JOURNAL OF BACTERIOLOGY, May 2000, p. 2855–2864 Vol. 182, No. 10
0021-9193/00/$04.00+0

Biosynthesis of Lipoteichoic Acid in *Lactobacillus rhamnosus*: Role of DltD in D-Alanylation

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Received 16 December 1999/Accepted 22 February 2000

The *dlt* operon (*dltA* to *dltD*) of *Lactobacillus rhamnosus* 7469 encodes four proteins responsible for the esterification of lipoteichoic acid (LTA) by D-alanine. These esters play an important role in controlling the net anionic charge of the poly (GroP) moiety of LTA. *dltA* and *dltC* encode the D-alanine–D-alanyl carrier protein ligase (Dcl) and D-alanyl carrier protein (Dcp), respectively. Whereas the functions of DltA and DltC are defined, the functions of DltB and DltD are unknown. To define the role of DltD, the gene was cloned and sequenced and a mutant was constructed by insertional mutagenesis of *dltD* from *Lactobacillus casei* 102S. Permeabilized cells of a *dltD*::*erm* mutant lacked the ability to incorporate D-alanine into LTA. This defect was complemented by the expression of DltD from pNZ123/*dlt*. In in vitro assays, DltD bound Dcp for ligation with D-alanine by Dcl in the presence of ATP. In contrast, the homologue of Dcp, the *Escherichia coli* acyl carrier protein (ACP), involved in fatty acid biosynthesis, was not bound to DltD and thus was not ligated with D-alanine. DltD also catalyzed the hydrolysis of the mischarged D-alanyl–ACP. The hydrophobic N-terminal sequence of DltD was required for anchoring the protein in the membrane. It is hypothesized that this membrane-associated DltD facilitates the binding of Dcp and Dcl for ligation of Dcp with D-alanine and that the resulting D-alanyl–Dcp is translocated to the primary site of D-alanylation.

Lipoteichoic acid (LTA) plays a vital role in the growth and physiology of gram-positive bacteria. It is postulated that this macroamphiphile modulates the activities of autolysins (4, 13), the binding of cations required for enzyme function (2, 20, 21, 27), and the electromechanical properties of the cell wall (37). The D-alanyl esters of LTA determine its net anionic charge and hence may regulate the functions of this polymer (32).

The biosynthesis of D-alanyl–LTA requires the 56-kDa D-alanine–D-alanyl carrier protein ligase (Dcl) and the 8.8-kDa D-alanyl carrier protein (Dcp) (33). Heaton and Neuhaus (19) isolated Dcp and showed that D-alanyl–Dcp donates its D-alanyl substituent to membrane-associated LTA. Debabov et al. (10) cloned, sequenced, and expressed the gene encoding this novel carrier protein, a homologue of the acyl carrier protein (ACP) involved in fatty acid biosynthesis. In addition to the genes encoding Dcl (DltA) and Dcp (DltC), the *dlt* operon contains two additional genes, *dltB* and *dltD*, encoding putative membrane proteins (10, 33).

The goal of this paper is to establish the function of the *Lactobacillus rhamnosus* protein encoded by *dltD*. To accomplish this goal, the gene was cloned, sequenced, and expressed in *Escherichia coli* with and without the sequence encoding the N-terminal hydrophobic domain. Additionally, the gene was inactivated and its protein product was examined for thioesterase activity and carrier protein binding specificity. The results support a role for this protein in hydrolyzing mischarged D-alanyl–ACP and as a facilitator of D-alanine ligation to the carrier protein Dcp.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and plasmids. All bacterial strains, phages, and plasmids used in this study are listed in Table 1.

Chemicals and reagents. [^{35}S]dATP (600 Ci/mmol in 5 mM Tris-HCl [pH 7.5]), D-[^{14}C]alanine (43 mCi/mmol), and dithiothreitol (DTT) were products of ICN Biochemicals, Inc. Medium supplies were obtained from Difco Laboratories. Tetracycline, ampicillin, carbenicillin, erythromycin, EDTA, Tris, bis-Tris, cetyltrimethylammonium bromide (CTAB), and chlorhexidine were obtained from Sigma Chemical Co. Solutions of phenol-chloroform were products of Amresco Inc. Metricel filter membranes (GN-6) and Econo-Safe scintillation cocktail were purchased from Gelman Sciences and RPI Corp., respectively. Restriction enzymes were obtained from a number of suppliers and were used according to the manufacturer's instructions. Isopropyl-β-D-galactoside (IPTG) and *Eco*RI (*Not*I) adapters were purchased from Gibco-BRL. The Genius System nonradioactive DNA labeling kit and DIG nucleic acid detection kit were purchased from Boehringer Mannheim GmbH. Gigapack II Plus packaging extracts, T4 DNA ligase, and PfuTurbo DNA polymerase were obtained from Stratagene. The Sequenase and PCR kits were purchased from United States Biochemical Corp. and MBI Fermentas, respectively. BioBlot nitrocellulose blotting membranes were purchased from Costar, Inc. The QIAprep spin miniprep, QIAquick nucleotide removal, and QIAquick gel extraction kits were obtained from Qiagen. Oligonucleotides were synthesized by Integrated DNA Technologies, Inc.

Growth of bacteria. *E. coli* was cultured in either Luria-Bertrani (LB) broth or 2× YT (2× yeast extract-tryptone) medium and plated on either LB agar or LB agarose (41). *L. rhamnosus* 7469 and *Lactobacillus casei* 102S were cultured in MRS medium (Difco). For electroporation, *L. casei 102S* was grown in a low-salt medium (LL) containing (grams/liter) tryptone, 15; yeast extract, 5; $MgSO_4$, 0.01; $MnSO_4$, 0.005; glucose, 10; and 5 mM potassium phosphate buffer (pH 7.0). Overnight cultures were diluted 1:30 with fresh LL medium, and the cells were grown for an additional 3 to 3.5 h at 37°C. The cells were collected by centrifugation, washed several times with water (4°C), and resuspended in 0.01 volume of cold 10% glycerol. Aliquots of cells were stored at −80°C. Tetracycline, ampicillin, and chloramphenicol were used to select and maintain plasmids in *E. coli* (10, 100, and 20 μg/ml, respectively). For *L. casei* 102S with plasmid and chromosomal integration, 10 μg of chloramphenicol/ml and 5 μg of erythromycin/ml, respectively, were used. Competent cultures of *E. coli* were prepared by the method of Hanahan (17). Bacteriophages were propagated by standard methods (41).

DNA techniques. *L. rhamnosus* 7469 and *L. casei* 102S were lysed by the method of Chassy and Giuffrida (7). Plasmids from *L. casei* 102S were isolated according to the method of O'Sullivan and Klaenhammer (36). Restriction digests, agarose and acrylamide gel electrophoreses, Southern blots, small- and large-scale plasmid and phage DNA preparations, transformations, and isolation of chromosomal DNA were performed by standard techniques (41). For the preparation of high-quality plasmids and DNA fragments, Qiagen kits were used. Digoxigenin labeling of the DNA probe, Southern blotting, and colony hybridizations were performed according to the conditions described by the manufacturer (Boehringer Mannheim GmbH). Both strands of DNA were sequenced by

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TABLE 1. Bacterial strains, phages, and plasmids used in this study[a]

