

## Biosynthesis of D-Alanyl-Lipoteichoic Acid by *Lactobacillus casei*: Interchain Transacylation of D-Alanyl Ester Residues

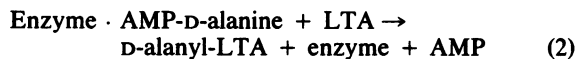
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**Lipoteichoic acid (LTA) from *Lactobacillus casei* contains poly(glycerophosphate) substituted with D-alanyl ester residues. The distribution of these residues in the in vitro-synthesized polymer is uniform. Esterification of LTA with D-alanine may occur in one of two modes: (i) addition at random or (ii) addition at a defined locus in the poly(glycerophosphate) chain followed by redistribution of the ester residues. A time-dependent transacylation of these residues from D-[<sup>14</sup>C]alanyl-lipophilic LTA to hydrophilic acceptor was observed. The hydrophilic acceptor was characterized as D-alanyl-hydrophilic LTA. This transacylation requires neither ATP nor the D-alanine incorporation system, i.e., the D-alanine activating enzyme and D-alanine:membrane acceptor ligase. No evidence for an enzyme-catalyzed transacylation reaction was observed. We propose that this process of transacylation may be responsible for the redistribution of D-alanyl residues after esterification to the poly(glycerophosphate). As a result, it is difficult to distinguish between these proposed modes of addition.**

The assembly of D-alanyl-lipoteichoic acid (D-alanyl LTA) requires a biosynthetic system for selectively acylating the glycerol-phosphate units of the poly(glycerophosphate) moiety with D-alanyl ester residues. These residues play a significant role in modulating the biological activities of this polymer (6, 9, 13). In *Lactobacillus casei* the incorporation of D-alanine into LTA is accomplished in the following two-step reaction sequence (14, 18, 20)



To provide a better understanding of this sequence, intermediates were sought that might participate in the incorporation of D-alanine or in the assembly of the poly(glycerophosphate) moiety. A series of membrane-associated lipophilic compounds containing ester-linked D-alanine was detected in membranes of *L. casei* (2). Characterization of these compounds indicated that they are D-alanyl-LTA with short chains of oligo(glycerophosphate). Fischer and coworkers (7, 8, 11) have also described a series of glycerophosphoglycolipids that are presumed to be intermediates in the assembly of LTA. One of those from *Bacillus licheniformis* is a D-alanyl-glycerophosphoglycolipid (5). Based on their ability to partition into chloroform, these compounds are designated D-alanyl-lipophilic LTA. The long-chained homologs, which do not partition into this solvent, are designated D-alanyl-hydrophilic LTA. Kinetic time courses of D-alanine incorporation into lipophilic LTA and hydrophilic LTA indicate that lipophilic LTA is not necessarily an intermediate in the transfer of D-alanine to

hydrophilic LTA. Both species of LTA act as acceptors for the activated D-alanine (2).

The D-alanyl ester residues are uniformly distributed along the poly(glycerophosphate) chain of the hydrophilic LTA (4, 6). Esterification of LTA with D-alanine via the two-step incorporation system described above may occur in one of two modes: (i) addition at random or (ii) addition at a defined locus in the poly(glycerophosphate) chain followed by redistribution of the D-alanyl ester residues. The observed uniform distribution of D-alanine was hypothesized to result from rapid transacylation of these residues. This redistribution could occur within the poly(glycerophosphate) chain (intrachain transacylation) or between chains (interchain transacylation). It is the purpose of this paper to demonstrate interchain transacylation of the D-alanyl ester residues. This reaction was detected by the transfer of D-[<sup>14</sup>C]alanyl ester residues from D-[<sup>14</sup>C]alanyl-lipophilic LTA to hydrophilic LTA. Transacylation of the D-alanyl ester residues represents a new feature of LTA.

