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Transfer of Phosphoethanolamine Residues from Phosphatidylethanolamine to the Membrane-Derived Oligosaccharides of *Escherichia coli*

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The membrane-derived oligosaccharides (MDO) of *Escherichia coli* are periplasmic constituents composed of glucose residues linked by β-1,2 and β-1,6 glycosidic bonds. MDO are substituted with phosphoglycerol, phosphoethanolamine, and succinic acid moieties. The phosphoglycerol residues present on MDO are derived from phosphatidylglycerol (B. J. Jackson and E. P. Kennedy, J. Biol. Chem. 258:2394–2398, 1983), but evidence as to the source of the phosphoethanolamine residues has been lacking. We now report that phosphatidylethanolamine, exogenously added to intact cells of *E. coli*, provides a source of phosphoethanolamine residues that are transferred to MDO. The biosynthesis of phosphoethanolamine-labeled MDO is osmotically regulated, with maximum synthesis occurring during growth in medium of low osmolarity.

The membrane-derived oligosaccharides (MDO) of *Escherichia coli* are a family of periplasmic oligosaccharides composed solely of glucose residues linked by β-1,2 and β-1,6 glycosidic bonds (12, 18, 21). MDO are multiply substituted with phosphoglycerol, phosphoethanolamine, and succinic acid moieties (12, 18, 21). The phosphoglycerol moieties present on MDO are derived from phosphatidylglycerol (6, 7), and about 90% of the total cellular turnover of phosphatidylglycerol may result from this reaction (19). Phosphoglycerol transferase I, catalyzing this reaction, has been studied in vitro (6) and in vivo (1) by measuring the transfer of phosphoglycerol residues from phosphatidylglycerol to the β-glucoside arbutin, a model substrate that substitutes for nascent MDO molecules. By analogy, it has been presumed that the source of the phosphoethanolamine residues of MDO is phosphatidylethanolamine, but direct evidence has been lacking. Recent attempts in this laboratory to develop an in vitro assay for the putative phosphoethanolamine transferase, with the use of model β-glucoside acceptors such as arbutin, have been unsuccessful. It should be noted, however, that the level of in vivo transfer of phosphoethanolamine moieties to arbutin represents only about 5% that of the level of transfer of phosphoglycerol residues (6a). The in vitro activities of our preparations with arbutin as substrate may, therefore, have been below the limit of detection.

A second approach to the elucidation of the source of phosphoethanolamine moieties of MDO was therefore pursued. Previous studies by Jones and Osborn (8) have shown that exogenous phospholipids may be added to gram-negative bacteria by a vesicle fusion technique. With this technique, we now find that phosphatidylethanolamine, added to cells of *E. coli*, provides a source of phosphoethanolamine moieties which are subsequently transferred to MDO.

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* K-12 mutants defective in a locus designated *mdoB* lack detectable phosphoglycerol transferase I activity (7). The bacterial strains employed were the isogenic pair PT245 (*mdoB14*) and PT247 (*mdoB*$^+$). This pair was constructed (7) by transducing the *E. coli* K-12 strain PC0950 (*thr25 purA54 mdoB*$^+$) to *thr*$^+$ with a P1 *vir* lysate of PT114 (*thr*$^+$ *mdoB14*). *E. coli* K-12 strain PC0950 was obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, Conn. *E. coli* K-12 strain PT114 is an *mdoB14* derivative of strain RZ60 (7).

Cells were grown in LB medium (14) at 37°C with vigorous shaking. Total cellular protein was determined by a modification of the method of Lowry (5).

Chemicals. Phosphatidylcholine was purchased from Sigma Chemical Co. (St. Louis, Mo.) and further purified by the method of Singleton et al. (20). The purified lipid was stored in ethanol at −20°C at a concentration of 100 mg/ml. Butylated hydroxytoluene was added to a final concentration of 0.1 mg/ml. Phosphatidyl-*sn*-1(3)-glycerol (derived from egg yolk phosphatidylcholine) and phosphatidylethanolamine (from *E. coli*) were purchased from Sigma. L-[G-^{3}H]serine was purchased from New England Nuclear Corp. (Boston, Mass.). Phospho[1,2-^{14}C]ethanolamine was synthesized by a procedure based on that of Plimmer and Burch (16). Authentic MDO was prepared from frozen cells (4 kg) of *E. coli* K-12 (Grain Processing Corp., Muscatine, Iowa) and purified to the stage just before chromatography on DEAE-cellulose. It was generously provided by A. C. Weissborn.

