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Role of the D-Alanyl Carrier Protein in the Biosynthesis of D-Alanyl-Lipoteichoic Acid

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D-Alanyl-lipoteichoic acid (D-alanyl-LTA) is a widespread macroamphiphile which plays a vital role in the growth and development of gram-positive organisms. The biosynthesis of this polymer requires the enzymic activation of D-alanine for its transfer to the membrane-associated LTA (mLTA). A small, heat-stable, and acidic protein that is required for this transfer was purified to greater than 98% homogeneity from *Lactobacillus casei* ATCC 7469. This protein, previously named the D-alanine–membrane acceptor ligase (V. M. Reusch, Jr., and F. C. Neuhaus, J. Biol. Chem. 246:6136–6143, 1971), functions as the D-alanyl carrier protein (Dcp). The amino acid composition, β-alanine content, and N-terminal sequence of this protein are similar to those of the acyl carrier proteins (ACPs) of fatty acid biosynthesis. The isolation of Dcp and its derivative, D-alanyl~Dcp, has allowed the characterization of two novel reactions in the pathway for D-alanyl-mLTA biosynthesis: (i) the ligation of Dcp with D-alanine and (ii) the transfer of D-alanine from D-alanyl~Dcp to a membrane acceptor. It has not been established whether the membrane acceptor is mLTA or another intermediate in the pathway for D-alanyl-mLTA biosynthesis. Since the D-alanine-activating enzyme (EC 6.1.1.13) catalyzes the ligation reaction, this enzyme functions as the D-alanine–Dcp ligase (Dcl). Dcl also ligated the ACPs from *Escherichia coli*, *Vibrio harveyi*, and *Saccharopolyspora erythraea* with D-alanine. In contrast to the relaxed specificity of Dcl in the ligation reaction, the transfer of D-alanine to the membrane acceptor was highly specific for Dcp and did not occur with other ACPs. This transfer was observed by using only D-[^{14}C]alanyl~Dcp and purified *L. casei* membranes. Thus, D-alanyl~Dcp is an essential intermediate in the transfer of D-alanine from Dcl to the membrane acceptor. The formation of D-alanine esters of mLTA provides a mechanism for modulating the net anionic charge in the cell wall.

The cell wall of gram-positive bacteria is composed primarily of two classes of polymers, peptidoglycan (murein) and teichoic acids (TA). Although the essential role of peptidoglycan in maintaining the structural integrity of the cell has been well documented, the functions of TA are less apparent. The TA family consists of chemically diverse anionic polymers consisting of poly(alditol phosphate)s and constitutes 30 to 70% of the cell wall (2, 3). Various substituents, e.g., D-alanine esters and glucosyl residues, are linked to the alcohol functions on the polymer backbone. Many gram-positive organisms contain two types of TA: (i) wall TA (WTA), which is covalently linked to peptidoglycan (45), and (ii) lipo-TA (LTA), which is anchored in the cytoplasmic membrane (17, 18). The widespread occurrence of TA and functionally similar anionic wall polymers has prompted the suggestion that they play an essential role in the physiology of the organism (15, 28, 32, 43).

In order to clarify the functions of LTA which are determined specifically by the addition of D-alanine esters, we have chosen *Lactobacillus casei* as the model for study (26). Not only does this organism lack WTA, but the LTA represents the simplest possible hydrophilic moiety: poly(GroP) with only D-alanine ester substituents (4, 10, 29). This hydrophilic chain is linked to either Glc(β1-6)Gal(α1-2)acyl→6Glc(α1-3)acyl$_2$ Gro (30%) or Glc(β1-6)Gal(α1-2)Glc(α1-3)acyl$_2$Gro (70%) (18, 33). In *L. casei*, this macroamphiphile is polydisperse, with chain lengths ranging from 5 to 50 residues (38). Mutants that are deficient in D-alanine ester content are characterized by aberrant morphology and defective cell separation (35).

The esterification of LTA with D-alanine residues provides a means for altering the anionic charge of the cell wall. The percentage of GroP units which are substituted with these residues varies from 10 to 70% (18). A unique feature of these positively charged esters is their ability to undergo interchain transacylation (9). Haas et al. (23) demonstrated the in vivo transacylation of D-alanine esters from LTA to WTA in *Staphylococcus aureus*. Thus, the mobility of the D-alanine esters appears to be an important aspect of their proposed functions. These include: (i) the regulation of autolytic activity (6, 19), (ii) the alteration of electromechanical properties of the cell wall (36), and (iii) the binding of Mg^{2+} for enzyme function (1, 27, 28, 30). None of these functions are mutually exclusive, and each may be important for a given organism, depending on the physiological circumstances. Establishing the biological role of these esters will be facilitated by defining the mechanism for D-alanine ester addition to LTA as well as constructing genetic systems for modulating the ester content of this polymer in vivo.