Strain, plasmid, or phage	Description	Source or reference
Strains		
L. rhamnosus 7469	Formerly named *L. casei* ATCC 7469	ATCC
L. casei 102S	*L. casei* ATCC 393 cured from plasmids	B. Chassy
L. casei 102S *dltD*::*erm*	Em^r; *dltD* interrupted by *erm*	This study
E. coli XL-1 Blue MRF′	Δ(*mcrA*) *183*Δ (*mcrCB-hsdSMR-mrr*) *173end1A supE44thi-1 recA1 gyrA96 relA1lac* [F′ *proAB* lac^qZΔM15 Tn*10*(Tet^r)]	Stratagene
E. coli BL21(DE3) pLysS	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm*(DE3)pLysS	Novagene
E. coli DH5α	F^- f80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*($r_k^- m_k^+$) *supE44* λ^- *thi-1 girA96 relA1*	Gibco-BRL
Phages (phagemids)		
λZAPII [Bluescript SK(−)]	Ap^r cloning vector	Stratagene
VCSM13	Helper phage	Stratagene
λZAPII(−)DE43	4.3 kb of *L. rhamnosus* DNA containing *dltA*, *dltB*, *dltC*, and partial *dltD*	10
λZAPII(−)A65	6.5 kb of *L. rhamnosus* DNA containing partial *dltB*, *dltC*, *dltD*, and downstream flanking region	This study
Plasmids		
pET-3d	Expression vector	Novagene
pDltD	Expression plasmid; *dltD* cloned into pET-3d	This study
pΔDltD	Expression plasmid; Δ*dltD* cloned into pET-3d	This study
pVE6006	Em^r Cm^r Ts shuttle vector	30
pNZ123	Cm^r shuttle vector	46
pΔDltD/*erm*	1.2-kb *Eco*ICR *erm* fragment of pVE6006 joined to *Bam*HI blunt-ended pΔDltD in direct orientation to Δ*dltD*	This study
pNZ123/*dlt*	4.895-kb fragment containing *dlt* operon cloned into *Hin*dIII of pNZ123	This study

[a] Em^r, erythromycin resistant; Cm^r, choramphenicol resistant; Ap^r, ampicillin resistant; ATCC, American Type Culture Collection.

the dideoxy chain termination method (42) with the use of Sequenase version 2.0 DNA polymerase (47).

Sequence analyses. The protein database searches were performed by the BLAST algorithm (1), and the deduced amino acid sequences were analyzed with the University of Wisconsin Genetic Computer Group sequence analysis software package, version 9.0 (11). Multiple alignments and consensus sequences were accomplished with PILEUP and determined with PRETTY, respectively. Comparisons of homologous proteins were accomplished with GAP. Remote homologies were detected by the method of Karplus et al. (22). The MacVector sequence analysis programs were used for the design of primers for PCR and sequencing and for the calculation of molecular masses, isoelectric points, and hydrophobicity profiles of proteins. The locations of protein and signal peptidase cleavage sites were predicted with the Signal P algorithm (34).

Construction of an *L. rhamnosus Ava*I library. A restriction map of the *dlt* chromosomal region was generated by Southern analyses of *L. rhamnosus* restriction digests with the 389-bp digoxigenin-labeled probe complementary to nucleotides (nt) 3029 to 3417 of the *dlt* operon (Fig. 1A) (10). The map shows four restriction fragments (*Ava*I, *Dra*I, *Bal*I, and *Hin*cII) which contain the downstream region of the *dlt* operon. The 6.5-kb *Ava*I fragment was selected for cloning. Chromosomal DNA fragments were purified after *Ava*I digestion and treated with DNA polymerase I Klenow fragment for 40 min at 37°C to generate blunt ends. *Eco*RI restriction sites were introduced by blunt-end ligation of phosphorylated *Eco*RI (*Not*I) adaptors. The resulting DNA was ligated into λZAPII *Eco*RI arms. This preparation was packaged with Gigapack II packaging extract and used to infect *E. coli* XL-1 Blue MRF′. Phages from the resulting plaques were transferred to Nytran maximum-strength membranes and screened with the above-mentioned 389-bp probe. A plaque from the hybridizing clones, designated λZAPII(−)A65, was purified, the phagemid was rescued by helper phage VCSM13, and single-stranded DNA was isolated for sequence analysis.

Construction of pDltD and pΔDltD. To construct the plasmids for *dltD* and Δ*dltD* expression, amplifications and mutations of the gene were accomplished by PCR. Each reaction was carried out with λZapII(−)A65 DNA and mutagenic primers. For the construction of pDltD, primer I, 5′-CGAACAAGCAacATGt cAAAACCGATC-3′ (the mutagenized nucleotides are lowercase; the restriction site is underlined), was complementary to the minus strand of the A65 insert and positioned a new *Afl*III site to span the ATG start codon of *dltD*. Primer II, 5′-GATGGCACCTAgAtCTGACTGGCC-3′, was complementary to the plus strand of the A65 insert and positioned a new *Bgl*II site 10 nt downstream of the termination codon of *dltD*. For the construction of pΔDltD, the first primer, 5′-GCGGCCAGCTaCATGTCGGCC-3′, was complementary to the minus strand of the A65 insert and positioned a new *Afl*III site to span the ATG codon encoding Met48 of DltD. The second primer was the same as primer II used for the construction of pDltD. Both DNA fragments were amplified (25 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C for 1.5 min), digested with *Afl*III and *Bgl*II, and ligated into the pET-3d vector (45) which had been digested with *Nco*I and *Bam*HI. The ligation mixtures were used to transform *E. coli* XL-1 Blue MRF′. Clones containing plasmids with *dltD* or Δ*dltD* inserts, designated pDltD and pΔDltD, respectively, were identified by restriction and sequence analyses (Fig. 1C). These plasmids were used to transform *E. coli* BL21(DE3) pLysS for expression studies.

Expression of *dltD* and Δ*dltD* in *E. coli*. *E. coli* BL21(DE3) pLysS containing either pDltD or pΔDltD was grown in 100 ml of LB broth with 100 μg of carbenicillin/ml at 37°C (300 rpm). At an optical density at 600 nm of 0.7, the cultures were induced with 0.4 mM IPTG, and the cells were harvested 2 h after induction. The expression of *dltD* and Δ*dltD* was observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (26). To prepare soluble and insoluble protein fractions for this analysis, cells were suspended in 0.1 culture volume of 50 mM Tris-HCl (pH 8.0) and disintegrated with a Labsonic U ultrasonic homogenizer (B. Braun Biotech, Inc.) for 3 min at 60-s intervals. The disrupted cells were centrifuged at 10,000 × *g* for 15 min, and the pellet was analyzed for insoluble proteins. The *E. coli* membranes were collected by ultracentrifugation of the supernatant fraction at 200,000 × *g* for 90 min and homogenized in a minimal amount of 25 mM Tris-HCl (pH 7.5) containing 10% glycerol. Membrane fragments were washed by three cycles of differential centrifugation at 10,000 × *g* and 200,000 × *g*. The washed membranes (18.5 mg of protein) were suspended in 1 ml of 30 mM Tris-HCl (pH 7.5) containing 1 mM $MgCl_2$ and 10% glycerol. Protein was measured with the Bradford reagent (Pierce Chemical Co.). Aliquots of membranes were frozen in liquid nitrogen and stored at −80°C.

For the preparation of ΔDltD, a pellet (inclusion bodies) as described above after centrifugation was dissolved in 8 M urea, centrifuged, and dialyzed against 25 mM Tris-HCl (pH 7.5). After dialysis, the pellet containing ΔDltD was collected by centrifugation and dissolved in a minimal volume of 10 mM NaOH. The protein solution was applied to a Sephacryl S-200 column (1 by 50 cm), and ΔDltD was eluted with 10 mM NaOH. The fraction containing ΔDltD was collected, concentrated on a Macrosep centrifugal concentrator (10K) (Filtron Technology Corp.), and stored in aliquots at −80°C.

