JOURNAL OF BACTERIOLOGY, Apr. 1987, p. 1702–1711
0021-9193/87/041702-10$02.00/0
Copyright © 1987, American Society for Microbiology
Vol. 169, No. 4

Assembly of D-Alanyl-Lipoteichoic Acid in *Lactobacillus casei*: Mutants Deficient in the D-Alanyl Ester Content of This Amphiphile

ALPHONSUS S. NTAMERE, DOUGLAS J. TARON,† AND FRANCIS C. NEUHAUS*

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

Received 23 May 1986/Accepted 30 December 1986

D-Alanyl-lipoteichoic acid (D-alanyl-LTA) from *Lactobacillus casei* ATCC 7469 contains a poly(glycerophosphate) moiety that is acylated with D-alanyl ester residues. The physiological function of these residues is not well understood. Five mutant strains of this organism that are deficient in the esters of this amphiphile were isolated and characterized. When compared with the parent, strains AN-1 and AN-4 incorporated less than 10% of D-[^{14}C]alanine into LTA, whereas AN-2, AN-3, and AN-5 incorporated 50%. The synthesis of D-[^{14}C]alanyl-lipophilic LTA was virtually absent in the first group and was approximately 30% in the second group. The mutant strains synthesized and selected the glycolipid anchor for LTA assembly. In addition, all of the strains synthesized the poly(glycerophosphate) moiety of LTA to the same extent as did the parent or to a greater extent. It was concluded that the membranes from the mutant strains AN-1 and AN-4 are defective for D-alanylation of LTA even though acceptor LTA is present. Mutant strains AN-2 and AN-3 appear to be partially deficient in the amount of the D-alanine-activating enzyme. Aberrant morphology and defective cell separation appear to result from this deficiency in D-alanyl ester content.

D-Alanyl-lipoteichoic acid (D-alanyl-LTA) is an example of an amphiphile that appears to play a vital role in bacterial growth (1, 3). A number of functions have been postulated for this polymer. These include regulation of autolytic activity (11, 23), binding of Mg^{2+} for enzyme function (29), and assembly of wall polymers (15, 20). One of the components of LTA which has been proposed to modulate these functions is the substituent D-alanine. Evidence from in vitro experiments indicates that these D-alanyl esters alter the abilities of LTA to inhibit autolysins (19), chelate Mg^{2+} ions (a function shared with wall teichoic acid) (2, 21, 24, 28), and function as a carrier for wall teichoic acid synthesis (17, 27). Fischer and co-workers (19) proposed that fluctuations of the D-alanyl ester content of LTA must be considered in its potential regulation of autolysins.

Substitution of LTA by D-alanine is not a universal feature of all LTAs. For example, LTAs from *Micrococcus varians*, *Streptococcus faecalis* 9790, and *S. lactis* Kiel 42172 are devoid of D-alanine (16). However, 30 to 93% of the glycerol phosphate units of LTAs from most organisms are substituted with D-alanyl ester residues (16). Growth of *Staphylococcus aureus* in the presence of increasing concentrations of NaCl, 0.2% to 7.5%, decreases the degree of esterification by D-alanine from 78 to 36% (18). In addition, growth of this organism at pH 8.1 instead of pH 7.1 results in a decrease of D-alanine ester content from 45 to 5% (32). Thus, not only growth in NaCl but growth at higher pHs induces wide variations in the esterification of LTA by D-alanine.

In *Lactobacillus casei*, incorporation of D-alanine into LTA is accomplished in the following two-step reaction sequence (4, 7, 10, 30, 36, 39):

$$\text{enzyme} + \text{D-alanine} + \text{ATP} \rightleftharpoons \text{enzyme} \cdot \text{AMP–D-alanine} + PP_i \quad (1)$$

$$\text{enzyme} \cdot \text{AMP–D-alanine} + \text{LTA} \rightarrow \text{D-alanyl-LTA} + \text{enzyme} + \text{AMP} \quad (2)$$

In reaction 1, D-alanine is activated in the presence of ATP and the D-alanine-activating enzyme to form an enzyme · AMP–D-alanine complex with the release of PP_i. In reaction 2, activated D-alanine is covalently linked to LTA in the presence of the D-alanine:membrane acceptor ligase. The precise function of the ligase in the D-alanine incorporation system has not been established.

It is our ultimate goal to determine the physiological function of the D-alanyl ester residues in the LTA of *L. casei* 7469. In this paper, we report the isolation and characterization of five mutant strains of this organism that are deficient in the ester content of this amphiphile. A study of these strains revealed defects in the D-alanine incorporation system. Aberrant morphology and defective cell separation appear to result from this deficiency in D-alanyl ester content.

(A portion of this study was presented at the 85th Annual Meeting of the American Society for Microbiology, Las Vegas, Nev., 3–7 March, 1985.)

MATERIALS AND METHODS

Materials. We thank Werner Fischer for glycerophospho (Gro-P)-6-Glc(β1-6)Gal(α1-2)Glc(α1-3)diacylglycerol and lipid extract isolated from *L. casei* DSM 20021 (34, 35). D-[1-^{14}C]alanine (56 mCi/mmol), [2-^{3}H]glycerol (2 Ci/mmol), UDP-[U-^{14}C]glucose (237 mCi/mmol), and D-[^{14}C]glucosamine hydrochloride (45 mCi/mmol) were purchased from International Chemical and Nuclear Corp., Irvine, Calif. D-Cycloserine and *n*-octyl-β-D-glucopyranoside were purchased from Calbiochem-Behring, San Diego, Calif. Sepharose 6B and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine were from Pharmacia Fine Chemicals Inc., Piscataway, N.J., and Sigma Chemical Co., St. Louis, Mo., respectively. Silica gel 60 thin-layer plates (250 μm thick [20 by 20 cm]) were obtained from E. Merck AG, Darmstadt, Federal Republic of Germany. Membrane filters (pore size, 0.45 μm) were obtained from the Millipore Corp., Bedford, Mass. Spurr embedding medium and Epon 812 were from Ladd Research Industries Inc., Burlington, Vt., and Tousimis Research Corp., Rockville, Md., respectively.

* Corresponding author.
† Present address: Department of Biotechnology, Amoco Research Center, Naperville, IL 60566.

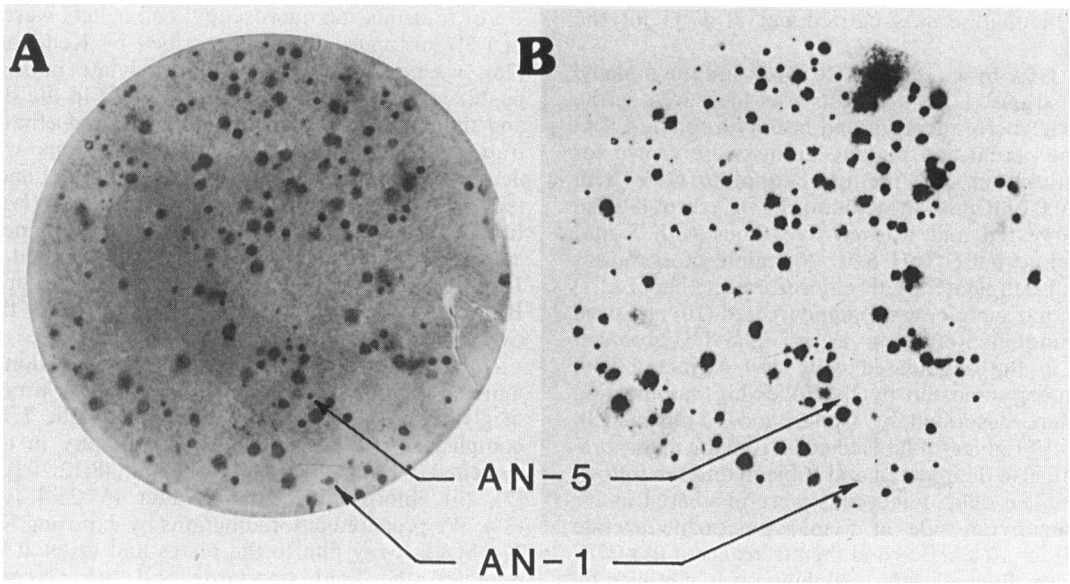

FIG. 1. Isolation of strains of *L. casei* 7469 deficient in D-alanyl ester formation. As described in the text, the colony autoradiographic screening procedure was used to detect putative mutant strains. We stained replica prints of the mutagenized parent with Coomassie blue to detect all colonies (A) and tested them for D-[^{14}C]alanine incorporation (B) as described in the text. The detection of AN-1 and AN-5 is shown.