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### MATERIALS AND METHODS

**Materials.** We thank Eugene Kennedy for a generous gift of phosphodiesterase II from *Aspergillus niger* (21). D-[1-<sup>14</sup>C]alanine (56.2 mCi/mmol) and DL-[<sup>3</sup>H]alanine (3 Ci/mmol) were purchased from International Chemical and Nuclear Corporation, Irvine, Calif. [2-<sup>3</sup>H]glycerol (200 mCi/mmol) was purchased from Amersham Corp., Arlington Heights, Ill. Wheat germ acid phosphatase was purchased from Worthington Biochemicals Corp., Freehold, N.J.

**Preparation of toluene-treated cells and membranes.** *L. casei* ATCC 7469 was grown by the method described by Childs and Neuhaus (4). The toluene-treated cells were prepared as previously described (4). Membranes were prepared from *L. casei* by the procedure described by Linzer and Neuhaus (14).

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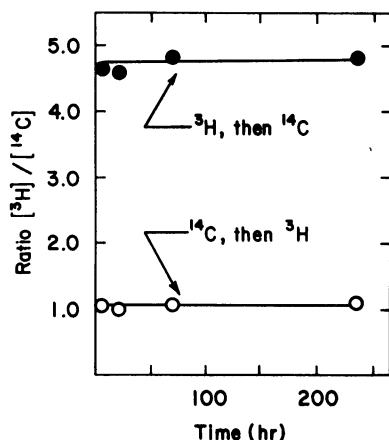


FIG. 1. Sequential degradation of D-[ $^{14}\text{C}$ ]alanyl-D-[ $^3\text{H}$ ]alanyl-hydrophilic LTA. Toluene-treated cells were incubated with D-[ $^{14}\text{C}$ ]alanine and DL-[ $^3\text{H}$ ]alanine as described in the text. The doubly labeled LTA was sequentially degraded with phosphodiesterase II-phosphatase. The reaction mixture for a given time point contained D-[ $^{14}\text{C}$ ]alanyl-D-[ $^3\text{H}$ ]alanyl-LTA (3,130 dpm of  $^{14}\text{C}$ , 3,490 dpm of  $^3\text{H}$  [bottom line] or 1,460 dpm of  $^{14}\text{C}$ , 7,260 dpm of  $^3\text{H}$  [top line]), 50 mM formate buffer (pH 4.5), 180  $\mu\text{g}$  of phosphodiesterase II, and 330  $\mu\text{g}$  of wheat germ acid phosphatase in a total volume of 760  $\mu\text{l}$ . The samples were incubated at 37°C for the indicated times. The labeled D-alanyl-glycerol fraction was isolated and assayed for radioactivity as described by Childs and Neuhaus (4).

**Preparation of D-[ $^{14}\text{C}$ ]alanyl-lipophilic LTA.** D-[ $^{14}\text{C}$ ]alanyl-lipophilic LTA was synthesized in toluene-treated *L. casei* cells. The reaction mixture contained 33 mM  $\text{MgCl}_2$ , 5 mM ATP (adjusted to pH 6.5 with NaOH), 40 mM piperazine acetate (pH 6.5), 1 mM dithiothreitol, 46  $\mu\text{M}$  D-[ $^{14}\text{C}$ ]alanine (56.2 mCi/mmol), and toluene-treated cells (300 mg [dry weight]) in a total volume of 8.5 ml. The chloroform-soluble fraction, which contained D-[ $^{14}\text{C}$ ]alanyl-lipophilic LTA was isolated by the Bligh and Dyer (1) extraction procedure. This fraction was dried under  $\text{N}_2$  and the residue was dissolved in 1 ml of chloroform. For D-[ $^{14}\text{C}$ ]alanyl-[ $^3\text{H}$ ]glycerol-labeled lipophilic LTA, *L. casei* was grown in the presence of [ $^3\text{H}$ ]glycerol (1 mCi/liter). The [ $^3\text{H}$ ]glycerol-labeled cells were toluene treated and incubated in the above reaction mixture, and the D-[ $^{14}\text{C}$ ]alanyl-[ $^3\text{H}$ ]glycerol-lipophilic LTA was isolated as described above.

