JOURNAL OF BACTERIOLOGY, June 1983, p. 1110–1116
0021-9193/83/061110-07$02.00/0

Vol. 154, No. 3

Biosynthesis of D-Alanyl-Lipoteichoic Acid: Role of Diglyceride Kinase in the Synthesis of Phosphatidylglycerol for Chain Elongation

DOUGLAS J. TARON, WARREN C. CHILDS III,† AND FRANCIS C. NEUHAUS*

Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois 60201

Received 28 December 1982/Accepted 28 February 1983

Lipophilic and hydrophilic D-alanyl-lipoteichoic acids are elongated in *Lactobacillus casei* by the transfer of *sn*-glycerol 1-phosphate units from phosphatidylglycerol to the poly(glycerophosphate) moiety of the polymer. These *sn*-glycerol 1-phosphate units are added to the end of the poly(glycerophosphate) which is distal to the glycolipid anchor; 1,2-diglyceride results from this addition. The presence of a diglyceride kinase was suggested by the ATP-dependent phosphorylation of 1,2-diglyceride to phosphatidic acid. Inorganic phosphate was used to initiate the synthesis of lipophilic lipoteichoic acid (LTA) and the elongation of both lipophilic and hydrophilic LTA. Three observations suggest that phosphate and other anions play a role in the in vitro synthesis of LTA and its precursors. First, the conversion of 1,2-diglyceride to phosphatidic acid by diglyceride kinase was stimulated. Second, the synthesis of phosphatidylglycerol was increased. Third, the elongation of lipophilic and hydrophilic LTA was enhanced. These observations indicated that one effect of phosphate might be to enhance the utilization of 1,2-diglyceride for the synthesis of phosphatidic acid. This phospholipid is a precursor of phosphatidylglycerol, the donor of *sn*-glycerol 1-phosphate for elongation of LTA.

Phosphatidylglycerol (PG) has been proposed to be the donor of *sn*-glycerol 1-phosphate (GroP) units of the poly(glycerophosphate) moiety of lipoteichoic acid (LTA) (8, 9, 13, 14) according to the following reaction:

$$\text{PG} + \text{LTA-poly(glycerophosphate)}_n \rightarrow \text{LTA-poly(glycerophosphate)}_{n+1} + \text{1,2-diglyceride}$$

Inorganic phosphate stimulates both the synthesis of PG and the elongation of D-alanyl-lipophilic LTA in vitro (4). These observations supported the proposed role for PG in the elongation of LTA.

During the elongation of LTA, one of the reaction products is 1,2-diglyceride. Significant amounts of this diglyceride might be expected to accumulate during chain elongation. Since large amounts of diglyceride are not commonly found in bacteria (25), it is proposed that the diglyceride is either degraded or reutilized for phospholipid synthesis. A diglyceride kinase similar to that found in *Escherichia coli* (24) could phosphorylate the diglyceride to phosphatidic acid, a known precursor of PG (25). This phosphorylation would result in a cycling of the diglyceride moiety of this phospholipid.

The goals of these experiments with *Lactobacillus casei* were to characterize further the effect of phosphate on the assembly of the LTA, to determine the site of the addition of GroP units to the growing polymer, and to suggest a fate for the 1,2-diglyceride. Toluene-treated cells were used to demonstrate the synthesis and elongation of D-alanyl-LTA as well as the synthesis of various phospholipids. These cells, which are permeable to GroP, ATP, and D-alanine, synthesized LTA and phospholipids in significant amounts. For detecting diglyceride kinase, membranes were used instead of toluene-treated cells. These membranes provided a system for introducing lipid substrates by repeated cycles of freezing and thawing.

MATERIALS AND METHODS

Materials. We thank Eugene Kennedy for a generous gift of phosphodiesterase II from *Aspergillus niger* (31), Werner Fischer for glycerophosphotrihexosyldiacylglycerol isolated from *Lactobacillus casei* DSM

† Present address: College of Dentistry, University of Illinois at the Medical Center, Chicago, IL 60612.

20021 (12), and J. Douglas Engel for [γ-^{32}P]ATP (carrier free). Nonradioactive phospholipids were obtained from Supelco, Inc., Bellefonte, Pa. 1,2-Diolein was obtained from Serdary Research Laboratories, London, Ontario. [*U*-^{14}C]glycerol 3-phosphate (171 mCi/mmol), 1,2-[^{14}C]dipalmitoyl phosphatidylcholine (115 mCi/mmol), and [2-^{3}H]glycerol (200 mCi/mmol) were purchased from Amersham Corp., Arlington Heights, Ill. D-[1-^{14}C]alanine (56.2 mCi/mmol) was purchased from International Chemical and Nuclear Corp., Irvine, Calif. Wheat germ acid phosphatase and phospholipase C were purchased from Worthington Biochemicals Corp., Freehold, N.J. and Sigma Chemical Co., St. Louis, Mo., respectively. Silica gel 60 thin-layer plates (250 μm [20 by 20 cm]) were obtained from E. Merck AG, Darmstadt, Germany. Activated silicic acid (Unisil, 100 to 200 mesh) was obtained from Clarkson Chemical Co., Inc., Williamsport, Pa. Sepharose 6B and DEAE-Sephadex were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. All other materials were reagent grade.