Isolation of strains of *L. casei* 7469 deficient in D-alanyl ester formation. The colony autoradiographic screening procedure described by Raetz (38) was used to detect mutants defective in D-alanyl ester formation. Two features provide the basis for isolating mutants deficient in the D-alanyl ester content of LTA. First, since *L. casei* contains only LTA and no wall teichoic acid (25), screening procedures using D-[^{14}C]alanine incorporation only reflect the D-alanyl ester content of LTA. Second, incorporation of D-alanine into peptidoglycan and conversion of D-alanine to L-alanine for protein synthesis can be inhibited by D-cycloserine. In contrast, incorporation of D-[^{14}C]alanine into LTA is insensitive to the action of this D-alanine analog (36). Thus, colonies defective in incorporation of D-[^{14}C]alanine that are screened in the presence of D-cycloserine will most likely be deficient in the D-alanyl ester content of their LTA.

An exponential culture of *L. casei* ATCC 7469 was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (100 μg/ml) for 60 min at 37°C as described by Miller (33). After a period of growth (16 h) at 37°C in AC medium (10), the mutagenized culture was plated on AC agar at a density of 200 to 250 CFU per plate. After 72 h at 37°C, a replica print of each plate was made on a 7.0-cm disk of no. 42 filter paper (Whatman, Inc., Clifton, N.J.). Each print was treated with 1 ml of a solution containing 10 mg of lysozyme per ml and 10 mM EDTA (pH 6.0) at room temperature for 20 min (38). After this treatment, the prints were subjected to two cycles of freezing and thawing and then blotted. To assay for D-alanyl ester formation, we incubated disks in a solution containing 30 mM piperazine acetate buffer (pH 6.5), 25 mM $MgCl_2$, 5 mM ATP, 2 mM D-cycloserine, and 0.13 mM D-[^{14}C]alanine (56 mCi/mmol) for 1 h at 37°C. We terminated the reactions by transferring the disks to 1.2 ml of 20% (wt/vol) trichloroacetic acid containing 20 mM DL-alanine at 4°C for 10 min. After drying the disks at 125°C, we prepared autoradiograms as described in Analytical Methods. The colonies were subsequently visualized with Coomassie brilliant blue R250. The stained colonies (Fig. 1A) which lacked the corresponding autoradiographic signals (Fig. 1B) were chosen as mutant candidates. The putative mutants, e.g., AN-1 and AN-5, were picked from the plate and grown in AC medium.

Preparation of toluene-treated cells and membranes. Toluene-treated cells from *L. casei* ATCC 7469 and the D-alanyl ester-deficient mutants were prepared as described previously (10). Membranes and supernatant fractions were prepared as described by Linzer and Neuhaus (30).

Assays for wall turnover and autolysis. Exponential-phase cells of *L. casei* ATCC 7469 and the mutant strains were transferred to AC medium supplemented with 1% glycerol in the absence of glucose. These cultures were labeled for 1.5 generations with D-[^{14}C]glucosamine (45 mCi/mmol). The cells were collected by centrifugation and washed three times with 0.5 mM piperazine acetate (pH 6.4). Radiolabeled cells were used for assays of wall turnover and autolysis.

To measure wall turnover in D-[^{14}C]glucosamine-labeled cells, we grew the parent and mutant strains in AC medium containing 2% glucose. Both the amount of intracellular radioactivity retained after filtration and washing on 0.45-μm filters and the amount of radiolabel released into the growth medium were monitored. The maximum rate of peptidoglycan turnover measured in the filtration assay was 20.8 ± 2% per generation. The maximum rate of turnover measured by released glycan was 25 ± 2%.

For the assay of autolysis, D-[^{14}C]glucosamine-labeled cells were suspended in 50 mM citrate buffer (pH 5.0) (13). The amount of released radiolabel was measured in the medium after removal of the cells by centrifugation.

D-Alanine incorporation assay. Incorporation of D-[^{14}C]alanine into either toluene-treated cells or membranes was performed in a reaction mixture which contained 33 mM $MgCl_2$, 5 mM ATP (adjusted to pH 6.5 with NaOH), 40 mM piperazine acetate buffer (pH 6.5), 1 mM dithiothreitol, 2.0 mM D-cycloserine, 0.13 mM D-[^{14}C]alanine, and either toluene-treated cells (32 mg [wet weight] per ml) or membranes (15 mg of protein per ml). For toluene-treated cells and membranes, 70- and 50-μl reaction volumes were used,

respectively. Incubation was carried out at 37°C for the indicated time.

Synthesis of LTA by *L. casei* ATCC 7469 and the D-alanyl ester-deficient strains. LTA synthesis was measured as the amount of [^{3}H]glycerol incorporated into hydrophilic LTA. Cultures of the parent and mutant strains were grown for three generations in AC medium supplemented with [^{3}H]glycerol (0.03 mCi/ml of medium). [^{3}H]glycerol-labeled cells were harvested and washed five times with 5 mM piperazine acetate buffer (pH 6.5). A sample of each was lyophilized and delipidated with chloroform-methanol (2:1) (45), and a second sample was toluene treated (10) and used in the D-alanine incorporation assay. The [^{3}H]glycerol-labeled LTA in the delipidated cells was extracted with *n*-octyl-β-D-glucopyranoside by the following modification of the procedure described by Childs and Neuhaus (10). D-[^{14}C]alanyl · [^{3}H]glycerol-labeled cells from the incorporation assay were also delipidated and subjected to the following procedure. The delipidated cells were incubated in 1% *n*-octyl-β-D-glucopyranoside in 5 mM piperazine acetate buffer (pH 6.5) for 16 h at 4°C and then transferred to a 37°C bath for 5 h with shaking. After cell debris was removed by centrifugation, the supernatant fractions were dialyzed against three changes of 5 mM piperazine acetate buffer (pH 6.5). The dialyzed extracts were concentrated by lyophilization and suspended in 0.25 ml of 5 mM piperazine acetate buffer (pH 6.5). We assayed samples for radioactivity to determine the amount of [^{3}H]glycerol incorporated into total LTA and phospholipid in the sample. The average percent yield as measured by the recovery of [^{3}H]glycerol in this fraction was 88% of the total incorporation. We filtered the remainder on Sephadex 6B to determine the amounts of acylated and deacylated LTAs (10). For comparison, samples were incubated with the phosphodiesterase-phosphatase from *Aspergillus niger* (10) and assayed for the amount of [^{3}H]glycerol in the poly(glycerophosphate) moiety of LTA. The average percent yield of phosphodiesterase-sensitive [^{3}H]glycerol phosphate was 55%. Each fraction was also assayed for hydrophilic LTA and chloroform-soluble lipid by the Bligh-Dyer extraction procedure (5). The average percent yield of hydrophilic LTA was 53%. These yields were used to correct the [^{3}H]glycerol incorporated into partially purified LTA after dialysis.

Synthesis of glycolipids. Glycolipid synthesis was examined in toluene-treated cells prepared from the parent and mutant strains. The reaction mixture described for the D-alanine incorporation assay contained 10.5 nM UDP-[^{14}C]glucose (237 Ci/mol) in place of D-[^{14}C]alanine. Isolation of the radiolabeled glycolipids was accomplished by the monophasic extraction procedure described by Bligh and Dyer (5) with partitioning into the aqueous and organic phases as described by Brautigan et al. (7). The chloroform–methanol-soluble compounds were chromatographed in solvent i (see below). After autoradiography, the plates were sprayed with α-naphthol as described in Analytical Methods.