A first step towards accomplishing this goal is the description of the genetic elements and biochemical components that are required for D-alanyl-LTA biosynthesis. The components include the 56-kDa activating enzyme, a 6-kDa heat-stable protein, membranes, ATP, and D-alanine (5, 7, 8, 25, 31, 34, 40). The enzyme which activates D-alanine is a member of a family of proteins that activates amino or fatty acids via a mechanism that utilizes the novel phosphate-binding loop, GXXGXPK, and is predicted to ligate the activated D-alanine to the 4′-phosphopantetheine prosthetic group of either (i) coenzyme A, (ii) a carrier protein, or (iii) an enzyme (25). In

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this article, we report that the heat-stable protein required for D-alanyl-LTA biosynthesis is the D-alanyl carrier protein (Dcp) and that it functions as the acceptor of activated D-alanine. It is our goal to establish that D-alanyl~Dcp is required for the biosynthesis of D-alanyl–membrane-associated LTA (D-alanyl-mLTA) and to show that the enzyme which activates D-alanine also functions as the D-alanine–Dcp ligase (Dcl).

MATERIALS AND METHODS

Bacterial strains and plasmids. Two strains were used: *L. casei* 7469 from the American Type Culture Collection and *Escherichia coli* XL1-Blue (Stratagene). Phagemid pDAE1, containing the gene for the D-alanine-activating enzyme (Dcl), has been described elsewhere (25).

Chemicals. D-[1-^{14}C]alanine (46 mCi/mmol in 2% ethanol) and [1-^{14}C]palmitic acid (17 mCi/mmol) used for the in vitro assays of carrier proteins were purchased from ICN Biomedicals, Inc., and Sigma Chemical Co., respectively. Isopropyl-thiol-β-D-galactoside (IPTG) was obtained from United States Biochemical Corp. Ammonium sulfate, Tris, bis-Tris, dithiothreitol (DTT), disodium ATP, EDTA, 3-cyclohexylamino-1-propanesulfonic acid (CAPS), and DEAE-cellulose were obtained from Sigma Chemical Co. Trichloroacetic acid (TCA) was purchased from Fisher Scientific. Media supplies for small-scale cultures (less than 30 liters) were obtained from Difco Laboratories. For the large-scale fermentation of *L. casei*, yeast extract from Marcor Development Corp. and NZ amine A from Quest International were used. The bicinchoninic acid protein reagent was purchased from Pierce Chemical Co. Metricel filter membranes (GN-6) and Econo-Safe scintillation cocktail were purchased from Gelman Sciences and Research Products International Corp., respectively. Polyvinylidene difluoride transfer membranes were obtained from Millipore Corp.

Large-scale fermentation of *L. casei*. Starter cultures (500 ml each) of *L. casei* were grown to log phase in LC medium (25) at 37°C without shaking. These cultures were used to inoculate two 13-liter fermentation vessels (Microferm; New Brunswick Scientific Co.) containing the same medium and cultured without aeration at 37°C to late log phase. These cultures were combined and used to inoculate 250 liters of the same medium in a 300-liter fermentor (Braun Biostat 300 D). This culture was grown for 9 h to late log phase (optical density at 600 nm, 5.2), cooled to 12°C, and harvested by using a tangential flow membrane system (Millipore Corp.). After final centrifugation (10,000 × *g*, 10 min), cell pellets were combined in 500-g portions and frozen at −20°C. The average yield of wet cell paste was 6 g per liter.