Preparation of phosphatidyl[^{3}H]ethanolamine. Phosphatidyl[^{3}H]ethanolamine (6.4 Ci/mmol), labeled specifically in the ethanolamine moiety, was prepared from L-[G-^{3}H]-serine and (dipalmitoyl) CDP-diglyceride in the presence of phosphatidylserine synthase and phosphatidylserine decarboxylase. Phosphatidylserine synthase (from *E. coli*) was prepared essentially by the method of Kanfer and Kennedy (10). Phosphatidylserine decarboxylase (from *E. coli*) was purified by the first four steps of the procedure described by Dowhan

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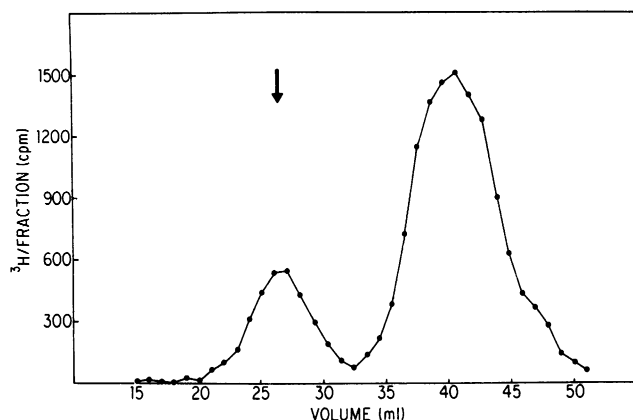

FIG. 1. Sephadex G-25 chromatography of trichloroacetic acid extract from cells of *E. coli* PT247. The neutralized extract, containing carrier MDO and prepared as described in the text, was concentrated and chromatographed on a Sephadex G-25 (Pharmacia) column (1.1 by 50 cm). The column was eluted with 0.15 M ammonium acetate (pH 7.0) in 7% (vol/vol) 1-propanol, and fractions (1 ml) were assayed for radioactivity. Results are expressed as ^{3}H (cpm) per fraction and represent the total radioactivity extracted from the 1-liter culture. The arrow indicates the volume at which the peak for authentic MDO is expected.

and co-workers (3). The reaction mixture contained CDP-dipalmitin (0.67 mM), potassium phosphate (0.1 M, pH 7.4), bovine serum albumin (1 mg/ml), Triton X-100 (1 mg/ml), L-[G-^{3}H]serine (6.4 Ci/mmol at a final concentration of 0.94 mM), phosphatidylserine synthase (0.89 mg/ml of protein), and phosphatidylserine decarboxylase (4,200 U/ml). The mixture was incubated for 1 h at 30°C with occasional shaking. The reaction was stopped by the addition of 5 volumes of 0.1 N HCl in methanol, followed by 15 volumes of chloroform and 30 volumes of 1 M $MgCl_2$. Phosphatidyl[^{3}H]ethanolamine was extracted into the chloroform phase. Subsequent analysis by thin-layer chromatography was performed on aluminum-backed plates of Silica Gel 60 F254 (E. Merck AG, Darmstadt, Federal Republic of Germany) with the solvent system chloroformmethanol-water (65:25:4, vol/vol). A typical preparation was found to contain approximately 99% phosphatidylethanolamine and 1% phosphatidylserine.

Preparation of phospholipid vesicles. Phospholipid vesicles were prepared by the method of Mimms and co-workers (15). Briefly, 500 to 1,000 nmol of phospholipid in chloroform was dried under nitrogen and placed under vacuum for 1 h to remove traces of solvent. The phospholipid composition (percent by weight) employed was 60% phosphatidylcholine, 20% phosphatidylglycerol, and 20% phosphatidylethanolamine. The final specific activity of phosphatidyl[^{3}H]ethanolamine in the total phospholipid preparation ranged from 1.1 to 6.4 Ci/mmol. Approximately 4×10^7 to 3×10^8 total cpm of ^{3}H were used in each experiment. A solution of 150 mM octyl glucoside (octyl β-D-glucopyranoside) in 10 mM Tris hydrochloride (pH 7.5) was added to the dried phospholipid preparation so that the final octyl glucoside/phospholipid mole ratio was 15:1. The dispersion was mixed vigorously by vortexing and stored overnight at 5°C. Phospholipid vesicles were prepared by passing the phospholipid and octyl glucoside dispersion (50- to 100-μl sample volume) over a small column (3.3 ml total bed volume) of Sephadex G-50 superfine (Pharmacia Fine Chemicals, Piscataway, N.J.). Phospholipid vesicles were eluted at the void volume with 10 mM Tris hydrochloride (pH 7.5). Vesicles were stored at 5°C and used within 4 h.