Synthesis of 1,2-[^{14}C]dipalmitin. 1,2-[^{14}C]dipalmitin was synthesized by the action of phospholipase C on 1,2-[^{14}C]dipalmitoyl phosphatidylcholine. [^{14}C]phosphatidylcholine (1.5 μCi) was dispersed into 800 μl of 10 mM imidazole buffer (pH 7.4) containing 5 mM $CaCl_2$. Phospholipase C (1.4 U) was added in a volume of 200 μl, and the reaction mixture was incubated for 4 h at 25°C (33). The reaction mixture was then subjected to the Bligh and Dyer (3) extraction procedure (minus the filtration step) described below. The incubation and extraction procedures were then repeated on the residue that resulted from drying the chloroform-soluble fraction under a stream of N_2. The 1,2-[^{14}C]dipalmitin was separated from unreacted [^{14}C]phosphatidylcholine by a modification of the procedure of Dittmer and Wells (7). The final chloroform-soluble fraction from the second incubation was passed over a column (0.6 by 3 cm) of activated silicic acid. The column was washed three times with 1 ml of $CHCl_3$ each time. The pooled effluents were dried under N_2 and redissolved in 1 ml of $CHCl_3$.

Preparation of toluene-treated cells and membranes. *L. casei* ATCC 7469 was grown, in some cases in the presence of [^{3}H]glycerol (1 mCi/liter), by the method of Childs and Neuhaus (6). The toluene-treated cells were prepared as previously described (6). Membranes were prepared from *L. casei* cells by the procedures of Linzer and Neuhaus (16) and Reusch and Neuhaus (27).

Incorporation assay for the synthesis of chloroform-soluble compounds in toluene-treated cells. The incorporation assay for the synthesis of chloroform-soluble compounds measures the incorporation of D-[^{14}C]alanine, ^{32}P from [γ-^{32}P]ATP, or [^{14}C]GroP into chloroform-methanol-extractable, chloroform-soluble compounds. The procedure described by Brautigan et al. (4) was used. The reaction mixture contained the following: 33 mM $MgCl_2$, 5 mM ATP (adjusted to pH 6.5 with NaOH), 40 mM piperazine acetate (pH 6.5), 1 mM dithiothreitol, 100 mM phosphate (adjusted to pH 6.5 with KOH), radiolabel, and toluene-treated cells (16 mg [wet weight]) in a total volume of 0.5 ml. D-[^{14}C]alanine (46 μM), [^{14}C]GroP (23 μM), and [γ-^{32}P]ATP (carrier free) were used as radiolabels. The mixture was incubated at 37°C for 30 min.

Labeled chloroform-soluble compounds were isolated by a modification of the monophasic extraction procedure of Bligh and Dyer (3) described by Brautigan et al. (4). The final chloroform-soluble fraction was transferred to a scintillation vial and assayed for radioactivity.

Assay for the synthesis of phosphatidic acid. The assay for the synthesis of phosphatidic acid measures the incorporation of 1,2-[^{14}C]dipalmitin or ^{32}P from [γ-^{32}P]ATP into phosphatidic acid. Either 1,2-diolein (200 μg) or 1,2-[^{14}C]dipalmitin (2×10^5 dpm) was first dispersed into 300 μl of buffer (40 mM piperazine acetate [pH 6.5] containing 33 mM $MgCl_2$) by cavitation. Membranes (4.0 mg of protein) were then added, and the suspension was subjected to three to six cycles of freezing and thawing (at −196 and 37°C, respectively) as described by Kalomiris et al. (15). The reaction mixture contained the membrane suspension, 33 mM $MgCl_2$, 40 mM piperazine acetate buffer (pH 6.5), 5 mM ATP, 1 mM dithiothreitol, 100 mM phosphate (pH 6.5), and 46 μM D-alanine in a final volume of 0.5 ml. This mixture was incubated at 37°C for 30 min. When 1,2-diolein was used, the mixtures also contained 25 μCi of [γ-^{32}P]ATP (carrier free), 10 mM GroP, and 5 mM CTP.

Labeled chloroform-soluble compounds were isolated by the monophasic extraction procedure described for the incorporation assay. The phosphatidic acid was separated by thin-layer chromatography and visualized by autoradiography.

Synthesis and degradation of [^{14}C]glycerol · [^{3}H]glycerol-LTA. Doubly labeled LTA was used to determine the location of the newly added GroP units. [^{14}C]Glycerol · [^{3}H]glycerol-LTA was synthesized by incubating [2-^{3}H]glycerol-labeled, toluene-treated cells with [^{14}C]GroP (4.8 μCi) in a scale-up of the standard incorporation assay reaction mixture (total volume, 25 ml). This mixture was incubated for 50 min at 37°C. Isolation and purification of [^{14}C]glycerol · [^{3}H]glycerol-D-alanyl-LTA free of D-alanyl-lipophilic LTA, degradation with phosphodiesterase II-phosphatase, and assay for the degradation products were done as previously described (4, 6).