**Synthesis of D-[ $^{14}\text{C}$ ]alanyl-D-[ $^3\text{H}$ ]alanyl-LTA.** Doubly labeled LTA was synthesized in a two-stage incubation with D-[ $^{14}\text{C}$ ] and DL-[ $^3\text{H}$ ]alanine. In the first stage, toluene-treated cells were incubated with D-[ $^{14}\text{C}$ ]alanine. The reaction mixture contained 40 mM piperazine acetate (pH 6.5), 33 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol, 5 mM ATP (adjusted to pH 6.5 with NaOH), 0.56  $\mu\text{M}$  D-[ $^{14}\text{C}$ ]alanine ( $1.6 \times 10^6$  dpm), toluene-treated cells (605 mg [wet weight]) in a total volume of 22.8 ml. The mixture was incubated at 37°C for 30 min and then centrifuged at  $3,000 \times g$  for 5 min. The pellet was resuspended in 18.8 ml of piperazine- $\text{MgCl}_2$ -dithiothreitol buffer containing 5 mM ATP and 0.11  $\mu\text{M}$  DL-[ $^3\text{H}$ ]alanine ( $1.6 \times 10^7$  dpm). In the second stage, this mixture was incubated at 37°C for 30 min. The doubly labeled LTA was then isolated by the procedure described by Childs and Neuhaus (4). For comparison, the synthesis was also performed with the stages of incubation reversed.

**Transacylation assay.** The transacylation assay measures the transfer of D-[ $^{14}\text{C}$ ]alanyl ester residues from D-[ $^{14}\text{C}$ ]alanyl-lipophilic LTA to endogenous hydrophilic LTA. A sample of D-[ $^{14}\text{C}$ ]alanyl-lipophilic LTA was dried under

$\text{N}_2$  and dispersed by cavitation into 900  $\mu\text{l}$  of 40 mM piperazine acetate buffer (pH 6.5) containing 33 mM  $\text{MgCl}_2$ . The reaction mixture contained D-[ $^{14}\text{C}$ ]alanyl-lipophilic LTA (50,000 dpm) containing 400 pmol of D-[ $^{14}\text{C}$ ]alanine, 1 mM dithiothreitol, and membranes (2 mg) in a total volume of 1 ml. The mixture was incubated for various times at 37°C. To terminate the reaction, 1 ml of 2% octyl- $\beta$ -D-glucopyranoside was added and the detergent-treated reaction mixture was maintained for 2 h at 4°C with frequent mixing.

D-[ $^{14}\text{C}$ ]alanyl-hydrophilic LTA from the membrane was isolated from the detergent extract by the following procedure. The extract was centrifuged at  $104,000 \times g$  for 45 min. The supernatant fraction was dialyzed overnight against 2 liters of 5 mM piperazine acetate (pH 6.5). It was applied to a column of Sepharose 6B (1.5 by 80 cm) and eluted with 5 mM piperazine-hydrochloride (pH 6.5). The labeled fraction eluting near the void volume (4) was pooled and concentrated under reduced pressure to a volume of 3 ml. D-[ $^{14}\text{C}$ ]alanyl-lipophilic LTA was removed from this fraction by three cycles of Bligh and Dyer extraction. Partitioning of the extract was with water rather than 0.9% NaCl. The aqueous layer, which contained D-[ $^{14}\text{C}$ ]alanyl-hydrophilic LTA, was concentrated to a volume of 2 ml and the amount of D-[ $^{14}\text{C}$ ]alanyl-hydrophilic LTA was quantitated.

**Analytical methods.** The method of Lowry et al. (16) was used for protein determination with bovine serum albumin as the standard. For the separation of D-[ $^{14}\text{C}$ ]alanine, D-[ $^{14}\text{C}$ ]alanyl-glycerol, and D-[ $^{14}\text{C}$ ]alanyl-LTA, chromatography was performed with solvent A (butanol-propionic acid-water, 142:71:100 [vol/vol/vol]) on Whatman 3MM paper. The scintillation fluid described by Patterson and Greene (19) was used for the determination of radioactivity. A Sonifier cell disruptor (Branson Sonic Power Co.) was used at 30% output (45 W) for 5 min to disperse lipids by cavitation into aqueous solution.