Purification of Dcp from *L. casei*. The purification strategy for Dcp was patterned after that reported by Linzer and Neuhaus (31) for the D-alanine–membrane acceptor ligase and that reported by Rock and Cronan (41) for *E. coli* acyl carrier protein (ACP). Unless specified, all manipulations were performed at 4°C, and centrifugation steps were performed for 15 min at 10,000 × *g*. The frozen cell paste (500 g) was thawed in 1 liter of 100 mM Tris (pH 8) containing 1 mM EDTA and disrupted by 12 cycles of sonication (10 min with stirring followed by 40 min of cooling on ice). The extract was clarified by centrifugation, the pellet was suspended in 1 liter of the same buffer, and the suspension was sonicated for another cycle. One volume of isopropanol was slowly added to the combined extracts with stirring, and the suspension was allowed to stand several hours at −20°C. The supernatant fraction was collected by centrifugation and the volume was doubled by the slow addition of acetone. This mixture was allowed to stand at −20°C for several hours. The precipitate was collected by centrifugation, air dried at 23°C, and dissolved in a minimum amount of 10 mM bis-Tris (pH 6.5). This solution was brought to 65% saturation by the slow addition of pulverized ammonium sulfate, and the suspension was stirred on ice for 1 h. The soluble fraction was collected after a 30-min centrifugation, the pH was adjusted to 3.9 by the addition of glacial acetic acid, and the suspension was allowed to stand overnight. The pellet was collected by centrifugation for 1 h, suspended in 15 ml of 30 mM bis-Tris (pH 6.5), and neutralized by the slow addition of 1 M NaOH with constant stirring. The solution was clarified by centrifugation, heated to 75°C for 10 min, and centrifuged. This solution was dialyzed (Spectra/Por 2, 12.5-kDa molecular mass cutoff; Spectrum Corp.) against two changes of 30 mM bis-Tris (pH 6.5) containing 100 mM NaCl. The dialyzed fraction was applied to a column (2.5 by 15 cm) of DEAE-cellulose equilibrated with the dialysis buffer. The column was developed with a linear gradient of the dialysis buffer and 30 mM bis-Tris (pH 6.5) containing 350 mM NaCl. Active fractions were pooled, concentrated to 5 ml via ultrafiltration, and precipitated by the addition of 5 volumes of acetone (−20°C, 60 min). After centrifugation, this sample was dried under vacuum and dissolved in a minimum amount of 30 mM bis-Tris (pH 6.5). This fraction was subjected to further purification by preparative, nondenaturing, nonreducing polyacrylamide gel electrophoresis (native PAGE) after being diluted with 6 volumes of loading buffer (85 mM Tris [pH 6.8], 10% glycerol, 10 μM bromophenol blue). A model 491 Prep Cell (Bio-Rad) was used with a 4% polyacrylamide stacking gel and a 15% polyacrylamide resolving gel according to the conditions described by the manufacturer. Fractions were concentrated by using Diaflo YM10 ultrafiltration membranes (Amicon Corp.) and precipitated with 5 volumes of acetone. The pellets were dried under vacuum, dissolved in 30 mM bis-Tris (pH 6.5), and stored at −80°C.

Preparation of recombinant Dcl from *E. coli*. Dcl was purified from a culture (10 liters) of *E. coli* XL1-Blue containing phagemid pDAE1. This strain was grown in Luria-Bertani medium at 37°C (optical density at 600 nm, 0.7), induced with 0.4 mM IPTG, and cultured for 2 h. Cells were harvested, washed in cold 100 mM Tris-HCl (pH 8) containing 1 mM EDTA, suspended in 5 volumes of the same buffer, and disrupted by sonication. The extract was clarified by centrifugation (10,000 × *g*, 10 min), and the supernatant fraction was brought to 80% saturation with ammonium sulfate. This suspension was stirred on ice for 2 h, and the precipitate was collected by centrifugation (15,000 × *g*, 30 min) and dissolved in 100 mM potassium phosphate buffer (pH 7.0). This fraction was dialyzed against three changes of the same buffer and stored at −80°C. The D-alanine activating capacity of Dcl was determined by the method of Heaton and Neuhaus (25). A unit of Dcl is defined as 1 μmol of D-alanine hydroxamate formed per h. This preparation (specific activity, 13.5 U/mg) was stable for at least 12 months when stored at −80°C. The protein concentration was determined by using the bicinchoninic acid protein assay with bovine serum albumin as the standard.

Purification of *L. casei* membrane fragments. The mLTA used as the acceptor for measuring the incorporation of D-alanine was isolated in the form of purified membranes from *L. casei*. The extracts of late-log-phase cultures were prepared as described for the isolation of Dcl (25). Membranes were prepared according to the method of Reusch and Neuhaus (40). Low-speed centrifugation was used to remove glass beads after cell disruption, and the supernatant fraction was clarified by four cycles of centrifugation at 10,000 × *g* for 15 min. The

TABLE 1. Purification of Dcp from *L. casei*[a]

Fraction	Vol (ml)	Protein (mg)	Dcp		Purification (fold)	Recovery (%)
			nmol	nmol/mg		
Extract	2,160	31,600	798	0.025	1	100
Isopropanol-acetone	201	1,320	424	0.321	13	53
Ammonium sulfate (pH 3.9)	20.1	302	366	1.21	48	46
DEAE-cellulose[b]	0.96	20.6	271	13.2	523	34
Preparative PAGE[c]	0.32	0.99	63	64	2,540	7.9

[a] Frozen cell paste (500 g).
[b] Fig. 1, lane 1.
[c] Fig. 1, lane 3.

membrane pellet was collected by ultracentrifugation at 200,000 × *g* for 90 min and homogenized in a minimal amount of 30 mM bis-Tris (pH 6.5). Membrane fragments were washed by three cycles of centrifugation at 10,000 × *g*, collected by ultracentrifugation at 200,000 × *g*, and homogenized in 30 mM bis-Tris (pH 6.5). The washed membranes were suspended in 30 mM bis-Tris (pH 6.5) to a concentration of 20.7 mg of membrane protein per ml (bicinchoninic acid protein assay) and frozen at −80°C. This preparation of membrane fragments retained full D-alanine incorporation activity for at least 9 months when stored at −80°C.