Expression and purification of Dcp and Dcl. Dcp was expressed and purified according to the method of Debabov et al. (10). The conversion of apo-Dcp to holo-Dcp was carried out using recombinant holo-ACP synthase generously provided by R. H. Lambalot, R. S. Flugel, and C. T. Walsh (Harvard University) (10). Dcl was expressed according to the method of Heaton and Neuhaus (18) and purified from inclusion bodies. In brief, a pellet obtained after centrifugation of sonicated cells was dissolved in 6 M urea, centrifuged, and dialyzed against 50 mM potassium phosphate buffer (PPB) (pH 7.0) containing 2 mM DTT. After dialysis, the protein solution was applied to a Q-Sepharose fast flow column (2.6 by 5.0 cm) (Amersham Pharmacia Biotech), which was washed with 25 mM PPB (pH 7.0) containing 2 mM DTT until the optical density at 254 nm returned to

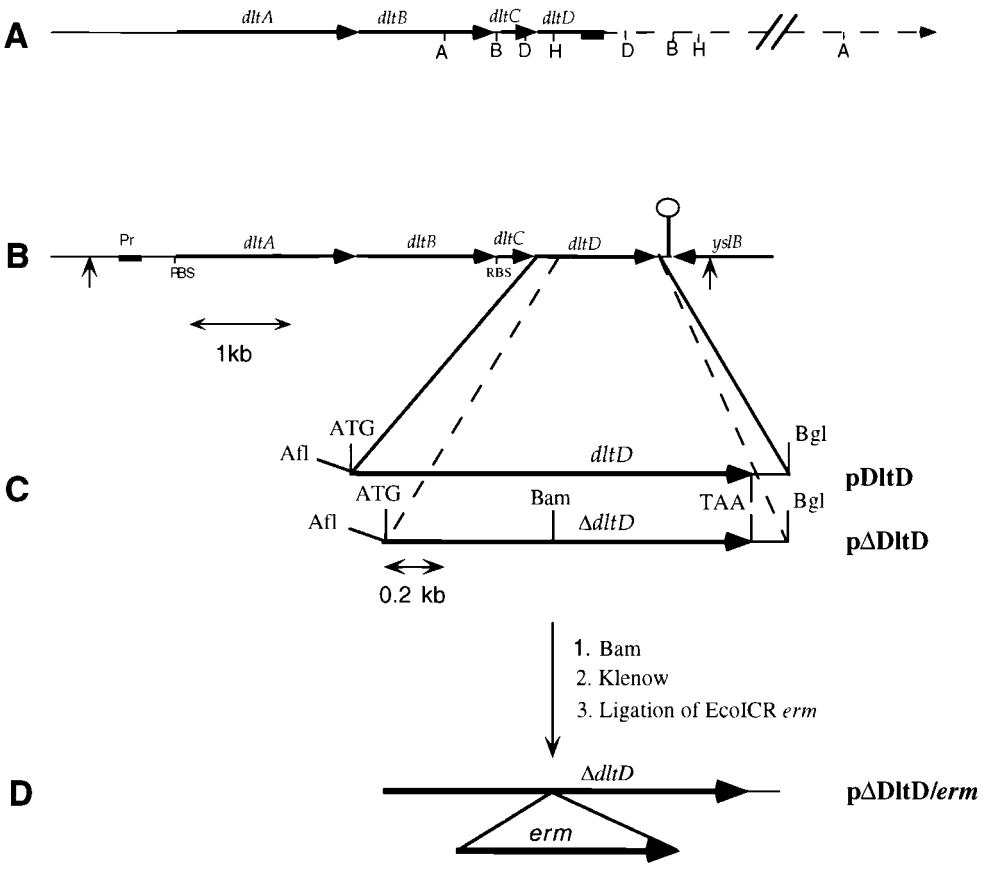

FIG. 1. Cloning strategy for the isolation, expression, insertional inactivation, and complementation of *dltD*. (A) *L. rhamnosus* chromosomal map of *dltD* and flanking regions. The site of probe hybridization to *dltD* is shown by the solid box. (B) Physical map of the *L. rhamnosus dlt* operon. ORFs are shown by arrows, and the terminator of transcription is shown by ♀. The positions of primers for the amplification of the *dlt* operon and its cloning into pNZ123 are shown by vertical arrows. (C) Inserts containing *dltD* and Δ*dltD* in pET-3d, designated pDltD and pΔDltD. The *Afl*III and *Bgl*II sites were introduced by PCR with the mutagenic primers described in Materials and Methods. (D) Cloning of the *Eco*ICR *erm* fragment from pVE6006 into the *Bam*HI site of Δ*dltD* to obtain the plasmid (pΔDltD/*erm*) for insertional inactivation. Pr, promoter; RBS, ribosome binding site; ATG, start codon; TAA, termination codon; A, *Ava*I; B, *Bal*I; D, *Dra*I; H, *Hin*cII, Afl, *Afl*III; Bam, *Bam*HI; Bgl, *Bgl*II.

baseline. Dcl was eluted with a 0 to 1.0 M NaCl gradient (10 volumes) in the same buffer. The first peak containing Dcl was collected, concentrated on a filtron centrifugal concentrator (10K), and stored in aliquots with 10% glycerol at −80°C. After purification, the Dcl had a specific activity of ca. 1,700 U/mg of protein (hydroxamate assay [3]) and a purity greater than 95% as estimated by SDS-PAGE.

D-Alanine incorporation assay. For the assay of D-alanine incorporation into LTA, *L. casei* 102S was permeabilized with toluene at 4°C according to the method of Childs and Neuhaus (8). Alternatively, membranes from *L. casei* 102S were isolated according to a procedure described previously (19) using a French pressure cell for the disruption of cells. The incorporation of D-alanine into either the permeabilized cells or membranes was carried out according to previously described protocols (8, 19) in a total volume of 50 μl. For the incorporation of D-alanine into LTA using membranes, 1.1 nmol of recombinant Dcp and 5 U of Dcl from *L. rhamnosus* were used.

Construction of *dltD*::*erm* mutant of *L. casei* 102S. For the insertional inactivation of the *dltD* gene, Δ*dltD* interrupted by *erm* was used (Fig. 1D). For its preparation, the 1.175-kb *Eco*ICR fragment of pVE6006, which encodes Em^r, was cloned into the *Bam*HI site of pΔdltD blunt-ended by Klenow DNA polymerase. The resulting plasmid, pΔDltD/*erm*, was isolated from *E. coli* DH5α. Plasmid pΔdltD/*erm* (30 μg of DNA per 100 μl of cells) was electroporated into *L. casei* 102S (1.8 kV; R = 129 Ω; 2-mm-gap cuvette) (BTX Electroporation System). After the pulse, the cells were immediately diluted with 2 ml of ice-cold MRS broth and incubated for 10 min on ice and then for 1.5 h at 37°C. Integrants were selected by plating them on MRS plus erythromycin at 37°C under 5 to 10% CO_2.

Complementation of *dltD*::*erm* in *L. casei* 102S. For complementation of the *dltD* mutation, the *dlt* operon of *L. rhamnosus*, including its promoter, was cloned into pNZ123 (Fig. 1B). For the construction of pNZ123/*dlt*, a 4.895-kb *dlt* fragment was amplified by PCR (40 cycles of 94°C for 1 min, 42.5°C for 1 min, and 72°C for 12 min) with PfuTurbo DNA polymerase, with primers (5′-GATATATTTAAGCTTTTTCGATGGTCCG-3′) and (5′-GCAGCAGGTATTaaGCTTTGCAGTCGAAGGAGC-3′) and chromosomal DNA from *L. rhamnosus* 7469 as the template. In these primers, the *Hin*dIII site is underlined and bases not complementary to the *dlt* sequence are shown in lowercase letters. The resulting PCR fragment was digested with *Hin*dIII and ligated into pNZ123. The ligation mixture was used to transform *L. casei* 102S and the *L. casei* 102S *dltD* mutant as described above. Cm^r clones containing the recombinant pNZ123/*dlt* were identified by restriction and PCR analyses.