## RESULTS

**Random distribution of D-alanyl esters.** In *Streptococcus faecium* (3) and *L. casei* (23), the elongation of LTA is accomplished by the distal addition of *sn*-glycerol 1-phosphate from phosphatidylglycerol. This mode of addition was determined in *L. casei* from the gradient of label generated in the poly(glycerophosphate) chain when toluene-treated cells were incubated with [ $^{14}\text{C}$ ]glycerol 3-phosphate in the presence of 100 mM  $\text{P}_i$ . The ability to detect a gradient of [ $^{14}\text{C}$ ]GroP label in poly(glycerophosphate) suggested that a similar approach could be used with D-[ $^{14}\text{C}$ ]alanine.

As a test of this suggestion, toluene-treated cells of *L. casei* were incubated with two isotopically labeled D-alanines in a two-stage experiment. In the first stage, the treated cells were incubated for 30 min with D-[ $^3\text{H}$ ]alanine. In the second stage, they were washed free of the D-[ $^3\text{H}$ ]alanine and incubated with D-[ $^{14}\text{C}$ ]alanine for an additional 30 min. For comparison, the experiment was repeated with the order of the labeled D-alanines reversed. The sequential degradation of these doubly labeled LTAs with phosphodiesterase II-phosphatase yielded a constant ratio of the two labeled D-alanines in the D-alanyl-glycerol fraction as a function of time (Fig. 1). This observation indicated that a gradient of labeled D-alanine is not formed. The absence of a gradient of labeled D-alanine along the poly(glycerophosphate) moiety of LTA suggested that D-alanine may be added to LTA in one of two possible modes: (i) the D-alanine is esterified to any available glycerol phosphate unit in the chain with equal probability, or (ii) the D-alanine is added to

a specific locus followed by redistribution of the D-alanyl ester residues along the polymer. The redistribution of the labeled D-alanine may be accomplished by intra- or inter-chain transacylation of the ester residues.

**Transacylation of D-alanyl ester residues.** For a test of the hypothesis that interchain transacylation of D-alanine occurs, D-[<sup>14</sup>C]alanyl-lipophilic LTA was incubated with membranes containing endogenous, unlabeled lipophilic and hydrophilic LTA. Transacylation was measured as the transfer of D-[<sup>14</sup>C]alanyl ester residues from lipophilic D-[<sup>14</sup>C]alanyl-LTA to the hydrophilic acceptor, presumably LTA. The addition of 1% octyl-β-D-glucopyranoside was used to terminate this reaction. The separation of the two species of LTA was accomplished by the Bligh-Dyer (1) extraction procedure. The time-dependent transfer of D-[<sup>14</sup>C]alanine from D-[<sup>14</sup>C]alanyl-lipophilic LTA to hydrophilic acceptor is shown in Fig. 2.

Two lines of evidence indicated that the D-alanine incorporation system is not responsible for the incorporation of D-alanine into hydrophilic acceptor. First, the omission of ATP or the addition of the D-alanine activating enzyme and the D-alanine:membrane acceptor ligase did not affect the transfer reaction. Second, the addition of unlabeled D-alanine to the reaction mixture did not inhibit the transfer of labeled D-alanine from D-[<sup>14</sup>C]alanyl-lipophilic LTA to hydrophilic acceptor. Thus, the incorporation of D-[<sup>14</sup>C]alanine esterified to hydrophilic acceptor is not due to the hydrolysis of the D-alanyl ester residues from lipophilic LTA followed by reincorporation of the labeled D-alanine into hydrophilic acceptor.