RESULTS

Transfer of vesicle phospholipid to cells of *E. coli*. The transfer of vesicle phospholipid to intact cells of *E. coli* was performed by the calcium-mediated fusion technique described by Jones and Osborn (8) with minor modifications. Cells of strain PT247 were harvested during the logarithmic stage of growth (from a 120-ml culture in LB medium) at a concentration of approximately 0.16 mg of total cellular protein per ml. Cells were suspended at a concentration of 9.6 mg of protein per ml in 2 ml of buffer containing 10 mM Tris hydrochloride (pH 7.5) and 20 mM $CaCl_2$. Phospholipid vesicles containing phosphatidyl[^{3}H]ethanolamine (3.11×10^8 cpm of ^{3}H at a specific activity of 6.4 Ci/mmol) were present at a total phospholipid concentration of approximately 140 μg/ml. The suspension was incubated for 1 h at 37°C with gentle shaking. After 1 h, 0.26 volume of 100 mM potassium EDTA was added, and the preparation was placed on ice for 20 min. Cells were separated from unfused vesicles by centrifugation at $10,000 \times g$ at 4°C through 10% Ficoll (wt/vol) in 10 mM Tris hydrochloride (pH 7.5) and 5 mM potassium EDTA. A second wash through buffer containing 10% Ficoll and 5 mM EDTA was performed, followed by a third wash with buffer alone (10 mM Tris hydrochloride, pH 7.5). After this extensive washing of cells, approximately 7.0% of the total radiolabeled phosphatidyl[^{3}H]ethanolamine was found to remain cell associated. The resulting radiolabeled cell pellet was suspended in modified LB medium (1.0 liter) at a concentration of approximately 20 μg of total cellular protein per ml. LB medium was modified so that the final NaCl concentration was 20 mM. Cells were then incubated at 37°C with vigorous shaking, and subsequent growth was monitored by measurement of A_{650}. Growth was allowed to proceed for 3.5 h (three generations). Cells were then harvested by centrifugation. At this time, approximately 70% of the initial cell-associated radioactivity had been released into the growth medium. After harvest, the cell pellets were extracted and analyzed for labeled MDO as described below.

Isolation of radiolabeled MDO. The MDO of *E. coli* are readily extracted with trichloroacetic acid or ethanol from intact cells and emerge in a well-defined peak after subsequent gel filtration chromatography on Sephadex G-25 (11). Further subfractionation of MDO may be accomplished by DEAE-cellulose chromatography, which yields three distinctive anionic fractions, designated MDO A, B, and C (21), with smaller and rather variable amounts of a less anionic fraction termed pre A. We examined cells for radiolabeled MDO with methods based on these properties. Cell pellets (derived from 1-liter cultures) were resuspended in 6 ml of 2% (wt/vol) bovine serum albumin. After resuspension, 6 ml of 8% (wt/vol) trichloroacetic acid was added, and the mixture was vortexed. Pellets were extracted at room temperature for 10 min with intermittent vortexing, and the supernatants were removed by centrifugation. Of the total cell-associated radioactivity, approximately 0.3% was extracted from cell pellets with trichloroacetic acid. The trichloroacetic acid extract was neutralized by the addition of NH_4OH, and 15 mg (glucose equivalent as determined by the phenol-sulfuric acid method [5]) of authentic MDO was added as carrier to the neutralized extract. The extract was then concentrated by rotary evaporation and fractionated on