Binding of Dcp to ΔDltD on NC membranes. To determine whether ΔDltD complexes with the carrier protein, ΔDltD in 10 mM NaOH was bound to nitrocellulose (NC) membranes. The membranes were washed with 25 mM PPB (pH 6.8) and blocked with 5% bovine serum albumin for 1.5 h, and the excess albumin was removed with the same buffer containing 0.1 M NaCl. The NC membranes were incubated with either D-[^{14}C]alanyl–Dcp or D-[^{14}C]alanyl–ACP or alternatively with either Dcp or ACP. D-[^{14}C]Alanyl–Dcp and D-[^{14}C]alanyl–ACP were prepared and purified according to previously described procedures (19). After incubation, the NC membranes were washed with 25 mM PPB containing 0.1 M NaCl. In those binding experiments containing Dcp or ACP, the NC membranes were incubated in 1 ml of reaction mixture containing 0.11 mM D-[^{14}C]alanine (43 mCi/mmol), 8.0 U of recombinant Dcl, 30 mM bis-Tris (pH 6.5), 10 mM ATP, 10 mM $MgCl_2$, and 1 mM DTT at 37°C for 1.5 h. The NC membranes were washed as described above, dried, and exposed for 3 to 5 days to X-OMAT (Kodak Co.) film using a Kodak Bio-Max intensifying screen.

Nucleotide sequence accession number. The sequence of *dltD* from *L. rhamnosus* together with that of *dltA* to *-C* and the adjacent chromosomal regions was reported to GenBank (accession no. AF192553).

RESULTS

Cloning and sequencing of the *dltD* gene. To clone *dltD* from *L. rhamnosus*, an *Ava*I genome library was constructed and screened with a digoxigenin-labeled probe complementary to a

previously determined partial *dltD* sequence (10) (Fig. 1A). A lambda clone [λZapII(−)A65] hybridizing with this probe was identified, and its 6.5-kb insert was isolated and sequenced. The complete sequence of *dltD* was determined from this insert. This gene encoded a putative protein of 423 amino acids (aa) whose mass and pI were 47.738 kDa and 9.54, respectively.

Since the *dlt* operons from at least two organisms, *Bacillus subtilis* and *Streptococcus mutans*, contained five genes (38; D. A. Boyd, D. G. Cvitkovitch, A. S. Bleiweis, M. Y. Kiriukhin, D. V. Debabov, F. C. Neuhaus, and I. R. Hamilton, submitted for publication), it was necessary to analyze the downstream sequence in *L. rhamnosus* for additional genes and for the transcriptional terminator. The analysis 3′ to *dltD* revealed a putative terminator 84 nt downstream of the *dltD* stop codon (Fig. 1B). It is a 12-nt inverted repeat followed by a series of T residues (TTTATTTT). At 104 nt 3′ to the terminator, another open reading frame (ORF) is encoded on the opposite strand (Fig. 1B). The deduced amino acid sequence of this ORF (136 aa) shows 31% identity (61% similarity) to the YslB protein of *B. subtilis*. Since it is encoded in the orientation opposite to that of the *dlt* operon and downstream of the terminator, this gene does not belong to the *dlt* operon. Thus, as in the case of *Staphylococcus aureus* and *Staphylococcus xylosus* (39), the *dlt* operon of *L. rhamnosus* contains only four genes.

Comparison of *L. rhamnosus* DltD with those from different gram-positive organisms. To establish the consensus sequences in DltDs, the deduced amino acid sequence from *L. rhamnosus* was aligned with those from seven gram-positive bacteria. The sequence of DltD from *L. rhamnosus* is 25 to 42% identical to those of the other DltDs. Two mostly conserved sequences were identified, PXXGSSEXXXXDXXXP (positions 69 to 83) and KKXXXXXSPQWFXXXG (positions 123 to 138) (Fig. 2A). Proteins of known function with primary sequence identity to these consensus sequences were not identified by BLAST search. However, there is some similarity of the first consensus sequence to the signature sequence of thioesterases, GXSXG (5), and the corresponding sequence of myristoyl-ACP-specific thioesterase from *Vibrio harveyi* (AASLS) (28). The sequences of DltDs from a variety of organisms are compatible with the structure of amino- and phosphotransferases as determined by the method for remote-homology detection (22). According to the CATH classification (35), these proteins belong to the alpha beta class three-layer (alpha-beta-alpha) sandwich architecture. Interestingly, as will be shown below, DltD catalyzes the hydrolytic cleavage of the mischarged D-alanyl–ACP. Thioesterases, which catalyze this activity, have the same architecture.

It was proposed that DltD from *S. aureus* has an N-terminal signal peptide with a cleavage site weakly conserved among four DltDs (39), whereas that from *B. subtilis* was proposed to have a noncleavable signal peptide functioning as an anchor (38). As shown in the hydropathy profile (Fig. 2B), DltD from *L. rhamnosus* also has a single N-terminal hydrophobic domain. However, based on the absence of a conserved cleavage site in all DltDs (eight homologues) as predicted by the Signal P algorithm (34), we proposed that this protein might not contain an N-terminal cleavable signal peptide and thus may contain a transmembrane anchor domain. To establish whether this domain is cleaved, it was necessary to express DltD with and without the domain.

Expression of *dltD* and Δ*dltD* in *E. coli*. To express DltD and ΔDltD (minus the 48 N-terminal residues), the corresponding DNA fragments were subcloned into the pET-3d expression vector; the plasmids pDltD and pΔDltD (Fig. 1C) were used to transform *E. coli* strain BL21(DE3) pLysS. After induction of the resulting strains with IPTG, the insoluble and membrane proteins were analyzed (Fig. 3). Both DltD and ΔDltD accumulated in the inclusion bodies. However, DltD but not ΔDltD accumulated in the membrane fraction. Thus, we have demonstrated that the N-terminal hydrophobic domain of DltD is not cleaved in *E. coli* and may be required for anchoring the protein in the membrane. While the growth of *E. coli* stopped after induction of *dltD*, this toxic effect was not observed in the expression of ΔDltD. ΔDltD was purified (99% purity by SDS-PAGE) from the inclusion bodies by treatment with 8 M urea, dialysis, and subsequent gel filtration on Sephacryl S-200 in 10 mM NaOH. All attempts to maintain the protein in solution in concentrations greater than 5 μg/ml (pH 6 to 8), even in the presence of the detergents Triton X-100 and CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, were unsuccessful. Thus, many of the experiments reported in this paper utilized ΔDltD (15 mg/ml) stored in 10 mM NaOH at −80°C.

Insertional inactivation of *dltD*. To define the role of DltD, a mutant strain was constructed by insertional mutagenesis. Because *L. rhamnosus* 7469 cannot be transformed, *L. casei* 102S (Table 1) was chosen for the mutagenesis experiment. Using a suicide plasmid with Δ*dltD* interrupted by the erythromycin resistance marker (pΔdltD/*erm* [Fig. 1D]), we obtained an Em^r mutant with inactivated *dltD*. It was confirmed by PCR that *erm* was integrated into *dltD* by allelic replacement. The doubling time of the mutant (1.31 h) was similar to that of the parent (1.21 h).

To establish that *dltD* in the mutant is defective, cells were permeabilized and assayed for D-[^{14}C]alanine incorporation into LTA. As shown in Table 2, the *L. casei* 102S *dltD*::*erm* mutant is defective for D-alanylation. Thus, *dltD* is required for the synthesis of D-alanyl–LTA in permeabilized cells. However, when purified membranes from the *dltD* mutant were incubated with recombinant Dcl (DltA) and Dcp (DltC), 50% of the activity observed for the parent membranes was detected (Table 2). This result suggested that the function of DltD could be partially circumvented in the isolated membrane system, and thus, DltD may not be the primary enzyme responsible for the D-alanylation of LTA.

The size of the mutant was increased on average to 1.6 times that of the parent. The mean length of the parent cells was 1.2 ± 0.25 μm, while the mean length of the mutant cells was 1.9 ± 0.45 μm (data not shown). No other defect could be observed by scanning electron microscopy. One of the unique phenotypes of this mutant was the higher susceptibility to the action of the cationic compounds CTAB and chlorhexidine (data not shown). Similar observations with the cationic antimicrobial peptides, e.g., defensins and protegrins, were also made with insertional mutants of *S. aureus* in the *dlt* operon (39). Therefore, the *dltD* mutant of *L. casei* 102S may have a higher polyanionic surface charge and thus bind more of the two cationic compounds.