The assay for transacylation measures the apparent transfer of D-[<sup>14</sup>C]alanine from lipophilic LTA to the hydrophilic acceptor LTA. A similar change in solubility of the D-alanyl ester residues might be expected if the added D-[<sup>14</sup>C]alanyl-lipophilic LTA were elongated to long-chain hydrophilic LTA. To determine whether this transfer of D-alanine is due to elongation of the added lipophilic LTA by endogenous phosphatidylglycerol, the following experiment was performed. Lipophilic LTA was labeled *in vivo* with <sup>3</sup>H in the

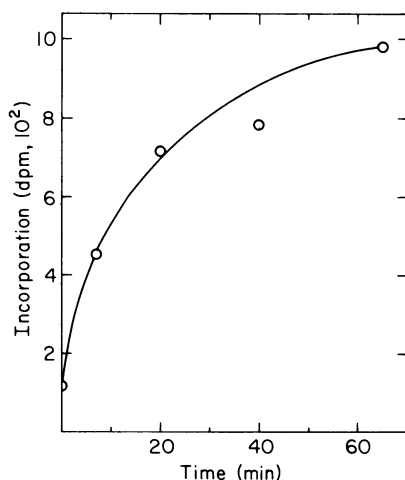


FIG. 2. Time course of transacylation. Membranes (2.6 mg of protein) were incubated with D-[<sup>14</sup>C]alanyl-lipophilic LTA containing 470 pmol of D-[<sup>14</sup>C]alanine (58,000 dpm) in the transacylation assay. At the indicated times, D-[<sup>14</sup>C]alanyl-hydrophilic LTA was isolated as described in the text. Samples containing hydrophilic LTA were chromatographed on Whatman 3MM paper in solvent A. The amount of radioactivity remaining at the origin was measured.

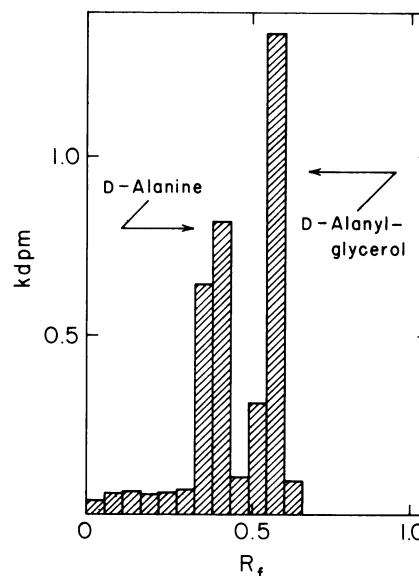


FIG. 3. Formation of D-[<sup>14</sup>C]alanyl-glycerol from the product of transacylation. A sample of the product isolated from the reaction mixture in the transacylation assay (text) labeled with D-[<sup>14</sup>C]alanine (7,160 dpm) was incubated for 72 h with phosphodiesterase II-phosphatase as described in the legend to Fig. 1. The degradation products were analyzed for D-[<sup>14</sup>C]alanyl-glycerol by paper chromatography in solvent A.

oligo(glycerophosphate) moiety and *in vitro* with <sup>14</sup>C in the D-alanyl ester residues. The D-[<sup>14</sup>C]alanyl · [<sup>3</sup>H]glycerol-lipophilic LTA was dispersed into solution by cavitation and incubated with unlabeled membranes. Elongation of the lipophilic LTA would result in both the <sup>3</sup>H and <sup>14</sup>C labels becoming hydrophilic. In contrast to elongation, transacylation of D-[<sup>14</sup>C]alanyl ester residues would result in only the <sup>14</sup>C label becoming hydrophilic. None of the tritium label of the glycerol (<10 dpm) was incorporated into the product of transacylation, whereas the <sup>14</sup>C of D-alanine was incorporated into the hydrophilic acceptor (1,190 dpm). Thus, elongation of the poly(glycerophosphate) moiety of the labeled lipophilic LTA did not occur in this system. Therefore, the incorporation of D-[<sup>14</sup>C]alanine from D-[<sup>14</sup>C]alanyl-lipophilic LTA into hydrophilic acceptor was not due to elongation of the D-[<sup>14</sup>C]alanyl-lipophilic LTA.