Chromatography of chloroform-soluble compounds. The chloroform-soluble fractions containing the radioactively labeled lipids were dried under N_2. The residues were redissolved in $CHCl_3$, and samples were applied to silica gel 60 thin-layer plates. The plates were developed with a solvent consisting of butanol-acetic acid-water (120:30:50, vol/vol/vol). Autoradiograms were prepared by exposing Kodak XR-5 XOMAT X-ray film to the plate at −80°C.

Analytical methods. For the identification of phosphatidic acid, CDP-diglyceride, PG, and 1,2-diglyceride, the labeled lipids were compared with authentic standards by thin-layer chromatography in three solvent systems: (i) butanol-acetic acid-water (120:30:50, vol/vol/vol); (ii) $CHCl_3$-CH_3OH-acetic acid (65:25:13.5, vol/vol/vol); (iii) $CHCl_3$–CH_3OH–7 M NH_4OH (60:35:5, vol/vol/vol). The standards were visualized by exposing the plates to I_2 vapor after autoradiography. Glycerophosphotrihexosyldiacylglycerol was compared with D-[^{14}C]alanyl- and [^{14}C]GroP-labeled lipophilic compounds by thin-layer chromatography in solvent i. The glycerophosphotrihexosyldiacylglycerol standard was visualized by

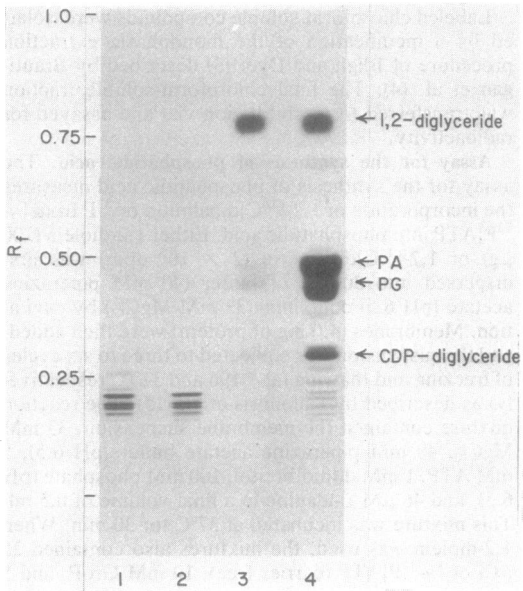

FIG. 1. Incorporation of D-[^{14}C]alanine (lanes 1 and 2) and [^{14}C]GroP (lanes 3 and 4) into chloroform-soluble compounds. The incorporation assay used is described in the text. PA, Phosphatidic acid. As visualized by I_2 staining, identical amounts of phospholipid were applied to all tracks.

lightly spraying the plate with a solution of 3.2% (wt/vol) α-naphthol in methanol–H_2SO_4–water (25:3:2, vol/vol/vol) and heating the plate at 110°C for 25 min (W. Fischer, personal communication). The method of Lowry et al. (19) was used for protein determination. Bovine serum albumin was used as the standard. The scintillation fluid described by Patterson and Greene (22) was used for the determination of radioactivity with a Packard model 2425 Tri-Carb liquid scintillation spectrometer. A Branson Sonifier cell disruptor was used at 30% output (45 W) for 5 min to disperse lipids into aqueous solution by cavitation.

RESULTS

In this study, two classes of LTA were defined on the basis of their solubility. Lipophilic LTA is short-chained lipoteichoic acid which partitions into the chloroform phase of the biphasic extraction system (3, 4). Hydrophilic LTA is longer chained and partitions into the aqueous phase of this system. The D-alanyl-lipophilic compounds previously described by Brautigan et al. (4) are D-alanyl-lipophilic LTA.

GroP incorporation into PG. To study the assembly of the poly(glycerophosphate) moiety of LTA in toluene-treated cells of *L. casei*, labeled GroP was used as a precursor of PG, the presumed donor of the GroP units of LTA. Incubation of these cells with [^{14}C]GroP resulted in the labeling of a number of chloroform-soluble compounds (Fig. 1). The labeling of these compounds was dependent on ATP. The addition of 100 mM phosphate strongly stimulated the incorporation of labeled GroP into PG, phosphatidic acid, and CDP-diglyceride, whereas it had little effect on the labeling of 1,2-diglyceride. Other anions, e.g., sulfate, chloride, and nitrate, also enhanced the formation of these phospholipids (Table 1). No difference was observed when sodium phosphate was used in place of potassium phosphate. Phosphate gave the best stimulation and was used routinely. Since there was no apparent correlation between ionic strength and percent stimulation, it appeared that this stimulation resulted from an anionic effect. Thus, in the presence of ATP and phosphate, PG can be readily labeled with [^{14}C]GroP.