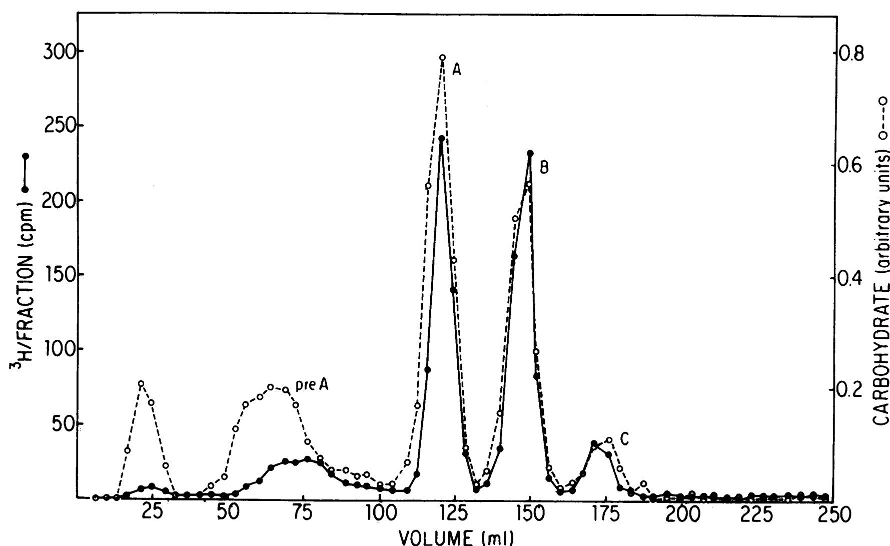

FIG. 2. DEAE-cellulose chromatography of [³H]MDO obtained from cells of *E. coli* PT247. Sephadex G-25 fractions containing [³H]MDO and carrier MDO were pooled and concentrated under nitrogen at 37°C. The concentrated sample was desalted by chromatography on a Sephadex G-15 column as described in the text. The desalted sample (10.7 ml) was applied to a DEAE-cellulose (Whatman DE52) column (1.1 by 19 cm). The column was washed with 30 ml of 10 mM Tris hydrochloride (pH 7.4) containing 7% (vol/vol) 1-propanol and was then eluted with a linear gradient of 0 to 0.25 M LiCl in 10 mM Tris hydrochloride (pH 7.4) containing 7% (vol/vol) 1-propanol in a total volume of 250 ml. Fractions (4 ml) were collected and assayed for radioactivity and carbohydrate by the phenol-sulfuric acid method (5). Results are expressed as ³H (cpm) per fraction (●) and represent the total radioactivity extracted from the 1-liter culture of strain PT247. Relative carbohydrate content of each fraction (○) was determined by measuring A_{487}. Pre A, A, B, and C designate subfractions of MDO (21).

a Sephadex G-25 column. Two radiolabeled peaks were observed (Fig. 1). The first peak represented approximately 20% of the total trichloroacetic acid-extractable material and was eluted at a position expected for MDO.

Further analysis of the radiolabeled material that eluted with authentic MDO was performed by DEAE-cellulose column chromatography (Fig. 2). Sephadex G-25 fractions were pooled and concentrated under nitrogen at 37°C. The concentrated sample was then desalted by chromatography on a Sephadex G-15 (Pharmacia) column (1.1 by 52 cm) and eluted with 10 mM Tris hydrochloride (pH 7.4) in 7% (vol/vol) 1-propanol. The desalted sample was then applied to a DEAE-cellulose column. Fractions eluted from the column were assayed for radioactivity and for total carbohydrate (which was almost entirely that of the added carrier MDO). The pattern of radioactivity closely paralleled that of the carrier MDO, offering strong evidence that the labeled material was indeed MDO.

Identification of phospho[³H]ethanolamine substituents on MDO. If the radioactive moiety transferred from exogenously added phosphatidyl[³H]ethanolamine to the MDO of *E. coli* is phospho[³H]ethanolamine in phosphodiester linkage, treatment of the radiolabeled MDO with periodate in the presence of sulfuric acid should result in the release of phospho[³H]ethanolamine from the preparation, as reported for authentic MDO by Kennedy et al. (12). [³H]MDO subfractions A and B (eluted from DEAE-cellulose as described in the legend to Fig. 2) were concentrated in a stream of nitrogen at 37°C and then desalted on a Sephadex G-15 (Pharmacia) column (1.1 by 52 cm) which was eluted with 7% propanol (vol/vol). The desalted fractions contained 630 cpm of ³H and 2.7 mg of glucose equivalent (as determined by the phenol-sulfuric acid method). Authentic phospho[1,2-¹⁴C]ethanolamine (500 cpm of ¹⁴C; specific activity, 37,700 cpm/μmol) was added to fractions containing desalted [³H]MDO, and the mixture was again concentrated under nitrogen at 37°C. The sample was treated with periodate and sulfuric acid at 100°C for 1 h as described previously (12). The sample was cooled and neutralized with barium hydroxide. After removal of barium sulfate, the products were chromatographed on a Dowex-1-formate column as described by Kennedy and co-workers (12). Approximately 70% of the recovered ³H was found to chromatograph in a sharp peak with the phospho[1,2-¹⁴C]ethanolamine standard (Fig. 3).