To confirm that this mutation is in fact responsible for the loss of D-alanylation, a plasmid containing *dltA* to *-D* and its native promoter was used to complement the *dltD*::*erm* mutant (Table 2). The permeabilized mutant with this plasmid gave a 2.8-fold-higher amount of D-alanine incorporation than permeabilized *L. casei* 102S, whereas membranes prepared from this complemented mutant (*dltD*::*erm*/*dltD*$^+$) gave approximately the same activity as the parent membranes (*dltD*$^+$) (Table 2).

Binding properties of DltD. Given the basic pI of DltD (9.5), we proposed that this protein might bind Dcl (pI 5.2) and Dcp (pI 3.8). Evidence for this proposal was observed in studies of the ligation reaction catalyzed by Dcl (Fig. 4). The addition of ΔDltD to the reaction mixture containing Dcl and Dcp re-

A.

```
                    49                                                  94
L. rhamnosus        sanvlkgeti kneamaen.. .yvPfiGSSE lsrmDafhPs vlaqkyhr.d
S. pyogenes         tdssfkngli krqalsdetc rfvPffGSSE wsrmDsmhPs vlaerykr.s
S. mutans           spssfkstnk kmralsdkrh lfvPffGSSE wqriDsmhPs vlaerynr.s
L. lactis           ntptftgysi kkqaysdpe. .flPvlGSSE mehvDsfhPs ayfskyns.g
S. xylosus          tdqvlkgtlv qdkmyqsda. .yyPiyGSSE lekdDpfnPa illnnrnqvs
S. aureus           tdqvlkgtli qdklyesnk. .yyPiyGSSE lgkdDpfnPa ialnkhn.an
L. monocytogenes    nesviqgldi qkkalqegn. .ylPiyGSSE lsrvDpfhPs vvskkynq.g
B. subtilis         npsmfqglyl qdqmlkdpt. .ylPiyGSSE lsrlDefhPs nyfqvnne.g
            Consensus -+--------- ---------- ---P--GSSE ----D---P- ----------

                    95                                                 143
L. rhamnosus        yrPfLmGmaG sqsLthflsi nalth.vegK KavmvlSPQW FvpgGvrkaq
S. pyogenes         yrPyLlGqkG stsLshyfgm qqignqiknK KavyviSPQW FvpkGtspia
S. mutans           yrPfLiGkrG sasLshyygi qqitnemqkK KaifvvSPQW FtaqGinpsa
L. lactis           fiPfLvGqpG tttLthffym nsvadelknr KivfviSPQW FskqGivese
S. xylosus          kkPfLiGtgG stdLvnavel gsqygnlkgK KmafivSPQW FtknGltqdn
S. aureus           kkafLlGagG stdLinavel asqydklkgK KltfiiSPQW FtnhGltnqn
L. monocytogenes    ytPfLlGrpG tqsLshfldv nalgddlkgK KvavvlSPQW FqpkGvsdps
B. subtilis         ftPyLvGkgG sqsLihslnf aahmdqlkgK KivfivSPQW FikrGsdeqh
            Consensus --P-L-G--G ---L------ ---------K K-----SPQW F---G-----
```

B.

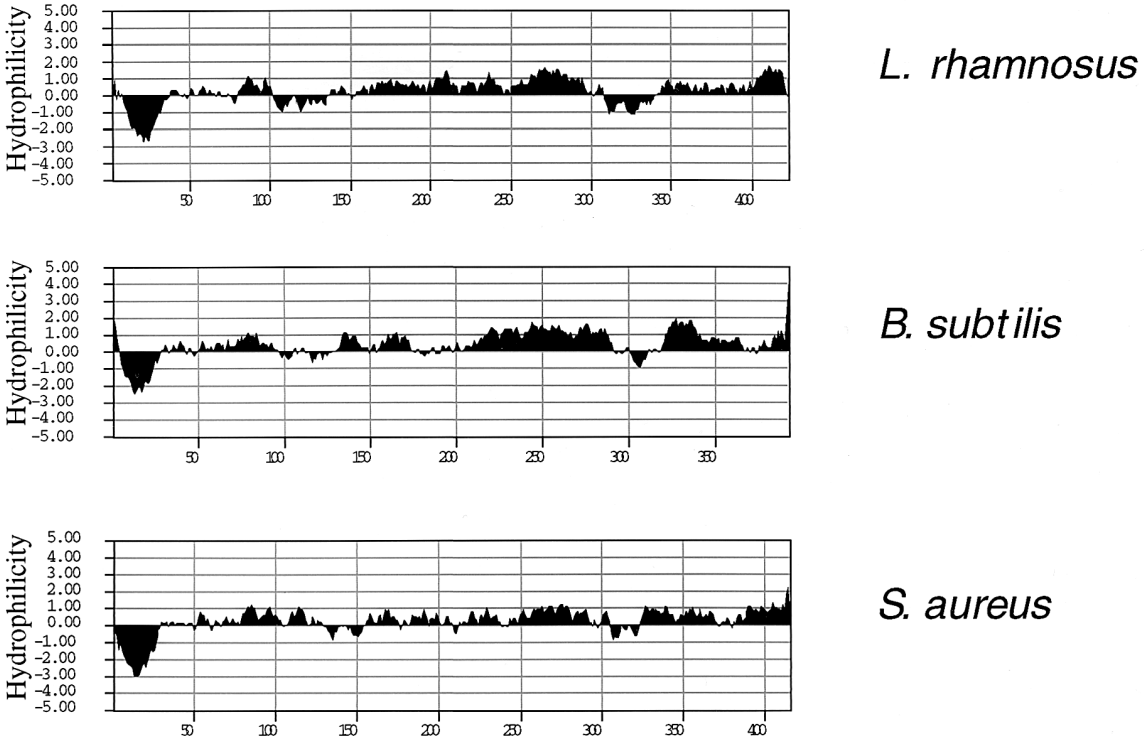

FIG. 2. Consensus sequences of DltD (A) and hydropathy plots of DltDs from *L. rhamnosus*, *B. subtilis*, and *S. aureus* (B). The multiple alignment was performed with the PILEUP program, and the consensus sequences were determined by the PRETTY program. In this alignment, the sequence of *L. rhamnosus* DltD (aa 49 to 144) was compared with those of seven other gram-positive bacteria: *Streptococcus pyogenes*, contig 320 (B. A. Roe, http://dna1.chem.uokhnor.edu/strep.html); *S. mutans*, GenBank accession no. AF049357 (44) and AF051356 (Boyd et al., unpublished); *S. aureus*, accession no. AF101234 (39); *S. xylosus*, accession no. AF032440 (39); *L. lactis*, accession no. U81621 (12); *Listeria monocytogenes*, accession no. AJ012255 (P. Trieu-Cuot, E. Abachin, C. Poyart, and P. Berche, unpublished data); and *B. subtilis*, accession no. X73124 (15). (B) Hydropathy plots performed by the method of Kyte and Doolittle (25).

sulted in a twofold increase in the initial rate of ligation of Dcp with D-alanine. However, when ACP (pI 3.9) from *E. coli* was substituted for Dcp, no increase in the rate of ligation was observed in the presence of ΔDltD (Fig. 4). It was concluded that ΔDltD stimulates the ligation of Dcp with D-alanine but not that with ACP. Because ΔDltD is sparingly soluble in pH 6 to -8 buffers, it was impossible to increase the concentration of the protein in these reaction mixtures. To circumvent this

