (Fig. 3). The kinetics of cyclopropane fatty acid synthesis for phosphatidyl glycerol and cardiolipin were nearly the same, but the small difference was reproducible and may be significant. After about 3 hr of stationary growth, all of the phospholipids seemed to be equally rich in cyclopropane fatty acids, as has also been observed in E. coli B (10).

Interconversions of phospholipids. Figure 4 shows what appears to be the conversion of phosphatidyl glycerol to cardiolipin during growth. The conversion seems to be quantitative, as was recently shown in T. thiooxidans (28). This observation probably explains the loss of phosphatidyl glycerol reported by Kanfer and Kennedy (11). These investigators did not detect cardiolipin (probably because of their chromatographic methods) and hence lost this fraction from their calculations. This conversion has also been found in E. coli B (unpublished data). The
enzyme which caused the conversion has been demonstrated (29).

**Cyclopropane fatty acid synthetase activity.** The obvious explanation for the synthesis of cyclopropane fatty acids during only a short part of the growth cycle is that the enzyme responsible for this synthesis is induced or derepressed at this time. Thus, we have assayed this activity throughout the growth cycle of *E. coli* AB301. Zalkin et al. (34) have demonstrated cyclopropane fatty acid synthetase activity in cell-free preparations from *S. marcescens* and *C. butyricum*. O'Leary (24) has shown the same activity in *Aerobacter aerogenes* and *L. arabinosus*. This activity was demonstrated in *E. coli* AB301 by either of the assay systems described in Materials and Methods. Assay 1 was routinely used because of the nonlinear behavior of assay 2. Assay 1 was linear for at least 15 hr (0.2 mg of protein from 4-hr cells), whereas assay 2 was linear for only 10 min under the same conditions. Assay 2 had the same rate as assay 1 for 10 min, then dropped to about 7% of the original rate; addition of S-adenosyl methionine-methyl-14C restored the initial rate. The only enzyme known to be present in such extracts of *E. coli* which degrades S-adenosyl-methionine in these amounts is homocysteine-S-adenosyl-methionine methylase (26). This enzyme transfers the methyl group of S-adenosyl-methionine to homocysteine to form methionine (26). This transfer disrupts assay 2 because the resulting methyl-labeled methionine cannot be reactivated as it can be in assay 1. This enzyme is found in K-12 strains of *E. coli*, but not in B or W strains (26). It seems that this enzyme is responsible for the nonlinearity of assay 2, because extracts of *E. coli* B gave identical cyclopropane fatty acid synthesis with either assay system for 85 min.

The amount of enzyme activity was constant for cells from any growth phase tested (Table 1). Thus, the hypothesis of induction or derepression as a method of control is unlikely. The activity was the same as that found in *E. coli* B (specific activity = 8.34 × 10^-6) and close to that found in *S. marcescens* (34). The lack of difference shown in Table 1 was not due to inhibitors, because assays of mixtures of the supernatant fluids gave activities which were the sum of the activities of the components of the mixture. This experiment also ruled out differences which might be due to variations in the soluble substrate.

The limited stimulation of enzyme preparations from stationary phase cells observed upon the addition of lipid was only found when the preparations were fresh. When these preparations were stored, activity was lost and the endogenous lipid (about 15 μg per mg of protein) in the supernatant fluid was not limiting under the assay conditions used. The finding that the endogenous lipid present in such preparations is a substrate indicates that the unsaturated fatty acids remaining in stationary-phase cells are available for cyclopropane synthesis in vitro, not in vivo (Table 3).

Table 2 shows that most of the radioactivity incorporated into fatty acid in vitro is found in...
the cyclopropane fatty acids. Lactobacillic acid constituted a much higher proportion of the total cyclopropane fatty acid when the in vitro preparation was compared with the in vivo preparation (Table 3).

During growth, the only fatty acid changes observable by gas chromatography were the increase in cyclopropane fatty acids and the concomitant decrease in unsaturated fatty acids (Table 3). The cyclopropane fatty acid data obtained by gas chromatography (Table 3) agrees with the results of the isotope incorporation studies (Fig. 1).
TABLE 2. Distribution of label from \(^{14}C\) \(H_2\)-methionine incorporated in vitro into fatty acid\(^{a}\)

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Percentage of total (^{14}C) incorporated into fatty acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylene hexadecanoic</td>
<td>76</td>
</tr>
<tr>
<td>Lactobacillic acid</td>
<td>19</td>
</tr>
<tr>
<td>Unsaturated fatty acids</td>
<td>5</td>
</tr>
</tbody>
</table>

\(^{a}\) The total fatty acids were isolated from a reaction mixture (Table 1) by use of the 5.5-hr supernatant fluid plus added lipid. The unsaturated fatty acids were removed by the mercuric acetate technique (5), and the saturated fatty acids were separated on a dodecane impregnated silica gel thin-layer plate and counted (5). The only radioactive spots found on this plate were the cyclopropane fatty acids.