Electron microscopy. Cells were fixed in 0.1 M cacodylate buffer (pH 7.4) containing 2.5% glutaraldehyde for 1 h. The cells were then collected and washed three times in 0.1 M cacodylate buffer. For scanning electron microscopy, a sample of cells was dehydrated in a graded series of ethanol concentrations. Dehydration in 100% ethanol was performed twice. A drop of each sample in 100% ethanol was subjected to critical-point drying, and the cells were coated with gold-palladium. Micrographs of the cells were obtained with a JEOL JSM-35 CF scanning electron microscope.

For transmission microscopy, cell pellets were embedded in 1.5% ultrapure agar as described by Kellenberger et al. (26), washed once in 0.1 M cacodylate buffer (pH 7.4), postfixed with 1.25% osmium tetroxide in the dark at 4°C, and then dehydrated in a graded series of ethanol concentrations. The agar pellets (1- to 2-mm sections) were embedded in either Epon 812 or Spurr medium (42) and sectioned (silver-gold) on an MT-2 Ultra-Microtome (Ivan Sorvall, Inc., Norwalk, Conn.). The sections were stained in uranyl acetate and lead citrate (40, 44) and examined in a JEOL JEM-100 CX 11 transmission electron microscope at 80 kV. Because of the poor staining characteristics of the wall, we overexposed the prints to intensify the image.

Analytical methods. The protein concentration was determined by the method of Lowry et al. (31). Chromatography of glycolipids, phospholipids, and lipophilic LTA was accomplished by thin-layer chromatography in two solvent systems: (i) butanol-acetic acid-water (48:12:20 [vol/vol/vol]) (7), (ii) chloroform-methanol-water (65:25:4 [vol/vol/vol]) (34). We prepared autoradiograms by exposing Kodak XR-5 X-OMAT X-ray film to the plates and disks at −80°C. We detected the lipid standards and the glycerophosphotrihexosyldiacylglycerol either by iodine vapor or by spraying them with 3.2% (wt/vol) α-naphthol in methanol-H_2SO_4-H_2O (25:3:2 [vol/vol/vol]) and then heating them at 110°C for 20 min (34).

RESULTS

Incorporation of D-[^{14}C]alanine into toluene-treated mutant strains. Incorporation of D-[^{14}C]alanine into toluene-treated cells was determined in each of the putative strains of *L. casei* ATCC 7469 that appeared deficient in D-alanyl ester content by the autoradiographic screening procedure. Since this incorporation is inhibited by hydroxylamine and is insensitive to the action of D-cycloserine, it reflects formation of the D-alanyl ester residues of LTA (10). In Fig. 2, the time courses of incorporation of D-[^{14}C]alanine into the parent and mutant strains are compared. Strains AN-1 and AN-4 incorporated less than 10%, whereas AN-2, AN-3, and AN-5 incorporated approximately 50%. Thus, the mutant strains were divided into two groups: (i) AN-1 AN-4 and (ii) AN-2, AN-3, and AN-5. In the following series of experiments, the biochemical bases of the defects and the morphological aberrations in these strains will be defined. Because these mutants were generated by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, they may contain more than one mutation (33).

Synthesis of LTA by mutant strains. Strains deficient in D-alanyl ester formation may be defective in either (i) the synthesis of the LTA acceptor or (ii) the ability to alanylate this acceptor. To determine whether LTA acceptor synthesis is defective, we grew cells in [^{3}H]glycerol for three generations and quantitated [^{3}H]glycerol-labeled LTA by using a modification of the procedure described by Childs and Neuhaus (10). Table 1 summarizes the amounts of [^{3}H]glycerol incorporated into partially purified hydrophilic LTA of the mutant strains and the parent. The amounts of glycerol incorporated into the LTA of the parent and strains AN-1, AN-2, AN-3, and AN-5 were similar. For AN-4, the amount of glycerol incorporated into hydrophilic LTA was generally 10 times that observed for the parent. As illustrated for the parent and AN-4 (Fig. 3), there was no significant difference in the ratio of fatty acid-acylated LTA to fatty acid-deacylated LTA (4.9 versus 5.3). Similar gel filtration profiles were also obtained for AN-1, AN-2, AN-3,

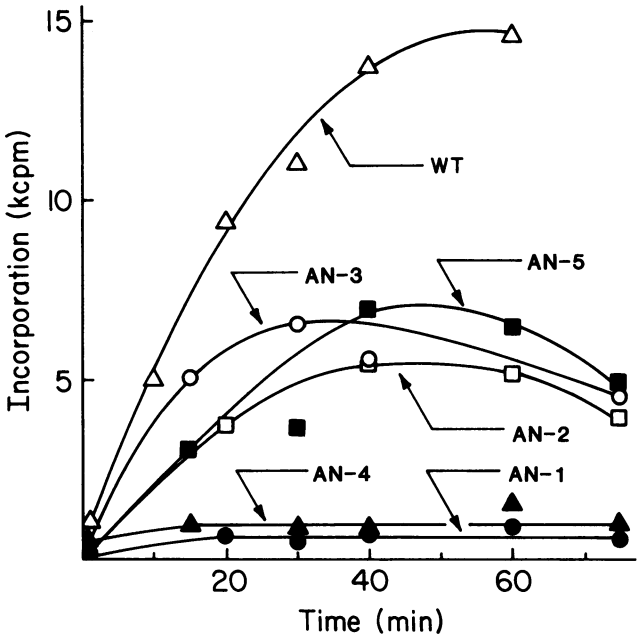

FIG. 2. Incorporation of D-[^{14}C]alanine into the parent and mutant strains. The D-alanine incorporation assay was used with toluene-treated cells of the parent (WT; △), AN-1 (●), AN-2 (□), AN-3 (○), AN-4 (▲), and AN-5 (■).

and AN-5. Thus, the amount of potential LTA acceptor does not appear to be limiting in the mutant strains. This conclusion assumes that the specific activity of the glycerol phosphate residues of the LTA is identical in each strain.

The ratio of D-[^{14}C]alanine to [^{3}H]glycerol in the partially purified LTA reflects the alanylation of the poly(glycerophosphate) moiety. To establish this ratio, we toluene treated [^{3}H]glycerol-labeled cells from each strain and incubated them with D-[^{14}C]alanine in the D-alanine incorporation assay. Table 1 summarizes the ratios of D-[^{14}C]alanine to [^{3}H]glycerol for each of the partially purified samples of hydrophilic LTA. The ratio ranged from <0.0008 for AN-4 to 0.055 for the parent. These data indicated that AN-1 and AN-4 synthesized the poly(glycerophosphate) moiety of LTA but were unable to effect its alanylation. The low ratio

TABLE 1. Synthesis of hydrophilic D-[^{14}C]alanyl · [^{3}H]glycerol-LTA in mutant strains and the parent strain of *L. casei* ATCC 7469[a]

Strain	[^{3}H]glycerol incorporated (10^3 cpm/mg)[b]	[^{14}C]D-alanine/[^{3}H]glycerol ratio (10^{-2})[b]
Parent	5.16	5.50
AN-1	5.54	0.51
AN-2	4.56	3.10
AN-3	6.61	2.88
AN-4	64.4	<0.08
AN-5	13.2	2.89

[a] The strains were grown for three generations in the presence of [^{3}H]glycerol as described in the text. The radioactivity represents the amount of [^{3}H]glycerol incorporated into the poly(glycerophosphate) moiety of partially purified hydrophilic LTA per milligram (dry weight) of cells. Samples of [^{3}H]glycerol-labeled cells were treated with toluene and used in the D-alanine incorporation assay as described in the text. The ratio is the ratio of counts per minute of D-[^{14}C]alanine to the counts per minute of [^{3}H]glycerol in partially purified hydrophilic LTA.