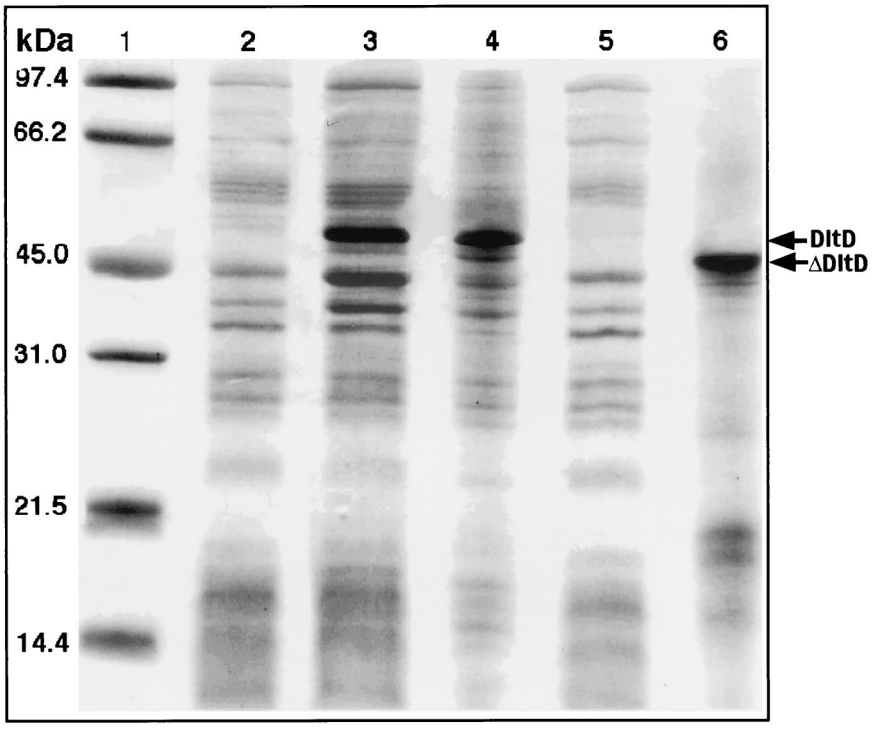

FIG. 3. SDS-PAGE analysis of DltD and ΔDltD expression in *E. coli*. Lane 1, molecular mass markers; lane 2, purified membranes without expression; lane 3, purified membranes after expression of DltD (pDltD); lane 4, DltD from inclusion bodies dissolved in 8 M urea; lane 5, purified membranes after expression of Δ*DltD* (pΔDltD); lane 6, ΔDltD from inclusion bodies dissolved in 8 M urea.

problem, a binding assay was designed using ΔDltD bound to NC membranes. When increasing amounts of ΔDltD were bound to these membranes and incubated with either D-[^{14}C] alanyl–Dcp or D-[^{14}C]alanyl–ACP, only D-[^{14}C]alanyl–Dcp was bound to ΔDltD (Fig. 5A). Thus, it was concluded that ΔDltD can selectively bind D-alanyl–Dcp.

It may be argued that ACP from *E. coli* is not a good reference protein for these experiments. Although ACP from *L. rhamnosus* has not been isolated, the corresponding protein has been isolated from *B. subtilis* (31). This ACP strongly resembles (sequence 61% identical) *E. coli* ACP. When *B. subtilis* ACP replaced *E. coli* ACP in our experiments, the observations were identical to those with ACP from *E. coli* (data not shown).

The ability of ΔDltD to bind Dcp and ligate D-alanine in the presence of Dcl was also tested. As shown in Fig. 5B, the addition of either Dcp or ACP to increasing amounts of ΔDltD, followed by washing with 0.1 M NaCl in phosphate buffer before incubation of the membrane in the presence of Dcl,

TABLE 2. Incorporation of D-[^{14}C]alanine into permeabilized cells and membranes from *L. casei* 102S (*dltD*$^+$) and its *dltD*::*erm* mutant

Genotype[a]	Incorporation of D-[^{14}C]alanine (cpm)[b]	
	Permeabilized cells	Membranes
dltD$^+$	960 ± 20	1,660 ± 20
dltD$^+$ (heat treated)	15 ± 4	40 ± 6
dltD::*erm*	55 ± 6	890 ± 20
dltD$^+$ + (pNZ123/*dlt*)	2,540 ± 20	1,790 ± 20
dltD::*erm* + (pNZ123/*dlt*)	2,680 ± 20	1,840 ± 20

[a] *dltD*$^+$ is the genotype of *L. casei* 102S (wild type).

[b] Cells (260 μg [wet weight]) were permeabilized with 0.6% toluene and incubated with ATP and D-[^{14}C]alanine according to the D-alanine incorporation assay described in Materials and Methods. Membranes (120 μg of protein) were incubated with ATP, D-[^{14}C]alanine, and recombinant Dcl and Dcp from *L. rhamnosus* according to the D-alanine incorporation assay.

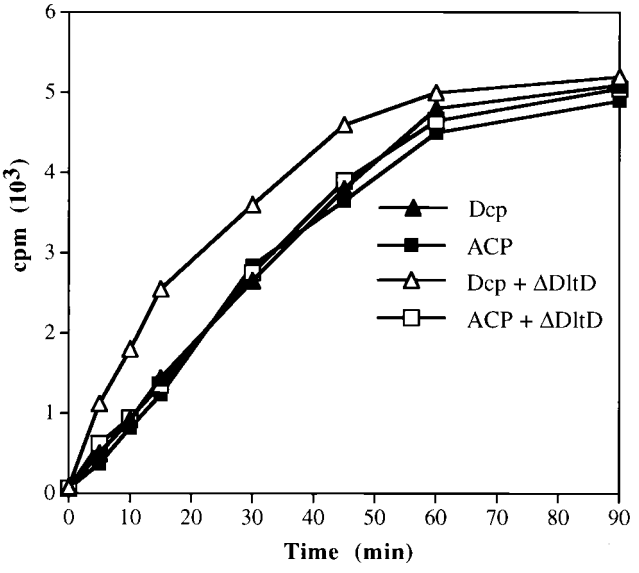

FIG. 4. Time courses of D-alanyl–Dcp and D-alanyl–ACP formation in the presence and absence of ΔDltD. The 400-μl reaction mixtures contained 5 nmol of the carrier protein, 12 U of Dcl, 10 mM $MgCl_2$, 10 mM ATP, 0.11 mM D-[^{14}C] alanine (46 mCi/mmol), 5 mM DTT, and 30 mM bis-Tris (pH 6.5). For the open symbols, 2 μg of ΔDltD was added to the mixtures. The amounts of D-alanyl–Dcp and D-alanyl–ACP were measured as described in Materials and Methods.