The biosynthesis of phospho[³H]ethanolamine-labeled MDO is osmotically regulated. Because the biosynthesis of MDO has been shown to be osmotically regulated (11), we examined the effect of extracellular osmolarity on the amount of phospho[³H]ethanolamine transferred from phosphatidylethanolamine to MDO. Cells of strain PT245 were harvested at the logarithmic stage of growth (from a 60-ml culture grown in LB medium) and suspended in 1 ml of 10 mM Tris hydrochloride (pH 7.5) containing 20 mM $CaCl_2$ at a concentration of 9.9 mg of total cell protein per ml. Phospholipid vesicles were added such that the final phospholipid concentration was approximately 200 μg/ml, and a total of 4.22×10^7 cpm of phosphatidyl[³H]ethanolamine (specific activity, 1.1 Ci/mmol) was present. The suspension was incubated as described above. After the removal of unfused vesicles, the cell suspension was divided, and equal volumes were inoculated into two cultures (250 ml each) for subsequent growth at 37°C. One culture contained LB medium with NaCl at a final concentration of 20 mM, and the other culture contained NaCl at a final concentration of 300 mM. Growth was allowed to proceed for 3.5 h (three generations). Growth rates and final cell densities were similar for the two cultures. Cells were then harvested and extracted with 4% trichloroacetic acid as described above. The analysis of [³H]MDO extracted from the two cultures

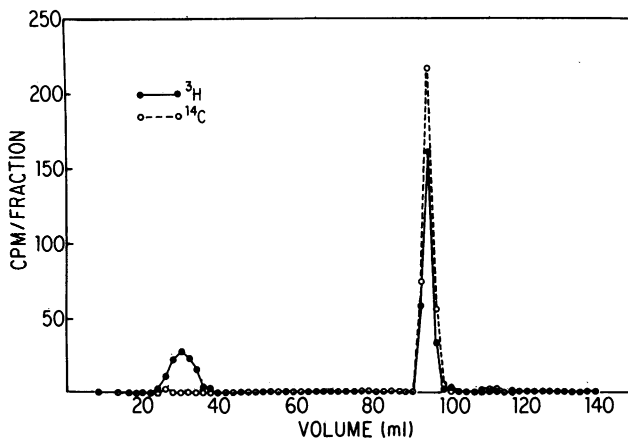

FIG. 3. Identification of phospho[^{3}H]ethanolamine substituents on MDO extracted from cells of *E. coli* PT247. Fractions from DEAE-cellulose chromatography containing [^{3}H]MDO subfractions A and B (see Fig. 2) were pooled, concentrated, and desalted as described in the text. Authentic phospho[1,2-^{14}C]ethanolamine was added to the sample, which was then treated with periodate and sulfuric acid as described in the text. The products of periodate-sulfuric acid treatment were examined by chromatography on a Dowex-1-formate (Bio-Rad Laboratories, Richmond, Calif.) column (1.1 by 22 cm) as described by Kennedy and co-workers (12). The column was washed with 30 ml of 0.1 M NH_4OH just before use. After application of the sample (8.6 ml), the column was washed with 5 ml of water and then eluted with a linear gradient of water to 0.04 N formic acid in a total volume of 140 ml. Fractions (2.0 ml) were collected and assayed for both ^{3}H (●) and ^{14}C (○). Results are expressed as total radioactivity (cpm) per fraction.

revealed a striking osmotic effect (Fig. 4). The amount of [^{3}H]MDO extracted from the culture grown in the presence of 300 mM NaCl represented less than 16% of the amount extracted from cells grown in the presence of 20 mM NaCl. The magnitude of this osmotic dependence is consistent with previous studies of MDO biosynthesis (11).

DISCUSSION

As first demonstrated by Jones and Osborn (8), phospholipid vesicles provide a means of incorporating exogenous phospholipid into the intact gram-negative cell. Although the efficiency of this transfer process appears to be much greater for bacterial strains defective in outer membrane lipopolysaccharide structure, it has been demonstrated that transfer does occur in wild-type strains (e.g., 5 to 20% of the level of transfer for lipopolysaccharide-defective mutants) of both *E. coli* (17) and *Salmonella typhimurium* (8). Importantly, previous studies have indicated that exogenous phospholipid is subsequently translocated to the inner cytoplasmic membrane. For example, phosphatidylserine, exogenously added to cells of *S. typhimurium*, has been shown to be a substrate for phosphatidylserine decarboxylase, an inner membrane enzyme (9). In addition, Wu and co-workers (2, 13) have demonstrated an extensive turnover of exogenously added phosphatidylglycerol in cells of both *E. coli* and *S. typhimurium*, consistent with the translocation of this phospholipid to the inner cytoplasmic membrane.