Assay of *L. casei* Dcp and *E. coli* ACP. The concentration of Dcp was determined by the amount of D-alanine ligated after 90 min. The reaction mixture (50 μl) for the ligation assay contained 30 mM bis-Tris (pH 6.5), 10 mM ATP, 10 mM $MgCl_2$, 1 mM DTT, 1.35 U of Dcl, 0.11 mM D-[^{14}C]alanine (46 mCi/mmol), and a sample of carrier protein. For the ligation of *E. coli* ACP with D-alanine, the final $MgCl_2$ concentration was adjusted to 30 mM. The mixtures were incubated at 37°C before the reaction was terminated by the addition of 0.9 ml of 10% TCA. The precipitate was collected on GN-6 Metricel filters (25 mm; pore size, 0.45 μm) and washed with three 1-ml and one 10-ml portions of 10% TCA. The filters were dissolved in ethyl acetate and counted in a scintillation cocktail.

The amount of *E. coli* ACP (Sigma Chemical Co. lot 40H9610) was measured by ligation with palmitic acid using a modified method of Rock and Cronan (41). The reaction mixture (50 μl) contained 50 mM Tris (pH 8.0), 1 mM DTT, 10 mM $MgCl_2$, 10 mM ATP, 400 mM LiCl, 0.29 mM [1-^{14}C]palmitic acid (17 mCi/mmol), 0.76 mU of acyl-ACP synthase (Sigma Chemical Co.), 5% glycerol, 2% Triton X-100, and a sample of ACP. The mixture was incubated at 37°C, and 50-μl aliquots were deposited on dry Whatman 3MM filter disks. The disk was immediately washed with five 1-ml and one 10-ml portions of chloroform-methanol-acetic acid (3:6:1, vol/vol/vol) to remove nonligated [^{14}C]palmitic acid, and the dried filters were placed in scintillation fluid for counting. The amount of *E. coli* ACP measured in this commercial preparation was 22.2 nmol/mg of protein.

Preparation of D-[^{14}C]alanyl~Dcp. The D-alanine-labeled derivative of Dcp was prepared by ligating 8.5 nmol of Dcp (Table 1, DEAE-cellulose fraction) with D-[^{14}C]alanine (46 mCi/mmol). The reaction mixture of the ligation assay was scaled up from 50 μl to 1 ml, incubated at 37°C for 90 min, and applied to a column of DEAE-cellulose (1 by 5 cm). The column was developed as described for the purification of Dcp. The purified fractions of D-[^{14}C]alanyl~Dcp were pooled, concentrated 40-fold by using Centricon-10 filters (Amicon Corp.), and frozen at −80°C.

Analytical native PAGE and electroblotting. In addition to measuring the amount of D-alanyl~Dcp formed in the ligation assay, the purification of Dcp was monitored by native PAGE. Samples were electrophoretically separated on 15% polyacrylamide gels by using nondenaturing and nonreducing conditions as described above for the preparation of Dcp. For sequence analyses, the proteins were electroblotted onto polyvinylidene difluoride transfer membranes by using a semidry blotting apparatus (Millipore Corp.) in a CAPS-methanol buffer system (pH 11) according to the instructions of the manufacturer. The blot was rinsed in Milli-Q water and stained briefly with Coomassie blue R-250. The Dcp band was excised from the membrane and submitted for amino acid analysis and N-terminal microsequencing. These analyses were performed at the Macromolecular Structure Facility in the Department of Biochemistry at Michigan State University.

RESULTS

Isolation of the D-alanyl carrier protein: a cytosolic component required for D-alanyl-mLTA biosynthesis. In 1973, Linzer and Neuhaus (31) reported the partial purification of a small, heat-stable, and acidic protein from *L. casei* that is required for the incorporation of D-alanine into mLTA. This protein was described as the D-alanine–membrane acceptor ligase. However, sequence comparisons of the enzyme which activates D-alanine (25) suggested that an acyl carrier protein may function in the incorporation system. This suggestion was supported by a preliminary observation that the D-alanine-activating enzyme ligated D-alanine to the ACP from *E. coli*. To further test this proposal, the isolation of an ACP-like protein from *L. casei* was undertaken. The purification of this protein was monitored by three methods: (i) the ligation of D-[^{14}C]alanine to the carrier protein, (ii) the incorporation of D-[^{14}C]alanine into mLTA, and (iii) native PAGE. Since the carrier protein is ligated with D-alanine and is required for D-alanyl-mLTA biosynthesis, it has been named the D-alanyl carrier protein (Dcp). The enzyme which catalyzes this ligation is the D-alanine–Dcp ligase (Dcl).