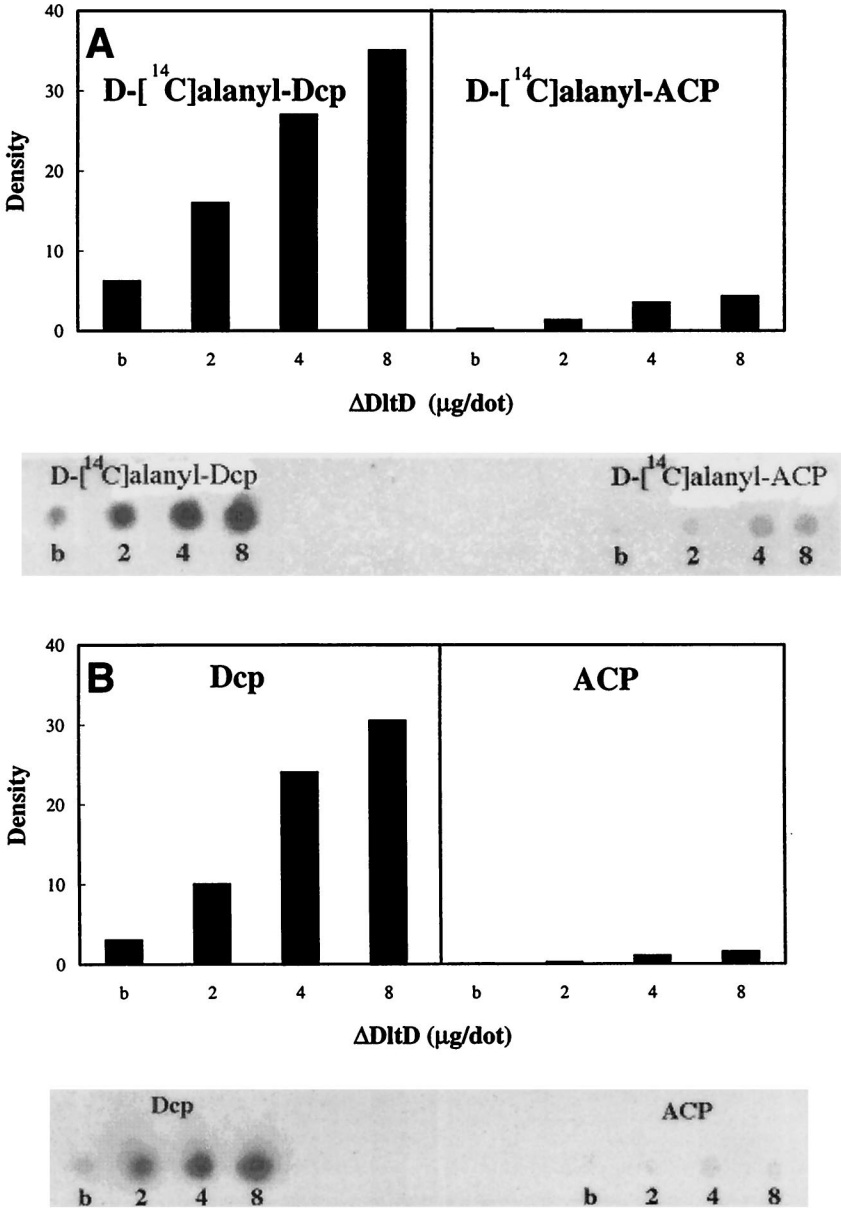

FIG. 5. Binding of D-[^{14}C]alanyl–Dcp and D-[^{14}C]alanyl–ACP (A) and ligation of D-[^{14}C]alanine to Dcp and ACP (B) in the presence of ΔDltD on NC membranes. (A) Either D-[^{14}C]alanyl–Dcp or D-[^{14}C]alanyl–ACP was incubated with the NC membrane containing increasing amounts of ΔDltD, and the amount bound was quantified as described in Materials and Methods. (B) Either Dcp or ACP was incubated with the membrane containing ΔDltD, and after washing, the filter was incubated in the presence of Dcl, ATP, and D-[^{14}C]alanine as described in Materials and Methods. b, 8 μg of ΔDltD previously treated at 100°C for 2 min.

ATP, and D-[^{14}C]alanine, showed that only in the case of Dcp is D-[^{14}C]alanine ligated to the carrier protein. These results suggest that in the presence of ΔDltD, the ligation of D-alanine catalyzed by Dcl is specific for Dcp.

Thioesterase activity of DltD. If DltD functioned as a thioesterase specific for D-alanyl–ACP, the results of binding specificity could be interpreted in terms of the hydrolytic release of the radiolabeled D-alanine from D-[^{14}C]alanyl–ACP. It was previously observed that the formation of D-alanyl–ACP was inhibited while the formation of D-alanyl–Dcp was not affected when membranes were added to the D-alanine incorporation assay (19). To test whether this inhibition is correlated with the presence of DltD, purified membranes (DltD$^+$, DltD$^-$, and DltD$^-$/DltD$^+$) were incubated with either Dcp or ACP in the presence of Dcl, ATP, and radiolabeled D-alanine. The formation of D-alanyl–ACP is inhibited only when parent membranes (DltD$^+$) or complemented membranes (DltD$^-$/DltD$^+$) are used (Fig. 6). The results with ACP are distinct from those observed with Dcp. In the case of D-[^{14}C]alanyl–Dcp, no effect on its formation is observed in those reaction mixtures containing either DltD$^+$, DltD$^-$, or DltD$^-$/DltD$^+$ membranes (Fig. 6). The inhibition of D-alanyl–ACP formation exerted by the addition of membranes showed either that DltD prevents ligation of D-alanine to ACP or that DltD has thioesterase activity for D-alanyl–ACP.

Thioesterase activity associated with DltD was directly tested by incubating D-[^{14}C]alanyl–ACP with purified membranes (DltD$^+$, DltD$^-$, or DltD$^-$/DltD$^+$). As shown in Fig. 7, D-

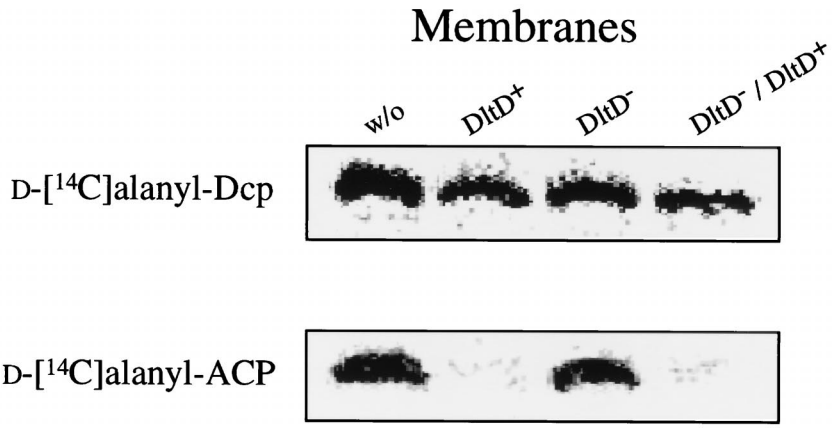

FIG. 6. Formation of D-alanyl–Dcp and D-alanyl–ACP in the presence of $DltD^+$, $DltD^-$, and $DltD^-/DltD^+$ membranes. Nondenaturing PAGE (15%) was used to monitor the amount of either D-[^{14}C]alanyl–Dcp or D-[^{14}C]alanyl–ACP according to the method of Heaton and Neuhaus (19). The reactions were performed in 250-μl mixtures containing 8 U of Dcl, 5 nmol of either Dcp or ACP, 5 mM $MgCl_2$, 5 mM DTT, 30 mM bis-Tris buffer (pH 6.5), 10 mM ATP, and 110 μM D[^{14}C]alanine (46 mCi/mmol) in the absence of membranes (w/o) and in the presence of membranes (130 μg) from *L. casei* 102S (*dltD*$^+$), *L. casei* 102S (*dltD*::*erm*), and the *dltD* mutant which had been complemented with pNZ123/*dlt* (*dltD*::*erm*/*dltD*$^+$). All reaction mixtures were incubated for 1 h at 37°C.

alanyl–ACP was hydrolyzed by membranes containing $DltD^+$ or membranes from the complemented mutant ($DltD^-/DltD^+$). In contrast, membranes from the *dltD*::*erm* strain did not effect the hydrolysis of this thioester. These results suggest that DltD has thioesterase activity with specificity for the mischarged carrier protein, D-alanyl–ACP.

DISCUSSION

The *dlt* operon encodes four proteins which are required for the D-alanylation of LTA. The first gene encodes Dcl, which shares homology with over 200 nonribosomal peptide synthetases in a wide variety of organisms (18). These synthetases are characterized by several conserved signature sequences, the most common of which is the adenylation domain, GXXGXPK (23, 24). The second gene (*dltB*) encodes a putative transporter that has sequence similarity to a variety of proton antiporters involved in the efflux of tetracycline, quaternary ammonium compounds, and glycerol phosphate (33). One DltB homologue, AlgI, is a transporter involved in the acetylation of alginate in *Pseudomonas aeruginosa* (14). The 8.8-kDa protein (Dcp) encoded by the third gene is homologous to the ACPs involved in fatty acid and polyketide biosyntheses. The fourth gene, *dltD*, encodes a membrane protein which has no known homologue. The fact that *dltD* is translationally coupled with *dltC* in all reported organisms (eight) indicates a close functional or structural relationship between the two proteins (33). We hypothesized that one of the DltD functions is to ensure the ligation of D-alanine to the appropriate carrier protein, Dcp.