In the present study, we show that phosphatidylethanolamine, exogenously added to intact *E. coli* cells, provides a source of phosphoethanolamine moieties that are subsequently transferred to the MDO of these cells. The finding that this reaction displayed an osmotic dependence provides strong evidence that this transfer process occurred within growing cells. Although the fraction of radiolabeled phosphoethanolamine residues transferred to MDO was quite small (e.g., 0.3% of the total cell-associated phosphatidyl[^{3}H]ethanolamine), the use of phosphatidyl[^{3}H]ethanolamine of high specific activity permitted this demonstration. A possible explanation for this low level of transfer is that newly synthesized phosphatidylethanolamine provides a preferred substrate for the transfer reaction. It should be noted that newly synthesized phosphatidylglycerol appears

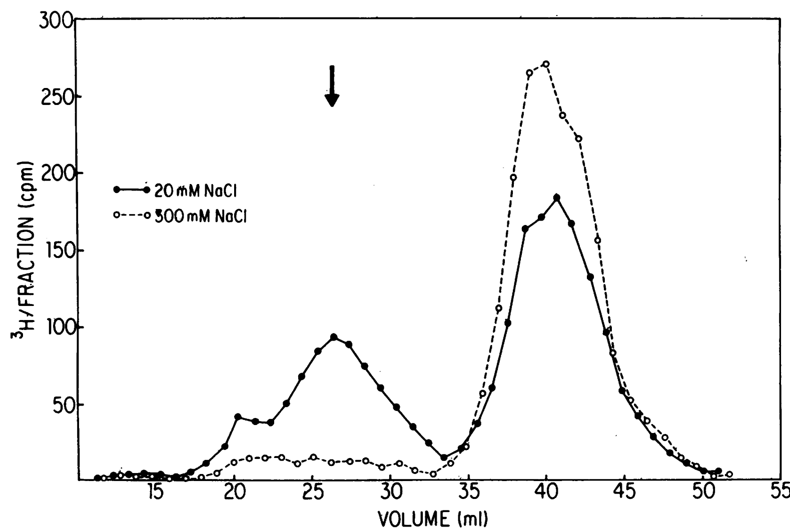

FIG. 4. Effect of osmolarity on the biosynthesis of phospho[^{3}H]ethanolamine-labeled MDO. Cells of strain PT245 were treated with labeled phosphatidylethanolamine and subsequently grown in LB medium containing either 20 or 300 mM NaCl as described in the text. Trichloroacetic acid extracts of cell pellets were examined by chromatography on Sephadex G-25 as described in the legend to Fig. 1. Fractions (1 ml) were assayed for ^{3}H. Results are expressed as ^{3}H (cpm) per fraction and represent the total radioactivity extracted from each 250-ml culture. The arrow indicates the volume at which the peak for authentic MDO is expected. Symbols: ●, culture containing 20 mM NaCl; ○, culture containing 300 mM NaCl.

to be a preferred source of phosphoglycerol residues that are subsequently transferred to MDO by phosphoglycerol transferase I (1).

The transfer of phosphoethanolamine residues to MDO from phosphatidylethanolamine may be catalyzed by an enzyme analogous to phosphoglycerol transferase I (6). It should be noted that *E. coli* PT245 (*mdoB14*) and PT247 ($mdoB^+$) both are capable of transferring phosphoethanolamine moieties to MDO. *mdoB* Mutants of *E. coli* lack detectable phosphoglycerol transferase I activity in vitro and synthesize MDO devoid of phosphoglycerol residues (7). Our results indicate that phosphoethanolamine transfer to MDO is not mediated by phosphoglycerol transferase I. This conclusion confirms that of a previous study by Fiedler and Rotering (4), who analyzed the substituents present on MDO produced by an *mdoB* mutant of *E. coli*. The MDO produced by this strain was found to lack phosphoglycerol residues, but phosphoethanolamine residues were present at levels approximately twofold higher than those of wild-type cells.

ACKNOWLEDGMENTS

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