The purification strategy for Dcp was patterned after that for the *L. casei* D-alanine–membrane acceptor ligase (31) and that for the *E. coli* ACP (41). Purification was indicated by fractionating an extract of *L. casei* with 50% (vol/vol) isopropanol. The Dcp remained soluble in this fraction until an additional volume of acetone was added. Precipitation of Dcp from a 65% solution of ammonium sulfate was accomplished by adjusting the pH to 3.9. The combination of these steps resulted in a preparation of Dcp which was 48-fold enriched relative to the extract (Table 1). Chromatography of the dialyzed ammonium sulfate fraction on DEAE-cellulose yielded a sample that was approximately 20% pure as determined by native PAGE (Fig. 1, lane 1). The final step in this purification consisted of a preparative, nonreducing, and non-

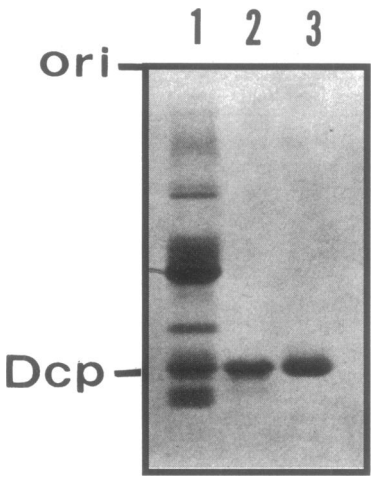

FIG. 1. Native PAGE analysis of purified *L. casei* Dcp fractions. Lane 1, DEAE-cellulose fraction; lanes 2 and 3, the two major fractions eluted from preparative native PAGE. PAGE conditions are described in Materials and Methods. The proportion of Coomassie-stained Dcp was measured by scanning laser densitometry.

TABLE 3. Amino acid compositions of Dcp from *L. casei* and ACP from *E. coli*

Amino acid	Dcp[a]	ACP[b]
Cysteic acid	0	0
β-Alanine	1	1
Aspartic	7	9
Threonine	3	6
Serine	2	3
Glutamic	9	18
Proline	1	1
Glycine	4	4
Alanine	4	7
Valine	4	7
Methionine	0	1
Isoleucine	3	7
Leucine	8	5
Tyrosine	0	1
Phenylalanine	2	2
Lysine	2	4
Histidine	0	1
Arginine	0	1

[a] Assuming 1 mol of β-alanine per mol of protein with rounding to the nearest integer value.
[b] From reference 43.

denaturing PAGE separation. This strategy yielded a fraction that was greater than 98% pure (Fig. 1, lane 3).

Requirements for the ligation of *L. casei* Dcp with D-alanine. The requirements for the formation of D-alanyl~Dcp are summarized in Table 2. For comparison, the requirements for the formation of *E. coli* D-alanyl~ACP are also presented. The synthesis of the aminoacylated carrier proteins was proportional to the amounts of carrier protein added. Both ligations required ATP and Dcl. The Mg^{2+} requirement for the formation of D-alanyl~Dcp was less stringent than that for *E. coli* D-alanyl~ACP. The concentration of Mg^{2+} which provided maximal D-alanyl~Dcp formation (10 mM) resulted in only 10% of the D-alanyl~ACP formed in 90 min. Optimal D-alanyl~ACP synthesis required 30 mM Mg^{2+}. Since D-alanyl~Dcp was formed in the absence of added Mg^{2+}, the effect of EDTA on this reaction was examined. The addition of 10 mM EDTA inhibited essentially all D-alanyl~Dcp formation. Another difference between the two carrier proteins was observed with the addition of DTT. The synthesis of D-alanyl~Dcp was stimulated 1.6-fold by the addition of 1 mM DTT, whereas this addition had no effect on the formation of D-alanyl~ACP. These results identify the requirements for the ligation of Dcp with D-alanine.

TABLE 2. Requirements for the ligation of D-alanine to carrier proteins and its transfer to mLTA via D-alanyl-Dcp

Component omitted	% Product formed[a]		
	D-Alanyl~Dcp[b,c]	D-Alanyl~ACP[b,d]	D-Alanyl-mLTA[e]
DTT	62	100	74
Mg^{2+}	54	2	72
+ 1 mM EDTA	13	NA[f]	80
+ 10 mM EDTA	2	NA	70
ATP	<1	<1	<10
Dcl	<1	<1	<10
Carrier protein	<1	<1	<10

[a] The complete systems were normalized to 100%.
[b] The ligation assay was performed as described in Materials and Methods.
[c] The reaction mixture contained 49 pmol of Dcp from *L. casei*.
[d] The reaction mixture contained 55 pmol of ACP from *E. coli* and 30 mM $MgCl_2$.
[e] The incubation mixture for D-alanyl-mLTA formation contained *L. casei* membranes (100 μg of protein), 2.3 pmol of Dcp, and the components of the ligation assay. The amount of D-alanyl-mLTA formed in 150 min was 19 pmol.
[f] NA, not assayed.