The present results suggest that DltD may be the protein responsible for discriminating between Dcp involved in the D-alanylation of LTA and ACP involved in fatty acid biosynthesis. This function may prevent gram-positive organisms from accumulating a pool of D-alanyl–ACP. In the absence of membranes containing DltD, Dcl ligates D-alanine to either Dcp or ACP. As shown previously (33), only Dcp participates in the incorporation of D-alanine into LTA. As in the case of other nonribosomal synthetic systems, it is essential that the D-alanylation machinery be able to purge the system of mischarged carrier proteins or to selectively bind the appropriate carrier protein for ligation with D-alanine.

To test the hypothesis that DltD binds D-alanyl–Dcp specifically, a binding assay was designed which allowed us to manipulate the concentration of ΔDltD and assess the binding of the carrier protein to DltD on an NC membrane. Only D-alanyl–Dcp bound to ΔDltD under these conditions. Thus, even though the pIs of Dcp and ACP are both acidic, only Dcp was ligated with D-alanine in the presence of ΔDltD bound to this membrane. Additional experiments indicated that DltD also possesses thioesterase activity for D-alanyl–ACP. Thus, not only does ACP not appear to be ligated with D-alanine in the presence of DltD on the membrane but DltD hydrolytically cleaves D-alanyl–ACP if it becomes ligated with D-alanine.

In most of the nonribosomal peptide synthetase modules, a thioesterase domain is encoded downstream of the synthetase

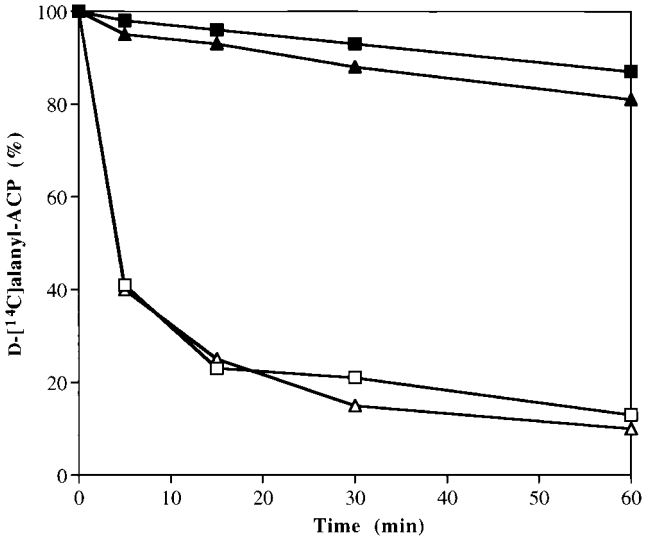

FIG. 7. Time courses of D-[^{14}C]alanyl–ACP hydrolysis in the absence of membranes (■) and in the presence of $DltD^+$(□), $DltD^-$(▲), and $DltD^-/DltD^+$(△) membranes. The 250-μl reaction mixture contained 6.5 nmol of D-[^{14}C]alanyl–ACP and 130 μg of membranes (except for the control) in 10 mM bis-Tris and 30 mM $MgCl_2$. Samples (50 μl) were removed from the incubation mixture (37°C) at the indicated times, and the amounts of D-[^{14}C]alanyl–ACP remaining were measured by precipitation with 10% trichloroacetic acid according to the method of Heaton and Neuhaus (19).

genes (43). It has been speculated that these domains encode a thioesterase which functions to edit the product or purge synthetases of aberrant materials that might otherwise block the synthesis of normal products (6). Alternatively, in polyketide synthesis, thioesterases may be responsible for the release and concomitant cyclization of the processed chain (16). While the homology search described in this paper produced only a distant similarity with thioesterases, the correlation of the insertional inactivation of *dltD* with the defective hydrolytic cleavage of D-alanyl–ACP strongly supports the conclusion that DltD catalyzes thioesterase activity. However, it is not clear whether the hydrolytic cleavage of mischarged ACPs is the primary or only function of the protein. If this were the only function of the protein, one might expect permeabilized cells of the *dltD* mutant to show activity for D-alanine incorporation into LTA. Since this is not the case, it is reasonable to suggest that the enzyme functions both as a hydrolase of mischarged ACP and as a facilitator of D-alanine ligation to Dcp.

It is possible that DltD is multifunctional and thus also facilitates or enhances the D-alanylation of LTA. If DltD plays a role in the direct D-alanylation of LTA, it will be necessary to establish whether the protein is on the inner or outer leaflet of the cytoplasmic membrane. It appears logical for the protein to be on the inner leaflet if it is to exert its selectivity for ligation of D-alanine to the carrier protein. However, if DltD is to enhance the D-alanylation of the LTA, one might speculate that the protein is located on the outer leaflet, the proposed site of D-alanylation. Thus, one of the goals of future experiments is to establish the topology of DltD. The present work only supports the conclusion that DltD is associated with the membrane and that the N-terminal sequence functions as a membrane-spanning domain for anchoring the protein.

One of the long-term goals of this research has been to determine the role of the membrane in the D-alanine incorporation system (29, 32, 40). Several suggested roles include (i) establishing a specific conformation of the LTA, (ii) anchoring an enzyme or protein that is required for D-alanine incorporation, and (iii) facilitating the formation of a specific LTA complex with other membrane constituents. The membrane protein, DltD, may function in one of these roles and thus is required for the incorporation of D-alanine into the LTA of permeabilized cells.

The importance of the *dlt* operon in the physiology of the gram-positive organism is illustrated by the variety of observed phenotypes. For example, in *Streptococcus gordonii* DL1 (Challis), insertional inactivation of *dltA* results in a loss of intrageneric coaggregation and in the formation of a variety of pleomorphs (9). In the case of *S. mutans*, Spatafora et al. (44) observed that a knockout mutation of the promoter of the *dlt* operon resulted in the defective synthesis of intracellular polysaccharides. On the other hand, insertional inactivation of *dltC* in this organism resulted in a loss of acid tolerance (Boyd et al., unpublished). In *B. subtilis*, deletion of either *dltA*, *-B*, *-C*, or *-D* resulted in mutants with enhanced autolytic activity (48). In *L. lactis* (12), it was found that mutants defective for DltD synthesis have enhanced UV sensitivity. As described in this paper, insertional inactivation of this gene in *L. casei* 102S results in an increase in cellular length and enhanced antimicrobial activity of CTAB and chlorhexidine. These phenotypes may be correlated to the decrease in D-alanylation of LTA, a result also observed in *S. aureus*, which leads to the enhanced sensitivity of these organisms to cationic antibiotics (39). It is apparent from these widely different phenotypes that the D-alanyl esters of LTA play a pleiotropic role in determining the properties of the cell surface.

ACKNOWLEDGMENTS

Dmitri V. Debabov and Michael Y. Kiriukhin made equal contributions to this paper.

The research was supported in part by Public Health Service grant RO1 GM51623 from the National Institute for General Medical Sciences.

We thank Bruce Chassy (University of Illinois) for the strain *L. casei* 102S and discussions of transformation in lactobacilli. We thank H. Ricardo Morbidoni and John E. Cronan, Jr. (University of Illinois) for ACP from *B. subtilis*. We are grateful to Ralph H. Lambalot, Roger S. Flugel, and Christopher T. Walsh (Harvard University) for the holo-ACP synthase and *E. coli* ACP. We are indebted to Sir James Baddiley and Werner Fischer (Universitat Erlangen) for discussions of these results. We also thank Eugene W. Minner for his generous help in the Electron Microscopy Facility of the Department of Neurobiology and Physiology (Northwestern University). We thank Andrei Gabrielian (Celera Genomics) for help in the protein sequence analyses. We are especially grateful to Michael P. Heaton (U.S. Meat Animal Research Center) for a critical reading of the manuscript and many discussions.

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