**Identification of the product of transacylation.** Because the hydrophilic acceptor was an endogenous component of the membrane complex, characterization of the product of transacylation as D-[<sup>14</sup>C]alanyl-LTA was necessary. In common with D-alanyl-LTA, the product of transacylation was nondialyzable, was eluted at the void volume of a Sepharose 6B column, and was retained at the origin of a paper chromatogram developed in solvent A (4). Degradation of this product by phosphodiesterase II-phosphatase gave D-[<sup>14</sup>C]alanine and D-[<sup>14</sup>C]alanyl-glycerol (Fig. 3). D-Alanyl-glycerol is one of the characteristic degradation products of D-alanyl-LTA resulting from this enzyme treatment (4, 6). The D-alanine results from the nonenzymatic hydrolysis of either D-alanyl-LTA or D-alanyl-glycerol.

If the product of transacylation is hydrophilic D-[<sup>14</sup>C]alanyl-LTA, degradation by phosphodiesterase II-phosphatase will yield D-[<sup>14</sup>C]alanyl-lipophilic LTA (2). Degradation of the labeled hydrophilic product with these enzymes resulted in increasing amounts of <sup>14</sup>C that partitioned into the chloroform phase of the Bligh-Dyer extraction system (Fig. 4).

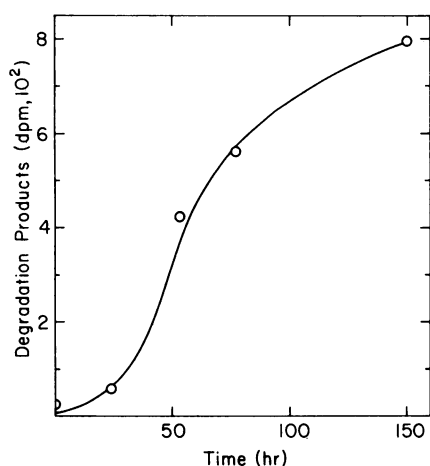


FIG. 4. Formation of D-[<sup>14</sup>C]alanyl-lipophilic LTA during the sequential degradation of the product of transacylation. Samples of the product labeled with D-[<sup>14</sup>C]alanine (10,000 dpm) were isolated from the reaction mixture in the transacylation assay (see the text). They were sequentially degraded with phosphodiesterase II-phosphatase as described in the legend to Fig. 1. At the indicated times, D-[<sup>14</sup>C]alanyl-lipophilic LTA was isolated by the Bligh and Dyer extraction procedure (1) and assayed for radioactivity.

When subjected to thin-layer chromatography, this material migrated as a series of bands with  $R_f$  values similar to those of D-alanyl-lipophilic LTA (2, 23). Thus, the D-[<sup>14</sup>C]alanyl-labeled hydrophilic acceptor can be degraded to labeled lipophilic LTA. These observations constituted strong evidence that the hydrophilic acceptor in the transacylation reaction is LTA.

**Role of enzyme in transacylation.** As a test for possible enzyme involvement in the process of transacylation, attempts were made to inactivate a possible transacylase. The transacylation assay was performed with membranes that had been pretreated with trypsin (0.13 mg/ml), iodoacetamide (1 mM), or heat (65°C for 15 min). Heat treatment of membranes at this temperature inactivates the D-alanine: membrane acceptor capacity by >95% in *L. casei* (V. M. Brautigan, Ph.D. thesis, Northwestern University, Evanston, Ill., 1977). None of these treatments inhibited transacylation of D-alanine from D-alanyl-lipophilic LTA to hydrophilic acceptor. The heat and trypsin treatments stimulated this reaction to a small extent. These results suggested that transacylation may not be the result of an enzyme-mediated reaction. For a test of this suggestion, D-[<sup>14</sup>C]alanyl-lipophilic LTA was dispersed into solution by cavitation and incubated with partially purified micellar hydrophilic LTA. Transacylation occurred in the absence of added membranes. In the 60-min assay period, 910 dpm of D-[<sup>14</sup>C]alanine from D-[<sup>14</sup>C]alanyl-LTA was incorporated into partially purified LTA under conditions in which 2,300 dpm of D-[<sup>14</sup>C]alanine was incorporated into membrane-associated LTA. Thus, these results further supported the suggestion that transacylation is probably not the result of an enzyme-catalyzed reaction.