[b] As described in the text, these values reflect only hydrophilic LTA.

observed with LTA from AN-4 also reflects the enhanced synthesis of the poly(glycerophosphate) moiety (Table 1). Strains AN-2, AN-3, and AN-5 synthesized an LTA with ratios of D-alanine to glycerol that are approximately 50% of that observed for the wild type. Thus, these strains contained LTA with a lower D-alanyl ester content. Although strains AN-1 and AN-4 were grouped together, they appeared to be different based on the amount of poly(glycerophosphate) moiety which was synthesized. The amount of [^{3}H]glycerol incorporated into the LTA of AN-1 was similar to that of the parent but only 8.6% of that observed for AN-4.

Incorporation of D-alanine into LTA by in vitro combinations of membrane fragments and supernatant fractions from mutant and parent strains. To define further the defect(s) in the D-alanine incorporation system of the mutant strains, we incubated (i) membrane fragments from each of the strains with supernatant fraction from the parent and (ii) supernatant fractions from each of the strains with membrane fragments from the parent and compared the activities with those from incubations containing homologous membranes and supernatant fractions (Table 2). The activities for each of the incubations containing homologous membranes and supernatant fractions (M + S) correlated with the incorporation of D-alanine into toluene-treated cells prepared from each of the strains (Fig. 2). When membranes from each of the mutant strains were tested with supernatant fraction from the parent ($M_m + S_p$), the activities also correlated with the homologous combinations. However, when supernatant fraction from each of the mutant strains was used with membranes from the parent ($M_p + S_m$), no correlation was observed. The defect(s) in strains AN-1 and AN-4 is clearly associated with a defect(s) in the ability of the membrane to function as an acceptor for D-alanine. The specific activity of the D-alanine-activating enzyme for each strain is also shown in Table 2. AN-2 and AN-3 appear to be partially deficient in this enzyme. Thus, the lower activities observed in the

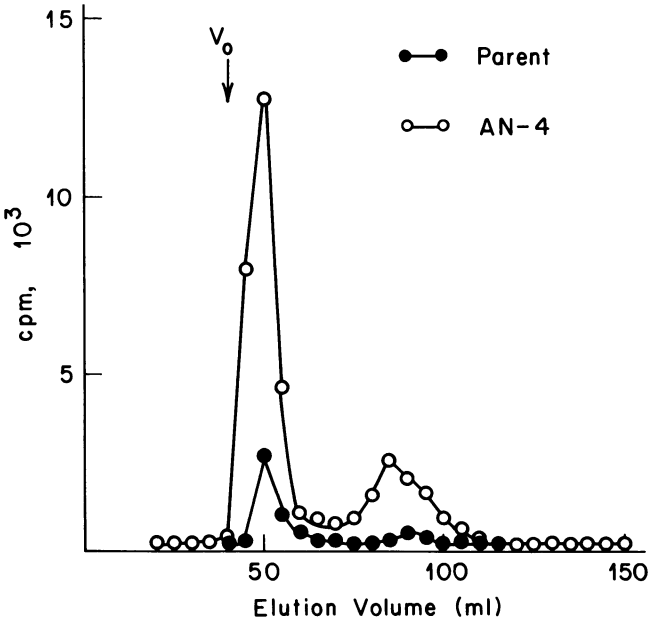

FIG. 3. Gel filtration of partially purified [^{3}H]glycerol-labeled LTA from the parent and AN-4. LTA prepared as described in the text was eluted from a Sepharose column 6B (1.5 by 66 cm) with 5 mM piperazine acetate (pH 6.5). Peak 1, fatty acid-acylated LTA; peak 2, fatty acid-deacylated LTA (10).

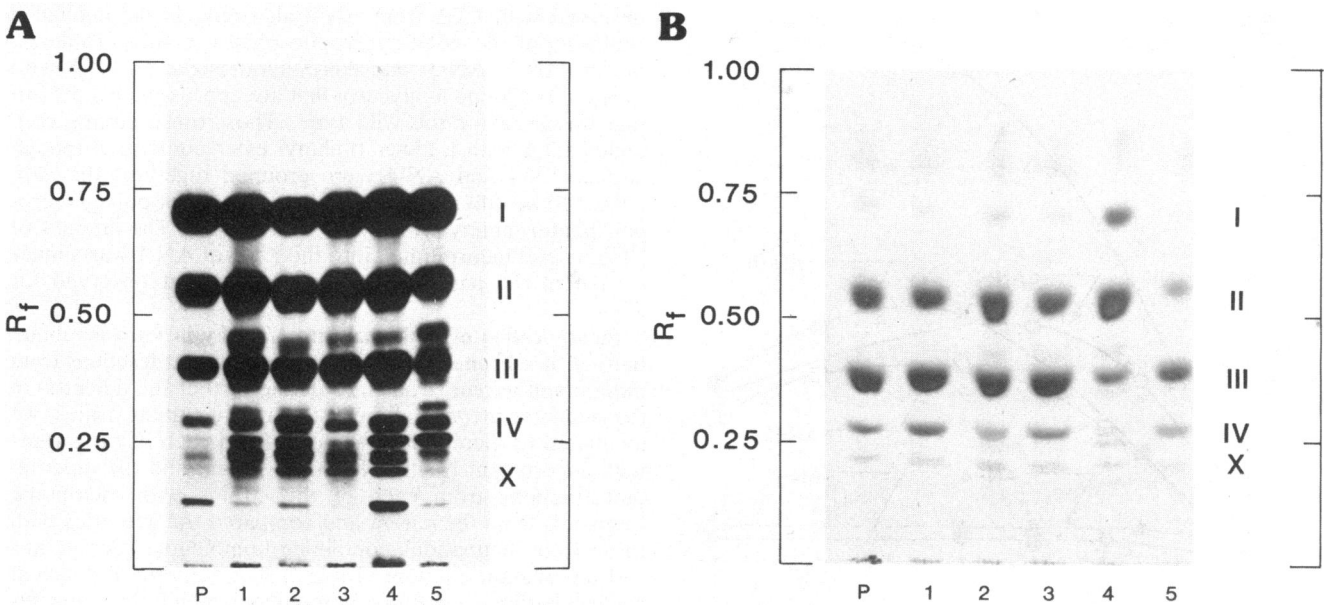

FIG. 4. Incorporation of [^{14}C]glucose from UDP-[^{14}C]glucose into the glycolipid intermediates of the mutant strains (A) and detection of α-napthol-sensitive compounds in toluene-treated cells of *L. casei* (B). Synthesis of the [^{14}C]glucose-labeled glycolipids was performed with the D-alanine incorporation assay with UDP-[^{14}C]glucose replacing D-[^{14}C]alanine and with toluene-treated cells as described in the text. I, II, III, IV, and X are Glc(α1-3)diacylglycerol, Gal(α1-2)Glc(α1-3)diacylglycerol, Glc(β1-6)Gal(α1-2)Glc(α1-3)diacylglycerol, Glc(β1-6)Glc(β1-6)Gal(α1-2)Glc(α1-3)diacylglycerol, and GroP-6-Glc(β1-6)Gal(α1-2)Glc(α1-3)diacylglycerol, respectively. P, Parent.

combined system using supernatant fractions from these strains may be the result of lower specific activities of the D-alanine-activating enzyme.

Synthesis and structure of the glycolipid anchor in mutant strains. The inability of LTA in the mutant strains to function as an acceptor of D-[^{14}C]alanine in the incorporation assay may be the result of a defect in the synthesis of the glycolipid anchor. In *L. casei*, the glycolipids are Glc(β1-6)Gal(α1-2)acyl→6Glc(α1-3)diacylglycerol and Glc(β1-6)Gal(α1-2)Glc(α1-3)diacylglycerol (35). Two approaches were used for characterizing the glycolipid anchors in the mutant strains. It was asked (i) whether the glycolipid is synthesized and (ii) whether the lipophilic LTA isolated from the hydrophilic LTA of each mutant strain is identical to that of the parent.