Amino acid analysis and limited N-terminal sequencing of Dcp. The amino acid composition of Dcp was similar to that reported for ACP of *E. coli*, a protein rich in aspartic and glutamic residues (Table 3). The percent sum of Asx and Glx in Dcp was 33% versus 35% for ACP. Both carrier proteins lacked cysteine and contained β-alanine. The detection of β-alanine in an acid-hydrolyzed sample indicates the presence of 4′-phosphopantetheine. Based on the β-alanine content, the estimated molecular mass of Dcp (6.2 kDa) was 30% less than that of the *E. coli* ACP (8.8 kDa). The N-terminal sequence of Dcp showed a similarity with sequences of bacterial ACPs and open reading frames (ORFs) (Fig. 2). The residues in the Dcp sequence which are identical in several ACPs include Glu-4, Lys-7, Lys-8, Val-10, and Leu-14. In addition, the residues corresponding to the Ile-6 and Ala-15 of Dcp are conserved in the prokaryotic ACPs. The N-terminal sequence with the highest homology to Dcp was the *Bacillus subtilis Ipa-3r* ORF. This putative protein has been recognized as an ACP-like homolog which is encoded as part of a five-gene operon in this organism (22). The second-best match to the Dcp N-terminal sequence was the unidentified *Lactococcus lactis* ORFX. This ORF flanks an insertion sequence element at the integration point for sex factor DNA which is associated with high-frequency transfer and cell aggregation (21). Since a role for LTA has been suggested in the formation of mating aggregates in *Lactobacillus plantarum* (39) and *Enterococcus faecalis* (14), this sequence has been included in the analysis. The comparison of the sequences in Fig. 2 may be useful for defining important residues in the N-terminal region of bacterial ACPs.

Effect of pH on the ligation of D-alanine to Dcp. An investigation of the pH optima for the ligation of D-alanine to Dcp and *E. coli* ACP provides an assessment of how the ionization status of the carrier protein substrate affects the Dcl activity. A comparison of the Dcl activities with Dcp and *E. coli*

Organism	Protein/ORF	Ref.	N-terminal sequence														
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Lactobacillus casei	Dcp	this study	M	A	D	E	A	I	K	K	T	V	L	D	I	L	A
Bacillus subtilis	Ipa-3r	(22)				M	D	F	K	Q	E	V	L	D	V	L	A
Lactococcus lactis	ORFX	(21)			I	T	P	Q	I	K	K	S	V	L	N	F	L	A
Rhodobacter sphaeroides	ACP	(11)	M	S	D	I	A	D	R	V	K	K	I	V	V	E	H	L	G
Rhizobium meliloti	ACP	(37)	M	S	D	I	A	E	R	V	K	K	I	V	I	D	H	L	G
Escherichia coli	ACP	(44)	M	S	T	I	E	E	R	V	K	K	I	I	G	G	Q	L	G
Vibrio harveyi	ACP	a	M	S	N	I	E	E	R	V	K	K	I	I	V	E	Q	L	G
Bacillus subtilis	ACP	b	M	A	D	T	L	E	R	V	T	K	I	I	V	D	R	L	G
Saccharopolyspora erythraea	ACP	(24)	M	D	R	K	E	I	F	E	R	I	E	Q	V	L	A	E	Q	L	G
Rhizobium meliloti	NodF	(12)	M	V	D	Q	L	E	S	E	I	I	G	I	I	K	N	R	V	E	S	E	G	G

FIG. 2. Comparison of the Dcp N-terminal amino acid sequence to related sequences. ACPs and related ORFs from prokaryotic organisms were analyzed with the University of Wisconsin Genetics Computer Group sequence analysis software package (version 7.2) (13). Alignment to Dcp was accomplished by using PILEUP. Unpublished sequences of ACPs were kindly provided by David M. Byers (a) and Ricardo Morbidoni (b). Ref., reference.

ACP revealed a pronounced difference (Fig. 3). The ligation of Dcp with D-alanine showed a broad pH optimum (pH 5 to 8). In contrast, the optimal pH for ligation of D-alanine to ACP was 6.6 to 7.0. A small activity peak was also observed for ACP (pH 4.5), which was not apparent with Dcp. The responses with these carrier proteins demonstrate that their interactions with Dcl are pH sensitive and reflect a difference in the ionization of the two carrier proteins.