GroP incorporation into lipophilic LTA via PG. The ability to label the PG pool with [^{14}C]GroP provided a method for studying the assembly of lipophilic LTA in toluene-treated cells. A series of [^{14}C]GroP-labeled compounds with mobilities similar to those of the D-[^{14}C]alanyl-lipophilic compounds became labeled when 100 mM phosphate was present (Fig. 1). It was previously suggested that the D-alanyl-lipophilic compounds are D-alanyl-LTA with short chains of poly(glycerophosphate) (4). Thus, the [^{14}C]GroP-labeled compounds may represent lipophilic LTA of various chain lengths and degrees of D-alanine substitution. One of these compounds has the same R_f as glycerophosphotrihexosyldiacylglycerol, a short-chained homolog of *L. casei* LTA isolated from strain DSM 20021 by Fischer and co-workers (10, 12, 21). As

TABLE 1. Incorporation of [^{14}C]GroP and ^{32}P from [γ-^{32}P]ATP into chloroform-soluble compounds

Reaction mixture[a]	Activity (% of control)[b]	
	[^{14}C]GroP	[γ-^{32}P]ATP
Complete	100	100
Without HPO_4^{2-}	9.9	11
Plus SO_4^{2-} [c]	61	78
Plus NO_3^-	79	68
Plus Cl^-	71	66
Without ATP	1.1	ND[d]
Without ATP and HPO_4^{2-}	1.1	ND

[a] The complete reaction mixture contained toluene-treated cells (16 mg [wet weight]), 33 mM $MgCl_2$, 40 mM piperazine acetate buffer (pH 6.5), 5 mM ATP (neutralized with KOH), 100 mM phosphate (pH 6.5), 1 mM dithiothreitol, 46 μM D-alanine, and 23 μM [^{14}C]GroP (171 mCi/mmol) or 25 μCi of [γ-^{32}P]ATP (carrier free) in a total volume of 500 μl. The mixture was incubated for 30 min at 37°C.

[b] For comparison, incorporation in the presence of 5 mM ATP and 100 mM phosphate was arbitrarily set at 100% (control). For [^{14}C]GroP, 100% represents 32,000 dpm of chloroform-soluble ^{14}C. For [γ-^{32}P]ATP, 100% represents 6,600 dpm of chloroform-soluble ^{32}P.

[c] All anions replacing phosphate were added as potassium salts to a concentration of 100 mM.

[d] ND, Not determined.

with PG, phosphate also stimulated the labeling of these lipophilic compounds by [^{14}C]GroP. This correlation suggested that as more ^{14}C-labeled PG is synthesized, the synthesis of labeled lipophilic LTA increases.

Site of addition of GroP units to LTA. The ability to label lipophilic LTA with [^{14}C]GroP suggested that it also may be possible to label LTA of any chain length and to determine the site of addition of these GroP units. Toluene-treated cells, which had been grown in the presence of [2-^{3}H]glycerol to label uniformly the LTA, were incubated in the presence of [^{14}C]GroP, ATP, and 100 mM phosphate for 50 min to generate a gradient of label. The omission of either phosphate or ATP resulted in little or no incorporation of [^{14}C]GroP into LTA. LTA was isolated as previously described (4, 6). Hydrophilic LTA was separated from labeled phospholipids and lipophilic LTA by the extraction procedure described for the incorporation assay. The resulting [^{14}C]glycerol · [^{3}H]glycerol-LTA was then degraded with phosphodiesterase II-phosphatase. This enzymatic procedure sequentially degrades from the distal end of LTA to the proximal end adjacent to the glycolipid anchor (4, 5, 11). There was a large intitial release of the [^{14}C]glycerol label relative to the [^{3}H]glycerol label (Fig. 2). This large initial ^{14}C/^{3}H ratio suggested that the [^{14}C]GroP units are added distally to the glycolipid anchor.

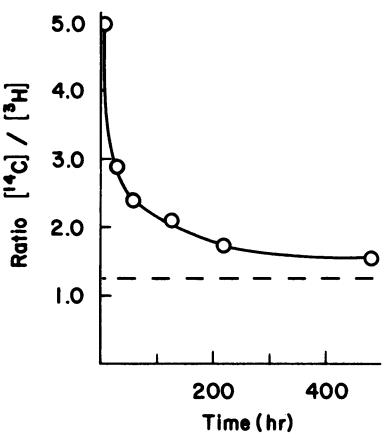

FIG. 2. Degradation of [^{14}C]glycerol · [^{3}H]glycerol-labeled LTA with phosphodiesterase II-phosphatase. Cells were uniformly labeled with [2-^{3}H]glycerol, treated with toluene, and incubated with [^{14}C]GroP as described in the text. The reaction mixture for a given time point contained: [^{14}C]glycerol · [^{3}H]glycerol-labeled D-alanyl LTA (^{14}C, 3,400 dpm; ^{3}H, 2,740 dpm), 50 mM formate buffer (pH 4.5), 180 μg of phosphodiesterase II, and 330 μg of wheat germ acid phosphatase in a total volume of 760 μl. The reaction mixtures were incubated at 37°C for the indicated times. The sample was applied to a column of DEAE-Sephadex (0.6 by 3 cm), and the column was eluted with 3 ml of formate buffer (pH 4.5) in 1-ml portions. The ^{14}C/^{3}H ratios in the original undegraded LTA (dashed line) and the degradation products collected in the effluent fractions (○) were then determined.