Synthesis of the glycolipids was examined in toluene-treated cells prepared from exponential cultures of the parent and mutant strains. Syntheses of [^{14}C]glucose-labeled mono-, di-, and trihexosyl diglyceride intermediates (I, II, and III) of the mutant strains were similar to those of the parent (Fig. 4). We identified these lipids by comparing their R_f values with those of the authentic glycolipids obtained from *L. casei* DSM 20021 (a strain derived from *L. casei* 7469) in two solvent systems (i and ii; data not shown). With

TABLE 2. Incorporation of D-alanine into LTA by in vitro combinations of membrane fragments and supernatant fractions from the parent and mutant strains

Strain	D-Alanine incorporation (10^2 cpm)[a]			D-alanine-activating enzyme (μmol/mg)[b]
	M + S	M_m + S_p	M_p + S_m	
Parent	7.1			2.85
AN-1	0.7	0.8	3.8	2.50
AN-2	5.7	3.8	1.6	0.96
AN-3	1.8	3.0	1.4	1.51
AN-4	0.2	0.3	4.0	2.56
AN-5	2.6	2.9	3.9	2.18

[a] In each incubation, 100 μg (protein) of membrane fragments was incubated in the D-alanine incorporation assay, as described in the text, with 100 μg of supernatant fraction.

[b] The D-alanine-activating enzyme was measured with the hydroxamate assay described by Baddiley and Neuhaus (4).

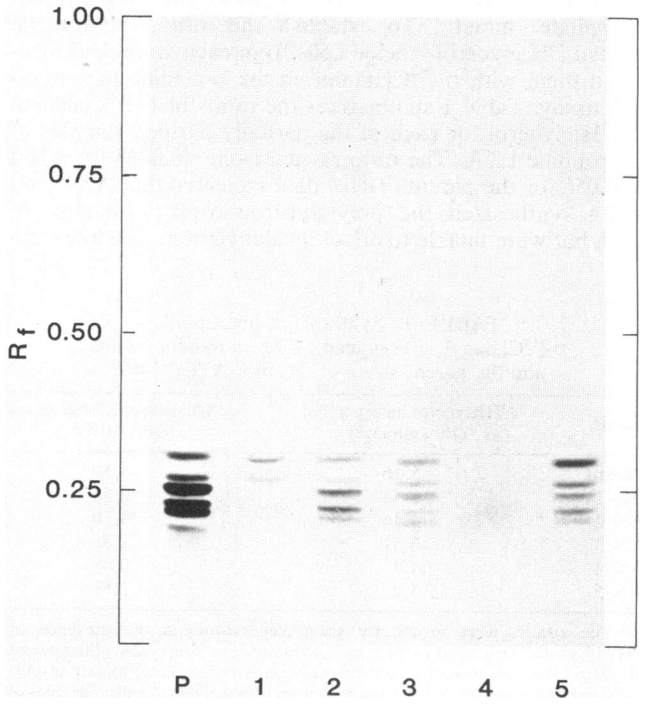

FIG. 5. Synthesis of D-[^{14}C]alanyl-lipophilic LTA in the parent (P) and mutant (AN-1, AN-2, AN-3, AN-4, and AN-5) strains. Synthesis of D-[^{14}C]alanyl-lipophilic LTA was measured as described by Brautigan et al. (7).

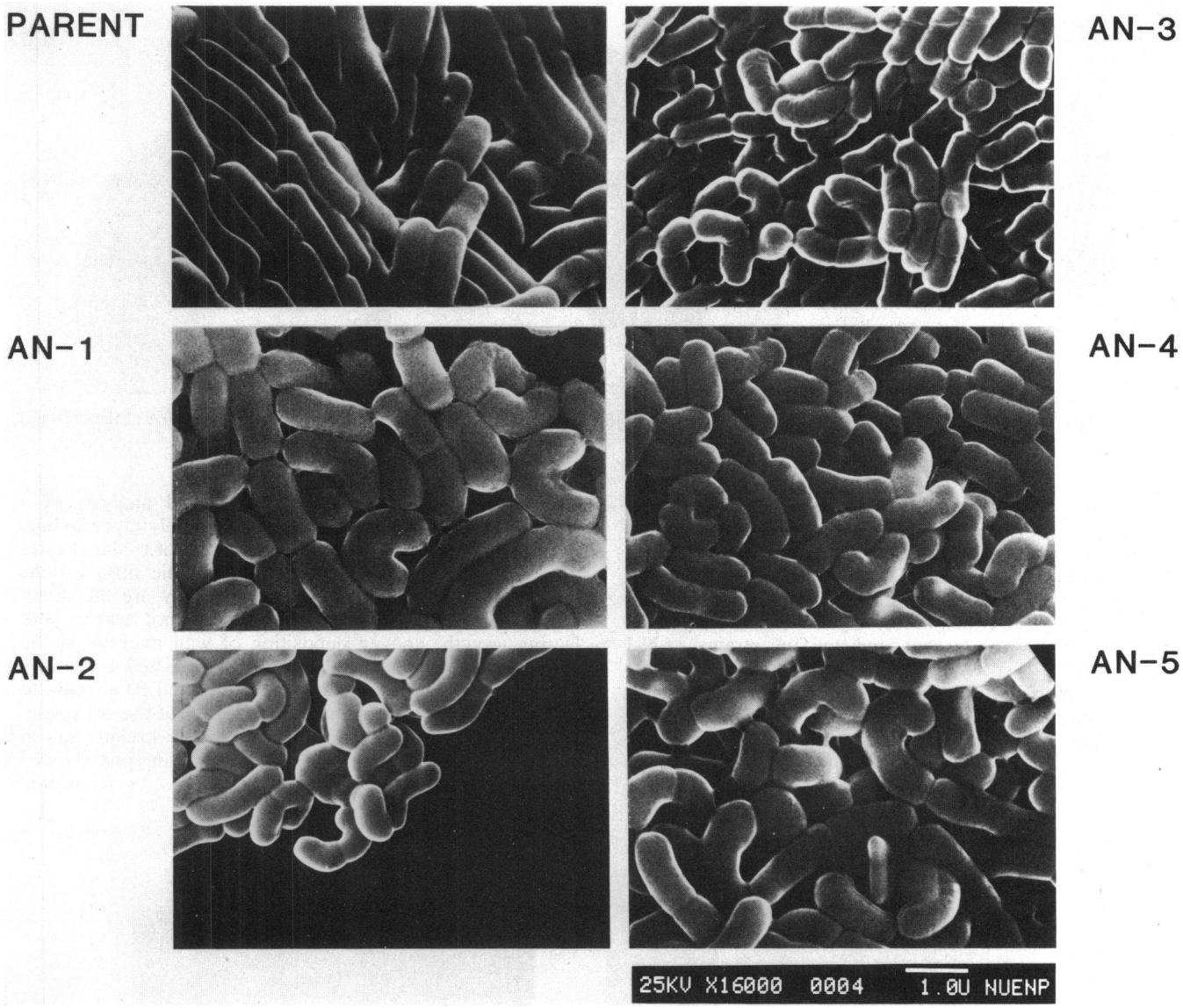

FIG. 6. Scanning electron micrographs of exponential-phase cells of *L. casei* and mutant strains AN-1, AN-2, AN-3, AN-4, and AN-5. Bar, 1 micrometer.

the exception of AN-4, the profiles (I, II, III, and IV) of the α-napthol-sensitive compounds in AN-1, AN-2, AN-3, and AN-5 were similar to that of the parent. In AN-4 (Fig. 4B), the amount of I was elevated, whereas the amounts of III and IV were decreased. This decrease may reflect increased utilization of glycolipid anchor in this strain (Table 1) for LTA synthesis even though the apparent rate of synthesis of glycolipids in vitro derived from UDP-[^{14}C]glucose was similar to that of the other strains (Fig. 4A).

We examined the lipophilic LTA derived from the hydrophilic LTA of each mutant strain to detect differences in the glycolipid anchor in the LTA of the parent and mutant strains. In each case, the [^{3}H]glycerol-labeled lipophilic LTA profile (7) derived from hydrophilic [^{3}H]glycerol-labeled LTA was identical to that found in the parent (data not shown).