## DISCUSSION

D-Alanyl ester residues have been reported to play an important role in modulating the physiological functions of LTA (13, 17). These include the binding of Mg<sup>2+</sup> (12), the inhibition of autolysins (9), and the assembly of wall poly-

mers on lipoteichoic acid carrier (6). Therefore, the esterification of D-alanine to LTA and its distribution along the poly(glycerophosphate) appear to be important to the function of LTA.

The D-alanyl ester residues are evenly distributed along the poly(glycerophosphate) chain in LTA isolated from *Staphylococcus aureus* (6). An even distribution of the D-alanyl esters in LTA was also observed when toluene-treated cells of *L. casei* were incubated with labeled D-alanine (4). In the two-stage experiment with D-[<sup>14</sup>C]alanine and D-[<sup>3</sup>H]alanine described in this paper, it was concluded that the two isotopically labeled D-alanines are randomly distributed along the poly(glycerophosphate) chains. Our results provide a mechanism for the redistribution of these D-alanine residues after their addition to LTA. This redistribution is hypothesized to occur by transacylation of the residues via interchain and possibly intrachain transfer.

Interchain transacylation was observed as a time-dependent transfer of D-[<sup>14</sup>C]alanine from D-[<sup>14</sup>C]alanyl-lipophilic LTA to hydrophilic LTA. This transfer requires neither ATP nor the D-alanine incorporation system. Furthermore, incubation of D-[<sup>14</sup>C]-alanyl-lipophilic LTA with partially purified hydrophilic LTA in the absence of membranes also results in the transfer of the D-[<sup>14</sup>C]alanine residues to the hydrophilic LTA. These results do not support the role of an enzyme in this transfer. One of the potential acceptors of the D-alanyl ester residues in the transacylation reaction is phosphatidylglycerol. Attempts to demonstrate the synthesis of D-alanyl-phosphatidylglycerol in toluene-permeabilized cells of *L. casei* have been unsuccessful (2). Thus, it would appear that this phospholipid is not synthesized in the transacylation reaction described in this report.

Transacylation reactions have been suggested for the alanyl ester of D-alanyl-glycerol (4) and the aminoacyl moiety of aminoacyl-tRNA (15). These reactions are not enzyme mediated, but rather are dependent on vicinal hydroxyl groups acting as nucleophiles. Shabarova et al. (22) recognized the enhanced reactivity of the alanyl ester by vicinal substituents. For example, a phosphate monoester further enhances the reactivity of the ester when compared with the hydroxyl group. In a similar manner, the phosphodiester groups of the poly(glycerophosphate) moiety may enhance the reactivity of the D-alanyl ester residues of LTA and thus facilitate interchain and intrachain transacylation of these residues.

As indicated in the introduction, esterification of LTA with D-alanine may occur in one of two modes: (i) addition at random or (ii) addition at a defined locus in the poly(glycerophosphate) chain followed by redistribution of the ester residues. We propose that this process of transacylation may be responsible for the redistribution of the D-alanyl residues after esterification to the poly(glycerophosphate). As a result, it is difficult to distinguish between these proposed modes of addition.

The results also emphasize a unique feature of the D-alanyl ester residues of LTA. This feature may be important for the incorporation of D-alanine into wall teichoic acid. Haas et al. (10) have reported the *in vivo* transfer of D-alanyl ester residues from LTA to wall teichoic acid in *Staphylococcus aureus*. Whether transacylation is responsible for this process remains to be established. This feature of D-alanyl-LTA may also play a role in the regulatory functions of this polymer. For example, perturbation of the D-alanyl ester content by either acylation or deacylation at one location in the membrane could be translated by inter- and intrachain transacylation of the alanyl esters to another location in the

membrane. Thus, transacylation may represent a unique way of transmitting a signal from one location in the membrane to another.

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