Effects of substrates on the thermostability of Dcl. The instability of the D-alanine-activating enzyme (Dcl) was one of the early difficulties encountered with this enzyme (33a). A preliminary analysis of the Dcl thermostability in the hydroxamate assay (an assay which measures D-alanine activation) indicated that the only substrate to have a significant stabilizing effect was ATP (data not shown). In order to further define the ligation reaction, the effects of substrates on the stabilization of Dcl were investigated. In the absence of substrates, 45% of the Dcl was irreversibly inactivated at 45°C in 15 min (Fig. 4A). This temperature was selected to measure the effects of substrates on the stability of the enzyme. The results showed that ATP, Dcp, or a combination of the two significantly enhanced the thermostability of Dcl (Fig. 4B). In contrast to Dcp, *E. coli* ACP facilitated the inactivation of Dcl at 45°C.

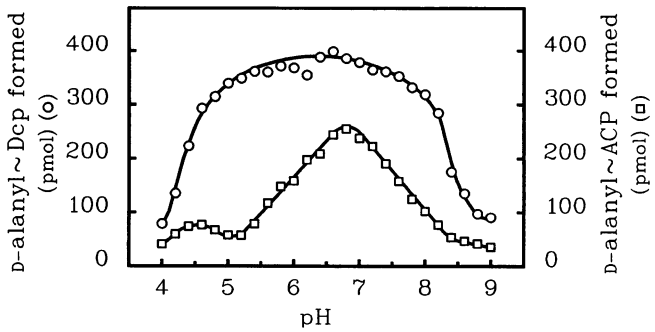

FIG. 3. Effects of pH on the ligation of *L. casei* Dcp (○) and *E. coli* ACP (□) with D-alanine. The ligation assay (30 min) was performed as described in Materials and Methods. The amount of Dcp added was 370 pmol, and the amount of *E. coli* ACP (based on the assay of palmitic acid ligation) was 550 pmol.

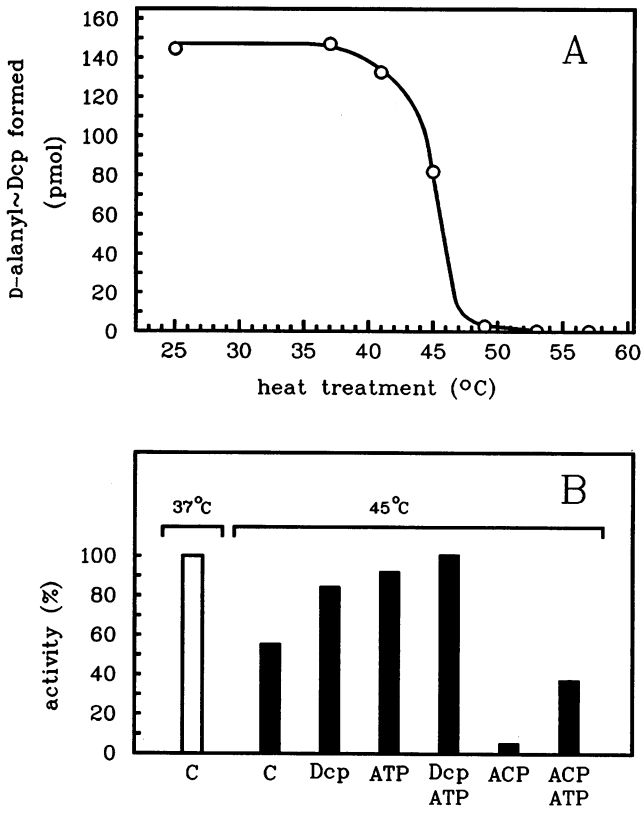

FIG. 4. Effects of substrates on the thermostability of Dcl. (A) Samples of Dcl (1.35 U each) were maintained at the indicated temperature for 15 min in 30 mM bis-Tris (pH 6.5). After this treatment, all samples were placed on ice until assayed at 37°C for ligation activity with Dcp as described in Materials and Methods. (B) Samples were treated as described above with the indicated additions at either 37 or 45°C. The amount of carrier protein added was 140 pmol, and the concentration of ATP was 10 mM. Unless specified otherwise, Dcp was used in the measurement of ligation activity. C, control (no addition).