Synthesis of D-[^{14}C]alanyl-lipophilic LTA. To define further the defects in D-alanyl-LTA synthesis in the mutant strains, we examined the synthesis of D-[^{14}C]alanyl-lipophilic LTA. With strains AN-1 and AN-4, 5 and 1%, respectively, of the parental levels of D-[^{14}C]alanyl-lipophilic LTA were observed. With strains AN-2, AN-3, and AN-5, 27, 20, and 36%, respectively, of these compounds were found (Fig. 5). These results are consistent with the observation that the D-alanine incorporation system in strains AN-1 and AN-4 not only failed to alanylate hydrophilic LTA (Table 1) but that the system also failed to alanylate lipophilic LTA.

Properties of mutant strains. A goal of this study was to associate deficiencies in the D-alanyl ester content of the LTAs of the mutant strains with unique growth characteristics, alterations in cell morphology, or defects in cell septation and separation. The doubling times at 32°C for the parent, AN-1, AN-2, AN-3, AN-4, and AN-5 were 120, 180, 150, 160, 270, and 160 min, respectively. The minimal growth-inhibitory concentrations of benzylpenicillin (2.2×10^{-6} M) and D-cycloserine (3.9×10^{-3} M) were similar for all of the strains. Autolysis of exponential-phase, D-[^{14}C]glucosamine-labeled cells of *L. casei* ATCC 7469 in 50

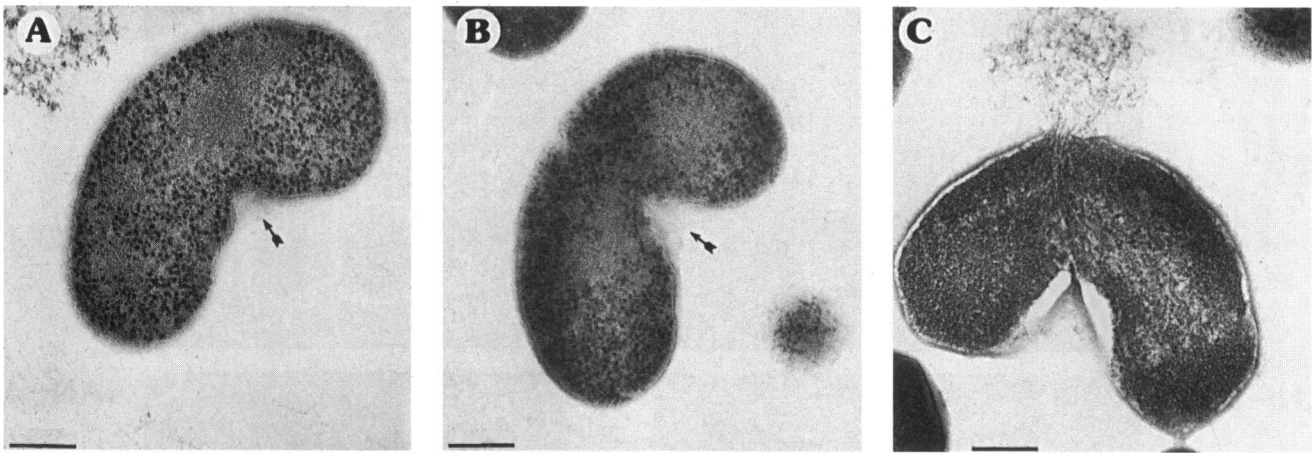

FIG. 7. Uneven deposition of wall material in AN-4. Two examples from this mutant (A and B) before rupture in 5 to 10% of the cells (C) are shown. Bar, 0.2 micrometer.

mM citrate (pH 5.0) was low (2% in 5 h) and was not significantly different from that detected in the mutant strains. The maximum turnover of peptidoglycan in D-[^{14}C]glucosamine-labeled cells was 20.8 ± 2.0% per generation in each mutant strain and the parent. Thus, no detectable differences in either autolysis or wall turnover were observed among the parent and mutant strains (AN-1 through AN-5).

The morphology of each mutant is shown in the scanning electron micrographs (Fig. 6). Many cells of the mutant strains were distinctly C shaped in exponential-phase cultures. In the case of AN-3, the fraction of C-shaped cells was greater in stationary-phase cells than in exponential-phase cells. For a more detailed morphological analysis of a mutant, we examined AN-4 because its poly(glycerophosphate) moiety of LTA had the lowest ratio of D-alanyl ester to glycerol phosphate (Table 1). Two basic observations were made with this mutant. Whether they are the direct result of D-alanyl ester deficiency was not established. During growth, uneven deposition of wall material in the cylinder occurs, which apparently leads to cell curvature. The progress of this deposition (Fig. 7A and B) appears to precede the rupture observed in 5 to 10% of the cells (Fig. 7C). The second observation made with AN-4 relates to cell separation. An examination of the separation zone showed various degrees of fragmentation (Fig. 8). As separation

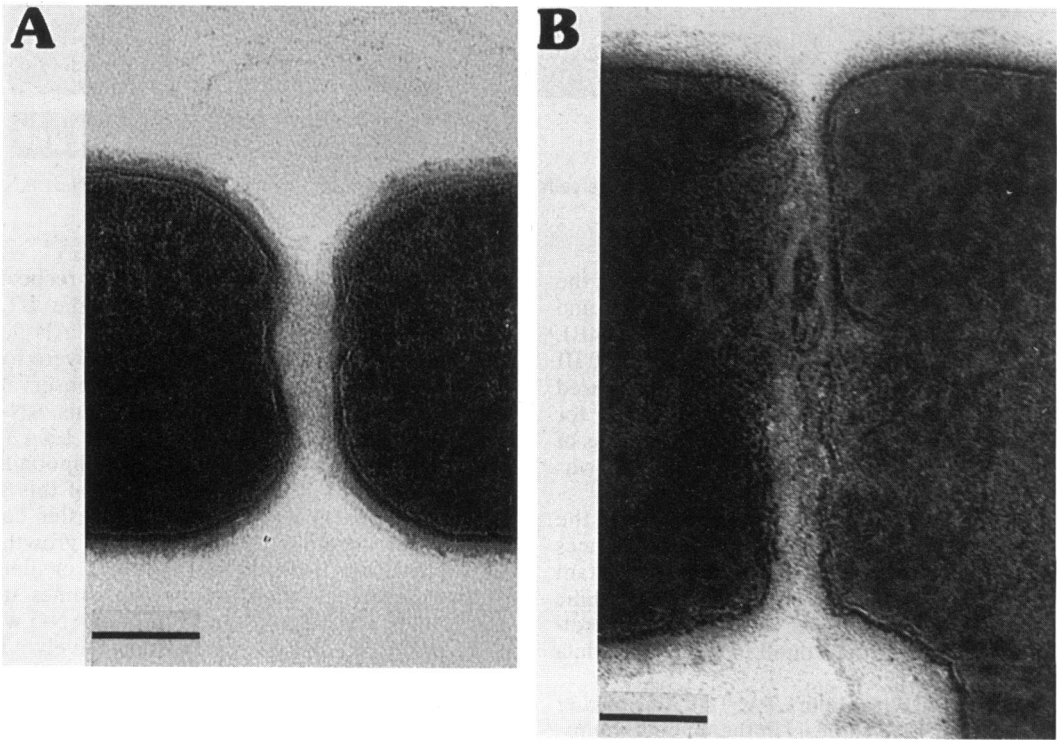

FIG. 8. Separation zones in the parent (A) and AN-4 (B). Bar, 0.1 micrometer.