Incorporation of ^{32}P from [γ-^{32}P]ATP into phospholipids. In previous experiments, $^{32}P_i$ was found to label PG and other chloroform-soluble compounds in toluene-treated cells of *L. casei* (4). It was suggested that $^{32}P_i$ was incorporated into a water-soluble intermediate, which was subsequently converted to PG in the presence of 100 mM phosphate (4). In the present experiments, incubating toluene-treated cells with [γ-^{32}P]ATP resulted in the labeling of PG, phosphatidic acid, and CDP-diacylglycerol, as well as several other chloroform-soluble compounds. Thus, ATP would appear to be one of the intermediates in the incorporation of $^{32}P_i$ into phospholipids. The labeling of chloroform-soluble compounds with [γ-^{32}P]ATP was enhanced by phosphate, sulfate, nitrate, or chloride (Table 1). Sodium phosphate and potassium phosphate gave the strongest stimulation. This stimulation was similar to that observed for the incorporation of [^{14}C]GroP into chloroform-soluble compounds.

Diglyceride kinase in *L. casei*. The production of 1,2-diglyceride in the elongation of LTA suggested the possibility that a 1,2-diglyceride kinase may exist in *L. casei*. Membranes were used as a system to which lipid substrates could be added to detect this enzymatic activity. Unlike toluene-treated cells, membranes required the addition of CTP and GroP for [γ-^{32}P]ATP labeling of PG. Incorporation of ^{32}P from [γ-^{32}P]ATP into PG and phosphatidic acid could be stimulated by the addition of 1,2-diolein to membranes and stimulated further by the addition of 100 mM phosphate (Fig. 3). The addition of phosphate in the absence of 1,2-diolein resulted in little stimulation of labeling. Thus, the 1,2-diolein appeared to be a substrate for the synthesis of PG. The appearance of [^{32}P]phosphatidic acid suggested that 1,2-diolein was phosphorylated by a diglyceride kinase. An additional assay with 1,2-[^{14}C]dipalmitin was also used to detect this kinase. Figure 4 (lane 5) shows the phosphate-stimulated, ATP-dependent synthesis of [^{14}C]phosphatidic acid from 1.2-[^{14}C]dipalmitin. The omission of phosphate or ATP resulted in little synthesis of phosphatidic acid. The conversion of this diglyceride to [^{14}C]phosphatidic acid is consistent with the presence of a 1,2-diglyceride kinase in *L. casei*. The stimulation of this kinase by 100 mM phosphate correlated with the phosphate-stimulated syntheses of PG, lipophilic LTA, and hydrophilic LTA.

DISCUSSION

Phosphate and other anions appear to play an important role in the in vitro elongation of LTA

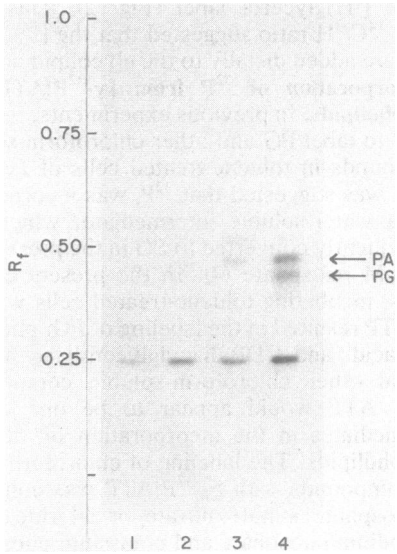

FIG. 3. Effect of 1,2-diolein on incorporation of ^{32}P from [γ-^{32}P]ATP into phospholipids. The complete reaction mixture (lane 4) contained 300 μl of the membrane–1,2-diolein suspension as described in the text, 40 mM piperazine acetate buffer (pH 6.5), 33 mM $MgCl_2$, 5 mM ATP, 1 mM dithiothreitol, 100 mM phosphate (pH 6.5), and 30 μCi of [γ-^{32}P]ATP (carrier free) in a total volume of 0.5 ml. The reaction mixture was varied as follows: lane 1, minus 1,2-diolein and phosphate; lane 2, minus 1,2-diolein, plus phosphate; lane 3, plus 1,2-diolein, minus phosphate. The compound migrating with an R_f of 0.25 was not identified. PA, Phosphatidic acid.

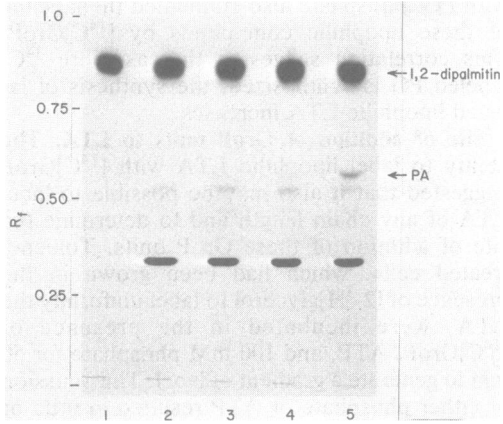

FIG. 4. Effect of ATP on the conversion of 1,2-[^{14}C]dipalmitin to phosphatidic acid. The complete reaction mixture (lane 5) contained 400 μl of the membrane–1,2-[^{14}C]dipalmitin suspension as described in the text, 40 mM piperazine acetate (pH 6.5), 33 mM $MgCl_2$, 5 mM ATP, 1 mM dithiothreitol, 100 mM phosphate (pH 6.5), and 46 μM D-alanine in a total volume of 1 ml. The lower two-thirds of the thin-layer chromatography plate was lightly sprayed with three coats of En^{3}Hance (New England Nuclear Corp., Boston, Mass.) before autoradiography. Lane 1 contains 20,000 dpm of the prepared 1,2-[^{14}C]dipalmitin. The reaction mixture was varied as follows: lane 2, minus ATP and phosphate; lane 3, minus ATP, plus phosphate; lane 4, plus ATP, minus phosphate. The compound migrating with an R_f of 0.3 was not identified. PA, Phosphatidic acid.