TABLE 3. Fatty acids of Escherichia coli AB301\(^{a}\)

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Percentage (w/w) of total fatty acid at culture age of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.5 hr</td>
</tr>
<tr>
<td>Myristic</td>
<td>5.2</td>
</tr>
<tr>
<td>Unidentified</td>
<td>1.3</td>
</tr>
<tr>
<td>Palmitoleic</td>
<td>32.3</td>
</tr>
<tr>
<td>Palmitic</td>
<td>39.6</td>
</tr>
<tr>
<td>Methylene</td>
<td></td>
</tr>
<tr>
<td>hexadecanoic</td>
<td>5.2</td>
</tr>
<tr>
<td>(\text{cis}-\text{vaccenic})</td>
<td>16.2</td>
</tr>
<tr>
<td>Lactobacillic</td>
<td>0.2</td>
</tr>
<tr>
<td>Total cyclopropane</td>
<td>5.4</td>
</tr>
</tbody>
</table>

\(^{a}\) Data obtained by gas chromatography. Hydroxy fatty acids were removed (5) prior to gas chromatography.

**DISCUSSION**

Cyclopropane fatty acids are synthesized at different rates in the various phospholipids of \(E.\) coli (Fig. 2). This finding indicates that an intact phospholipid is the substrate for this reaction in vivo as well as in vitro (32, 34). It has been postulated (33) that the acylation of added cyclopropane fatty acids may be an alternative pathway for the formation of phospholipids containing cyclopropane fatty acids. To account for the results in Fig. 2, this hypothesis would require the operation of a mechanism which would place certain fatty acids in a specific phospholipid. Such a specific mechanism has not been observed in bacteria, as the fatty acid compositions of the different phospholipids of a bacterial species are identical (8, 10, 32). This hypothesis is also contradicted by the currently accepted pathways for the formation of \(E.\) coli phospholipids (11, 12, 29).

The phospholipid alterations which occur during the transition from log phase growth to stationary phase growth may be thought of as the adaptation of the bacteria to new environmental conditions. Thus, several investigators have studied such changes in altered environments. It has been shown that the amount of cyclopropane fatty acid in \(E.\) coli can be increased by growth at high temperature (15, 23), in acid medium (15), and under low oxygen tension (15). The amount may be decreased by growth in a limiting carbon source (16, 23, 27), limiting sulfate (16), or limiting magnesium (16), but not by limitation of the nitrogen source (16, 23) or of phosphate (16). A small amount of cyclopropane fatty acid is always formed, even under the most adverse conditions. AB301 synthesizes considerable cyclopropane fatty acid when grown on limiting methionine (unpublished data). Methionine auxotrophs require much less methionine for normal growth than is needed for normal cyclopropane fatty acid synthesis (13; unpublished data).

The function of cyclopropane fatty acids is unknown. Law et al. (17) have postulated that the cyclopropane ring may be formed to protect double bonds from oxidation, but the control mechanism would be difficult to explain because low oxygen tension increases the amount of cyclopropane fatty acid (15). Another possible function of the cyclopropane fatty acids may be that these phospholipid alterations serve to protect the phospholipids from degradation at a time when resynthesis would be difficult owing to sluggish metabolism (stationary-phase growth). This is an attractive hypothesis because of the following observations: (i) the phospholipid biosynthetic rate of resting cells is only about 0.5\% that of growing cells (31); (ii) stationary-phase cells contain appreciable phospholipase activity, as assayed on rat liver phospholipids (25); (iii) phospholipid content does not decrease in stationary-phase cells (Fig. 1); and (iv) once formed, cyclopropane fatty acids are stable during log (unpublished data) and during stationary phases (17; unpublished data). Thus, it appears that stationary-phase cell phospholipids are somehow protected from degradation. Perhaps it is not a coincidence that the phospholipids (phosphatidyl glycerol and cardiolipin) which are poor in cyclopropane fatty acids during exponential growth show rapid turnover in this growth phase, but the more heavily cyclopropane substituted phospholipid phosphatidyl ethanolamine shows little turnover (10, 11). The conversion of phos-
phatidyl glycerol to cardiolipin may also be a protective mechanism, as Kanemasa et al. (10) have shown that the turnover of cardiolipin is less than that of phosphatidyl glycerol. The formation of amino acid esters of phosphatidyl glycerol could also function in this manner.

The finding that the amount of cyclopropane fatty acid synthetase activity is independent of the growth phase of the cells seems to indicate that some event other than enzyme synthesis or activation triggers the synthesis of cyclopropane fatty acids. Of course, it may well be that some type of control (e.g., feedback inhibition) occurs in vivo and is not apparent in vitro because of dilution of the ligand.

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