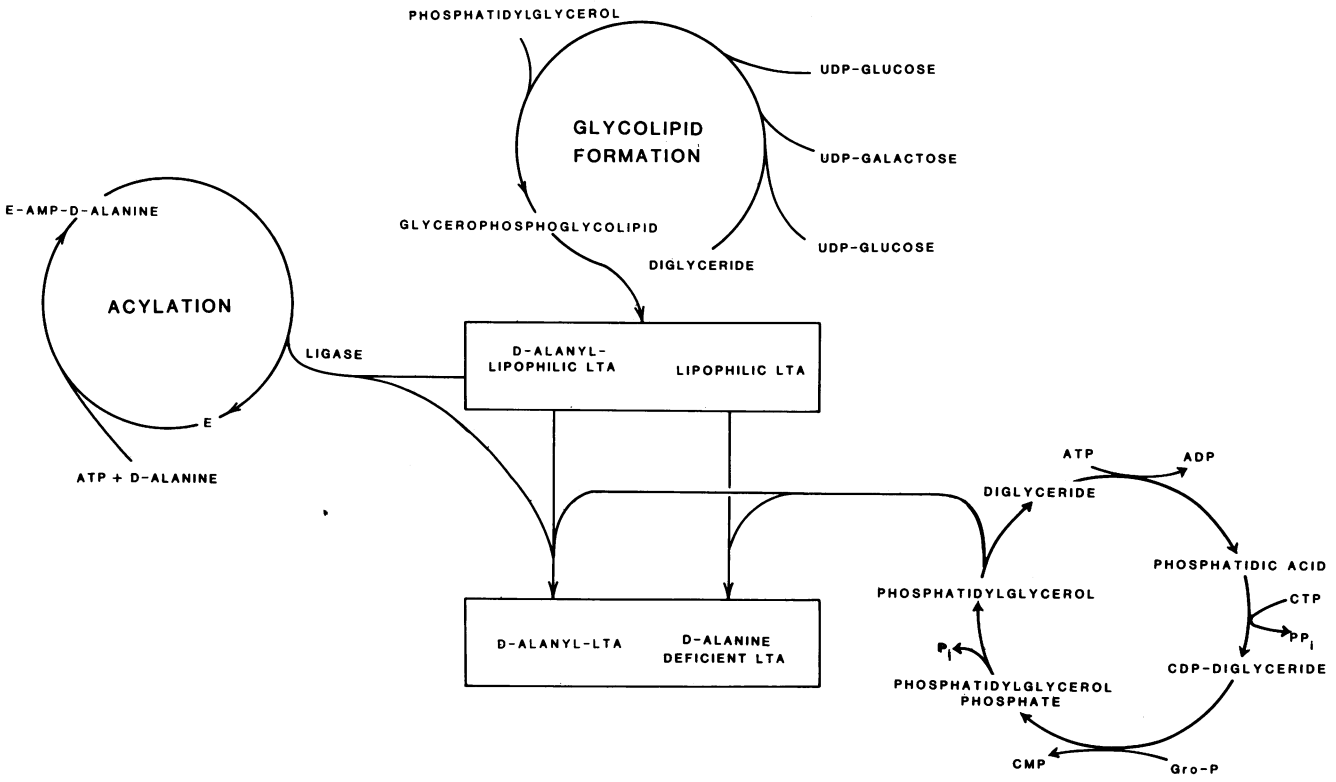

FIG. 9. Proposed pathway for assembly of D-alanyl-LTA and D-alanyl ester-deficient LTA in *L. casei*.

proceeded, the fragments in this zone disappeared. As in the case of other *Lactobacillus* strains (6, 22, 43), the walls of *L. casei* 7469 and the mutant strains are characterized by an inner electron-dense region and an outer poorly staining region that appears loosely organized and sloughs from the surface.

DISCUSSION

Five mutant strains of *L. casei* 7469 deficient in their D-alanyl ester content of LTA were isolated. These strains may allow one to define better the role of the esters in the function of this amphiphile. This communication represents the first isolation and description of such mutants.

To assess the defects in the mutant strains, we divided the assembly of LTA into three phases (Fig. 9): (i) synthesis and selection of the glycolipid anchor, (ii) synthesis of the poly(glycerophosphate) moiety, and (iii) D-alanylation (acylation of LTA by D-alanine) of lipophilic and hydrophilic LTAs. The mutant strains were able to achieve synthesis and selection of the glycolipid anchor. In addition, they were all able to synthesize the poly(glycerophosphate) moiety of LTA. It was concluded that the membranes derived from mutant strains AN-1 and AN-4 were defective for D-alanylation of LTA even though acceptor LTA was present. Two postulated functions of the membrane in the D-alanine incorporation system may be either defective or compromised in these strains. The membrane may (i) facilitate formation of a specific lipoteichoic acid complex with other membrane components or (ii) establish a specific conformation or topological organization of LTA. Mutant strains AN-2 and AN-3 appear to be partially deficient in the amount of D-alanine-activating enzyme. At this stage of our investigation, we are unable to define the defect in AN-5.

Some of the biochemical defects expressed in our mutant strains are similar to those expressed in a stabilized L form derived from *Streptococcus pyogenes* (8). In this L form, the membrane glycerol teichoic acid (LTA) lacks D-alanyl ester residues and is shorter (41). It was observed that the lipid chain rigidity of these membranes is greater than that of the membranes from the parent (9). This feature may be responsible for the absence of LTA acceptor capacity in the membrane from this organism even though the acceptor is present.

A variety of mutants defective for the synthesis of wall teichoic acid have been isolated and partially characterized. For example, Park and co-workers (37) isolated three groups of bacteriophage-resistant mutants from *S. aureus* that lack either wall teichoic acid or a specific component of teichoic acid. One of these, strain 52A5, is totally deficient in wall teichoic acid. It was concluded that "although a wall teichoic acid or its equivalent is not essential in *S. aureus*, the presence of teichoic acid provides a marked selective advantage to the organism." One of the major defects in this strain is incomplete cell separation. Similarly, a temperature-sensitive *rod* mutant (168ts-200b) from *Bacillus subtilis*, which forms spheres under nonpermissive conditions, is also defective for synthesis of wall teichoic acid (12). Loss of this polymer alters not only cell surface growth but also influences cellular division, separation, and morphology. Thus, difficulty in cell separation and aberrant morphology also appear to be characteristics of mutant strains deficient in wall teichoic acid.

A common property of both wall teichoic acid mutants and the D-alanyl ester-deficient LTA mutants from *L. casei* is a defect in cell separation. In the mutant strain AN-4, separation is characterized by various degrees of fragmentation even though the cells eventually divide. The role of LTA in

this process may be related to its postulated regulation of autolysin(s). Another property of the D-alanine ester-deficient mutant AN-4 is an apparent malfunction of cylindrical wall assembly. Uneven deposition of wall polymers leads to localized thickening. In a similar manner, the autolysin-deficient mutant βA177 of *B. subtilis* shows a curved (bent) morphology (14). Addition of (i) autolysin from the parent or (ii) lysozyme to the growth medium reduces the formation of bent cells and increases the growth rate. Hence, it was proposed that an autolysin was required for normal wall expansion (14). In the case of mutant strain AN-4 of *L. casei*, the similar bent or C-shaped morphology may result from improper regulation of autolysin function.

Fischer et al. (19) proposed that D-alanine-free LTA and D-alanyl-LTA are in vitro active and inactive forms of an inhibitor of autolysin(s). On the basis of this proposal, mutants deficient in the D-alanine ester content of LTA (e.g., AN-4) may have a higher amount of the active form (D-alanine-free LTA) of the autolysin inhibitor. It was not possible to detect differences in autolysis or wall turnover rates among the parent and mutant strains. The defect(s) may be expressed in topologically defined zones or regions of the cell and thus may not have major effects on the bulk rates of autolysis and wall turnover.

At this stage of our investigation it is not known whether the mutant strains resulted from multiple mutations introduced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (33) or whether a single mutation was responsible for the pleiotropic phenotype. In future experiments isogenic strains with a single mutation must be constructed to prove that a deficiency in D-alanyl ester content is associated with a given morphological or separation defect.

ACKNOWLEDGMENTS

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

We are indebted to Eugene W. Minner for his generous help in the Electron Microscopy Facility of the Department of Neurobiology and Physiology, Northwestern University. We thank Werner Fischer for helpful discussions.