by enhancing the synthesis of PG. GroP units from this phospholipid are added distally to the glycolipid anchor of the elongating LTA chains in toluene-treated cells. This addition results in the formation of 1,2-diglyceride. Evidence is presented for the presence of a diglyceride kinase in *L. casei*. It is proposed that this enzyme converts the resulting 1,2-diglyceride to phosphatidic acid, which can be utilized for the synthesis of PG, the donor of GroP for LTA elongation (Fig. 5).

Three observations suggested that inorganic phosphate and other anions stimulate one or more reactions in the assembly of lipophilic and hydrophilic LTA in toluene-treated cells of *L. casei*. First, inorganic phosphate greatly increased the incorporation of [^{14}C]GroP into lipophilic LTA. Second, it stimulated the elongation of D-[^{14}C]alanyl-lipophilic LTA (4). Third, as with lipophilic LTA, it enhanced the incorporation of [^{14}C]GroP into hydrophilic LTA. Thus, inorganic phosphate can be used as a tool for initiating the in vitro synthesis of lipophilic LTA and for elongating both lipophilic and hydrophilic LTA.

Inorganic phosphate was used to stimulate the incorporation of [^{14}C]GroP into hydrophilic LTA in toluene-treated cells to generate a gradient of label. Sequential degradation of this labeled LTA with phosphodiesterase II-phosphatase revealed that GroP units were added distally to the glycolipid anchor. This observa-

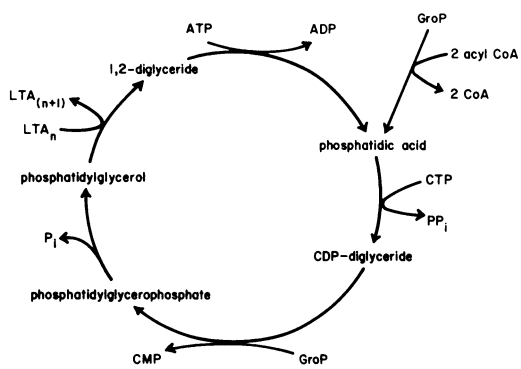

FIG. 5. Proposed diglyceride cycle for LTA biosynthesis in *L. casei*. acyl CoA, Acyl coenzyme A.

tion supports the conclusion of Cabacungan and Pieringer (5), who reported that GroP units were added distally in whole cells and isolated membranes of *Streptococcus faecium*. Thus, the mode of distal addition of GroP to LTA in toluene-treated cells of *L. casei* appears to be similar to that in *S. faecium* cells.

In addition to stimulating the elongation of LTA, inorganic phosphate in the presence of ATP was found to increase the synthesis of PG, phosphatidic acid, and CDP-diglyceride. Sulfate, chloride, and nitrate also stimulated the synthesis of these phospholipids, but to a lesser extent than phosphate did. This stimulation was similar to that observed for sulfate, which enhances the synthesis of phosphatidylethanolamine in *Bacillus megaterium* (23). The phospholipids stimulated by these anions occur in the pathway of PG assembly observed in *B. megaterium* (23) and *Escherichia coli* (25). Therefore, it seems likely that a similar or identical pathway for phospholipid synthesis occurs in *L. casei* (Fig. 5). The labeling of 1,2-diglyceride by [^{14}C]GroP implies that GroP is acylated. Acylation of [^{14}C]GroP by endogenous fatty acyl coenzyme A results in [^{14}C]phosphatidic acid, which is converted to [^{14}C]PG and 1,2-[^{14}C]diglyceride. In addition, PG can be labeled by the incorporation of [^{14}C]GroP into the glycerol headgroup. Thus, two routes for the incorporation of [^{14}C]GroP into PG exist. The phosphate-enhanced labeling of PG, phosphatidic acid, and CDP-diglyceride suggests that this anion stimulates one or more of the enzymes in this pathway. Alternatively, the enhanced labeling could result from the inhibition of ATPase (28).

In toluene-treated cells, ATP is required for the phosphate-stimulated synthesis of phospholipids. ATP may be a substrate for a diglyceride kinase which generates phosphatidic acid from 1,2-diglyceride. A kinase found in *E. coli* (24, 29, 30) catalyzes the phosphorylation of diglycerides via the following reaction:

$$\text{1,2-diglyceride} + \text{ATP} \rightarrow \text{phosphatidic acid} + \text{ADP}$$

In this organism, 1,2-diglyceride results from the transfer of GroP from PG to membrane-derived oligosaccharides (25). Mutant strains lacking a functional diglyceride kinase accumulate significant amounts of 1,2-diglyceride (26). Since PG is the donor of GroP units for LTA elongation in *L. casei*, 1,2-diglyceride might be expected to accumulate as the polymer is elongated. Alternatively, a diglyceride kinase could function to salvage the 1,2-diglyceride produced during LTA assembly (Fig. 5). In the present work, this enzyme was detected in *L. casei*. The stimulation of diglyceride kinase by phosphate correlates with the stimulation of phospholipid and LTA synthesis by this anion. This correlation suggests a possible involvement of this enzyme in phospholipid and LTA synthesis.

In a number of bacteria, PG is present in two distinct pools (1, 2, 17, 18) with different rates of metabolic turnover. In *B. megaterium*, one pool of PG undergoes rapid turnover whereas the other exhibits metabolic stability. It has been suggested that the former pool is the donor of GroP units for LTA synthesis (17, 18). Recently, Brautigan et al. suggested that toluene-treated cells of *L. casei* synthesize a unique population of PG in the presence of excess phosphate (4). This PG could donate GroP units to D-alanyl-lipophilic LTA, resulting in chain elongation. This suggestion is supported by the anionic stimulation of [^{14}C]GroP incorporation into PG as well as into lipophilic and hydrophilic LTA. Diglyceride kinase showed a similar anionic stimulation. Thus, this kinase may play a significant role in the rapid turnover of one of the PG pools and hence in the proposed diglyceride cycle involved in the elongation of LTA.

Inorganic phosphate is a useful tool for stimulating the in vitro elongation of LTA in toluene-treated cells of *L. casei* and the in vitro synthesis of PG both in membranes and in these treated cells. One enzyme in the proposed diglyceride cycle, diglyceride kinase, is stimulated by phosphate. The exact mechanism by which phosphate and other anions exert their effect is not known. Further studies on these anionic effects on individual enzymes in the proposed pathway may help to define this mechanism more precisely. The physiological role of phosphate in the in vivo synthesis of LTA is not understood. In contrast, phosphate has a major, well-defined effect on the in vivo synthesis of wall teichoic acid (32). It has been recognized that phosphate-limited cells of *Bacillus subtilis* synthesize only 10% of their phospholipid as PG, whereas cells which are not limited in phosphate synthesize 54% of their phospholipid as PG (20). These observations can be correlated with those of Button et al. (D. Button, M. K. Choudhry, and N. L. Hemmings, Proc. Soc. Gen. Microbiol. **2**:45, 1975), who reported a 10-fold decrease in LTA synthesis in phosphate-limited cells of *Bacillus licheniformis*. These results, together with those reported in this paper, suggest a possible role for phosphate in the in vivo regulation of the diglyceride cycle described in this organism.

ACKNOWLEDGMENTS

This investigation was supported in part by Public Health Service grant AI-04615 from the National Institute of Allergy and Infectious Diseases.

We thank Werner Fischer for discussions and help in characterizing the lipids from this strain of *L. casei*.