LITERATURE CITED

1. **Archibald, A. R.** 1974. The structure, biosynthesis and function of teichoic acid. Adv. Microb. Physiol. **11**:53–95.
2. **Archibald, A. R., J. Baddiley, and S. Heptinstall.** 1973. The alanine ester content and magnesium binding capacity of walls of *Staphylococcus aureus* H grown at different pH values. Biochim. Biophys. Acta **291**:629–634.
3. **Baddiley, J.** 1972. Teichoic acids in cell walls and membranes of bacteria. Essays Biochem. **8**:35–77.
4. **Baddiley, J., and F. C. Neuhaus.** 1960. The enzymic activation of D-alanine. Biochem. J. **75**:579–587.
5. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**:911–917.
6. **Boothby, D., L. Daneo-Moore, M. L. Higgins, J. Coyette, and G. D. Shockman.** 1973. Turnover of bacterial cell wall peptidoglycans. J. Biol. Chem. **248**:2161–2169.
7. **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.
8. **Chevion, M., C. Panos, R. Linzer, and F. C. Neuhaus.** 1974. Incorporation of D-alanine into the membrane of *Streptococcus pyogenes* and its stabilized L-form. J. Bacteriol. **120**:1026–1032.
9. **Chevion, M., C. Panos, and J. Paxton.** 1976. Membrane studies of *Streptococcus pyogenes* and its L-form growing in hypertonic and physiologically isotonic media: an electron spin resonance spectroscopy approach. Biochim. Biophys. Act **426**:288–301.
10. **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.
11. **Cleveland, R. F., A. J. Wicken, L. Daneo-Moore, and G. D. Shockman.** 1976. Inhibition of wall autolysis in *Streptococcus faecalis* by lipoteichoic acid and lipids. J. Bacteriol. **126**: 192–197.
12. **Cole, R. M., T. J. Popkin, J. Boylan, and N. H. Mendelson.** 1970. Ultrastructure of a temperature-sensitive rod$^-$ mutant of *Bacillus subtilis*. J. Bacteriol. **103**:793–810.
13. **Coyette, J., and J.-M. Ghuysen.** 1970. Wall autolysin of *Lactobacillus acidophilus* strain 63 AM Gasser. Biochemistry **9**:2952–2955.
14. **Fan, D. P., M. M. Beckman, and W. P. Cunningham.** 1972. Ultrastructural studies on a mutant of *Bacillus subtilis* whose growth is inhibited due to insufficient autolysin production. J. Bacteriol. **109**:1247–1257.
15. **Fiedler, F., and L. Glaser.** 1974. The synthesis of polyribitol phosphate. II. On the mechanism of polyribitol phosphate polymerase. J. Biol. Chem. **249**:2690–2695.
16. **Fischer, W., and H. U. Koch.** 1981. Alanine ester substitution and its effect on the biological properties of lipoteichoic acids, p. 181–194. *In* G. D. Shockman, and A. J. Wicken (ed.), Chemistry and biological activities of bacterial surface amphiphiles. Academic Press, Inc., New York.
17. **Fischer, W., H. U. Koch, P. Rösel, 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.
18. **Fischer, W., and P. Rösel.** 1980. The alanine ester substitution of lipoteichoic acid (LTA) in *Staphylococcus aureus*. FEBS Lett. **119**:224–226.
19. **Fischer, W., P. Rösel, and H. U. Koch.** 1981. Effect of alanine ester substitution and other structural features of lipoteichoic acids on their inhibitory activity against autolysins of *Staphylococcus aureus*. J. Bacteriol. **146**:467–475.
20. **Hancock, I., and J. Baddiley.** 1976. In vitro synthesis of the unit that links teichoic acid to peptidoglycan. J. Bacteriol. **125**:880–886.
21. **Heptinstall, S., A. R. Archibald, and J. Baddiley.** 1970. Teichoic acids and membrane function in bacteria. Nature (London) **225**:519–521.
22. **Higgins, M. L., J. Coyette, and G. D. Shockman.** 1973. Sites of cellular autolysis in *Lactobacillus acidophilus*. J. Bacteriol. **116**:1375–1382.
23. **Höltje, J.-V., and A. Tomasz.** 1975. Lipoteichoic acid: a specific inhibitor of autolysin activity in *Pneumococcus*. Proc. Natl. Acad. Sci. USA **72**:1690–1694.
24. **Hughes, A. H., I. C. Hancock, and J. Baddiley.** 1973. The function of teichoic acids in cation control in bacterial membranes. Biochem. J. **132**:83–93.
25. **Kelemen, M. V., and J. Baddiley.** 1961. Stucture of the intracellular glycerol teichoic acid from *Lactobacillus casei* ATCC 7469. Biochem. J. **80**:246–254.
26. **Kellenberger, E., J. Séchaud, and B. Blondel.** 1972. A micromethod for the embedding of small amounts of cells from liquid suspension. J. Ultrstruct. Res. **39**:606–607.
27. **Koch, H. U., W. Fischer, and F. Fiedler.** 1982. Influence of alanine ester and glycosyl substitution on the lipoteichoic acid carrier activity of lipoteichoic acids. J. Biol. Chem. **257**:9473–9479.
28. **Lambert, P. A., I. C. Hancock, and J. Baddiley.** 1975. Influence of alanyl ester residues on the binding of magnesium ions to teichoic acids. Biochem. J. **151**:671–676.
29. **Lambert, P. A., I. C. Hancock, and J. Baddiley.** 1977. Occurrence and function of membrane teichoic acids. Biochim. Biophys. Acta **472**:1–12.
30. **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.
31. **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.
32. **MacArthur, A. E., and A. R. Archibald.** 1984. Effect of culture pH on the D-alanine ester content of lipoteichoic acid in *Staphylococcus aureus*. J. Bacteriol. **160**:792–793.
33. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 125–129. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
34. **Nakano, M., and W. Fischer.** 1977. The glycolipids of *Lactobacillus casei* DSM 20021. Hoppe-Seyler's Z. Physiol. Chem. **358**:1439–1453.
35. **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.
36. **Neuhaus, F. C., R. Linzer, and V. M. Reusch, Jr.** 1974. Biosynthesis of membrane teichoic acid:role of the D-alanine activating enzyme and D-alanine: membrane acceptor ligase. Ann. N.Y. Acad. Sci. **235**:502–518.
37. **Park, J. T., D. R. D. Shaw, A. N. Chatterjee, D. Mirelman, and T. Wu.** 1974. Mutants of *Staphylococci* with altered cell walls. Ann. N.Y. Acad. Sci. **236**:54–62.
38. **Raetz, C. R. H.** 1975. Isolation of *Escherichia coli* mutants defective in enzymes of membrane lipid synthesis. Proc. Natl. Acad. Sci. USA **72**:2274–2278.
39. **Reusch, V. M., Jr., and F. C. Neuhaus.** 1971. D-Alanine: membrane acceptor ligase from *Lactobacillus casei*. J. Biol. Chem. **246**:6136–6143.
40. **Reynolds, E. S.** 1963. The use of lead citrate at high pH as an electron-opaque strain in electron microscopy. J. Cell Biol. **17**:208–212.
41. **Slabyj, B. M., and C. Panos.** 1973. Teichoic acid of a stabilized L-form of *Streptococcus pyogenes*. J. Bacteriol. **114**:934–942.
42. **Spurr, A. R.** 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26**:31–43.
43. **Thorne, K. J. I., and D. C. Barker.** 1969. Bactoprenol, ATPase and acetate activating enzymes of a vesicular fraction from *Lactobacillus casei*. Eur. J. Biochem. **11**:582–591.
44. **Venable, J. H., and R. Coggeshall.** 1965. A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. **25**:407–408.
45. **Wicken, A. J., J. W. Gibbens, and K. W. Knox.** 1973. Comparative studies on the isolation of membrane lipoteichoic acid from *Lactobacillus fermenti*. J. Bacteriol. **113**:365–372.