LITERATURE CITED

1. **Ballesta, J. P. G., C. L. DeGarcia, and M. Schaechter.** 1973. Turnover of phosphatidylglycerol in *Escherichia coli*. J. Bacteriol. **116**:210–214.
2. **Bevers, E. M., S. A. Singal, J. A. F. Op den Kamp, and L. L. M. van Deenen.** 1977. Recognition of different pools of phosphatidylglycerol in intact cells and isolated membranes of *Acholeplasma laidlawii* by phospholipase A_2. Biochemistry **16**:1290–1295.
3. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**:911–917.
4. **Brautigan, V. M., W. C. Childs III, and F. C. Neuhaus.** 1981. Biosynthesis of D-alanyl-lipoteichoic acid in *Lactobacillus casei*: D-alanyl-lipophilic compounds as intermediates. J. Bacteriol. **146**:239–250.
5. **Cabacungan, E., and R. A. Pieringer.** 1981. Mode of elongation of the glycerol phosphate polymer of membrane lipoteichoic acid of *Streptococcus faecium* ATCC 9790. J. Bacteriol. **147**:75–79.
6. **Childs, W. C., III, and F. C. Neuhaus.** 1980. Biosynthesis of D-alanyl-lipoteichoic acid: characterization of ester linked D-alanine in the in vitro-synthesized product. J. Bacteriol. **143**:293–301.
7. **Dittmer, J. C., and M. A. Wells.** 1969. Quantitative and qualitative analysis of lipids and lipid components. Methods Enzymol. **14**:482–530.
8. **Emdur, L. I., and T.-H. Chiu.** 1974. Turnover of phosphatidylglycerol in *Streptococcus sanguis*. Biochem. Biophys. Res. Commun. **59**:1137–1144.
9. **Emdur, L. I., and T.-H. Chiu.** 1975. The role of phosphatidylglycerol in the *in vitro* biosynthesis of teichoic acid and lipoteichoic acid. FEBS Lett. **55**:216–219.
10. **Fischer, W.** 1981. Glycerophosphoglycolipids: presumptive biosynthetic precursors of lipoteichoic acids, p. 209–228. *In* G. D. Shockman and A. J. Wicken (ed.), Chemistry and biological activities of bacterial surface amphiphiles. Academic Press, Inc., New York.
11. **Fischer, W., H. U. Koch, P. Rosel, and F. Fiedler.** 1980. Alanine ester-containing native lipoteichoic acids do not act as lipoteichoic acid carrier: isolation, structural and functional characterization. J. Biol. Chem. **255**:4557–4562.
12. **Fischer, W., R. A. Laine, and M. Nakano.** 1978. On the relationship between glycerophosphoglycolipids and lipoteichoic acids in gram positive bacteria. II. Structures of glycerophosphoglycolipids. Biochim. Biophys. Acta **528**:298–308.
13. **Ganfield, M.-C., and R. A. Pieringer.** 1980. The biosynthesis of nascent membrane lipoteichoic acid of *Streptococcus faecium* (*S. faecalis* ATCC 9790) from phosphatidylkojibiosyl diacylglycerol and phosphatidylglycerol. J. Biol. Chem. **255**:5164–5169.
14. **Glaser, L., and B. Lindsay.** 1974. The synthesis of lipoteichoic acid carrier. Biochem. Biophys. Res. Commun. **59**:1131–1136.
15. **Kalomiris, E., C. Bardin, and F. C. Neuhaus.** 1982. Biosynthesis of peptidoglycan in *Gaffkya homari*: reactivation of membranes by freeze-thawing in the presence and absence of walls. J. Bacteriol. **150**:535–544.
16. **Linzer, R., and F. C. Neuhaus.** 1973. Biosynthesis of membrane teichoic acid: a role for the D-alanine activating enzyme. J. Biol. Chem. **248**:3196–3201.
17. **Lombardi, F. J., S. L. Chen, and A. J. Fulco.** 1980. A rapidly metabolizing pool of phosphatidylglycerol as a precursor for phosphatidylethanolamine and diglyceride in *Bacillus megaterium*. J. Bacteriol. **141**:626–634.
18. **Lombardi, F. J., and A. J. Fulco.** 1980. Two distinct pools of membrane phosphatidylglycerol in *Bacillus megaterium*. J. Bacteriol. **141**:618–625.
19. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
20. **Minnikin, D. E., H. Abdolrahimzadeh, and J. Baddiley.** 1972. Variation of polar lipid composition of *Bacillus subtilus* (Marburg) with different growth conditions. FEBS Lett. **27**:16–18.
21. **Nakano, M., and W. Fischer.** 1978. Trihexosyldiacylglycerol and acyltrihexosyldiacylglycerol as lipid anchors of the lipoteichoic acid of *Lactobacillus casei* DSM 20021. Hoppe-Seyler's Z. Physiol. Chem. **359**:1–11.
22. **Patterson, M. S., and R. C. Greene.** 1965. Measurement of low energy beta-emitters in aqueous solution by liquid scintillation counting of emulsions. Anal. Chem. **37**:854–857.
23. **Patterson, P. H., and W. J. Lennarz.** 1971. Studies on the membranes of bacilli. I. Phospholipid biosynthesis. J. Biol. Chem. **246**:1062–1072.
24. **Pieringer, R. A., and R. S. Kunnes.** 1965. The biosynthesis of phosphatidic acid and lysophosphatidic acid by glyceride phosphokinase pathways in *Escherichia coli*. J. Biol. Chem. **240**:2833–2838.
25. **Raetz, C. R. H.** 1978. Enzymology, genetics, and regulation of membrane phospholipid synthesis in *Escherichia coli*. Microbiol. Rev. **42**:614–659.
26. **Raetz, C. R. H., and K. F. Newman.** 1978. Neutral lipid accumulation in the membranes of *Escherichia coli* lacking diglyceride kinase. J. Biol. Chem. **253**:3882–3887.
27. **Reusch, V. M., Jr., and F. C. Neuhaus.** 1971. D-Alanine: membrane acceptor ligase from *Lactobacillus casei*. J. Biol. Chem. **246**:6136–6143.
28. **Schnebli, H. P., and A. Abrams.** 1970. Membrane adenosine triphosphatase from *Streptococcus faecalis*. J. Biol. Chem. **245**:1115–1121.
29. **Schneider, E. G., and E. P. Kennedy.** 1973. Phosphorylation of ceramide by diglyceride kinase preparations from *Escherichia coli*. J. Biol. Chem. **248**:3739–3741.
30. **Schneider, E. G., and E. P. Kennedy.** 1976. Partial purification and properties of diglyceride kinase from *Escherichia coli*. Biochim. Biophys. Acta **441**:201–212.
31. **Schneider, J. E., and E. P. Kennedy.** 1978. A novel phosphodiesterase from *Aspergillus niger* and its application to the study of membrane-derived oligosaccharides and other glycerol-containing biopolymers. J. Biol. Chem. **253**:7738–7743.
32. **Ward, J. B.** 1981. Teichoic and teichuronic acids: biosynthesis, assembly, and location. Microbiol. Rev. **45**:211–243.
33. **Worthington Biochemicals Corporation.** 1977. Phospholipase C (*Clostridium perfringens*), p. 150–152. *In* L. A. Decker (ed.), Worthington enzyme manual. Worthington Biochemicals Corp., Freehold, N.J.