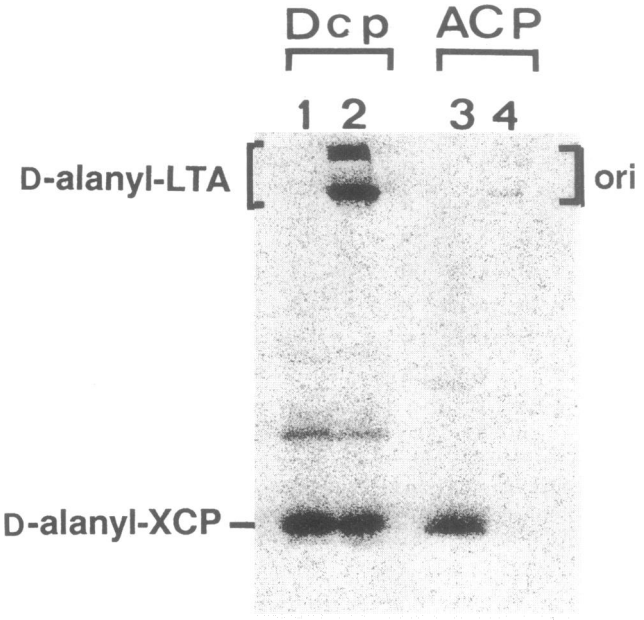

FIG. 5. Native PAGE of D-[^{14}C]alanine-labeled products from the incorporation system. The reaction mixtures of the ligation assay were increased 20-fold to accommodate 1 nmol of either Dcp (lanes 1 and 2) or ACP (lanes 3 and 4) and incubated in the presence or absence of *L. casei* membranes (2 mg of membrane protein) for 90 min. The reactions were terminated by precipitation in 5 volumes of acetone at −20°C for 30 min. The pellet was collected by centrifugation, dried under vacuum, and dissolved in 50 μl of 30 mM bis-Tris (pH 6.5). The sample was precipitated a second time, and the dried pellet was solubilized in loading buffer (85 mM Tris [pH 6.8], 10% glycerol, 10 μM bromophenol blue). The labeled products were electrophoretically separated on 15% polyacrylamide gel as described in Materials and Methods. The gel was stained with Coomassie blue R-250, and the D-[^{14}C]alanine-labeled products were visualized by phosphor imaging. Xcp is either Dcp or Acp.

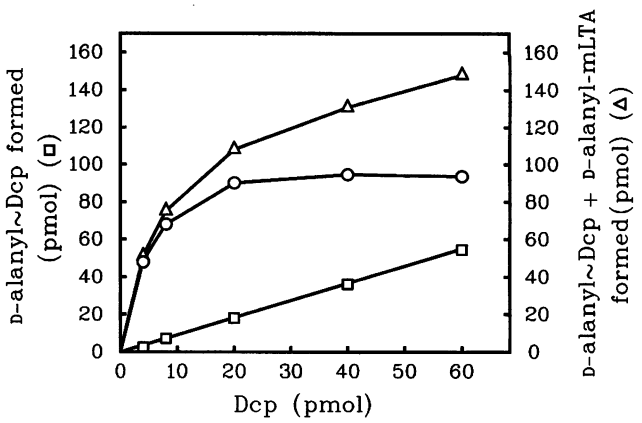

FIG. 6. Effect of Dcp concentration on the incorporation of D-[^{14}C]alanine into D-alanyl-mLTA. The formation of D-[^{14}C]alanyl~Dcp was measured by the ligation assay (see Materials and Methods). The combined formation of D-[^{14}C]alanyl~Dcp and D-[^{14}C]alanyl-mLTA was measured in the same reaction mixture with the addition of *L. casei* membranes. Samples were incubated at 37°C for 15 min before the reaction was terminated by the addition of TCA. The difference between the combined amount of D-[^{14}C]alanyl~Dcp and D-[^{14}C]alanyl-mLTA formed (Δ) and that of D-[^{14}C]alanyl~Dcp formed (□) represents the amount of D-[^{14}C]alanyl-mLTA (○).

The addition of ATP provided partial protection against this destabilizing effect of *E. coli* ACP. The effects of these carrier proteins on Dcl demonstrated that, although both serve as substrates in the ligation reaction, their interactions with Dcl are different.

Reconstitution of the system for incorporating D-alanine into mLTA. The ability to reconstitute the system which incorporates D-alanine is essential for elucidating the mechanism and components responsible for the synthesis of D-alanyl-mLTA. The D-[^{14}C]alanine-labeled products were extracted from the reconstituted *L. casei* system and analyzed by native PAGE (Fig. 5). The formation of both D-[^{14}C]alanyl~Dcp and D-[^{14}C]alanyl~ACP was observed in incubations without *L. casei* membranes (lanes 1 and 3). One of the major functional differences between Dcp and *E. coli* ACP is most apparent in lanes 2 and 4. Dcp participated in the transfer of D-alanine to *L. casei* mLTA, whereas *E. coli* ACP did not. The reaction mixture which contained Dcp produced two slowly migrating, D-alanine-labeled bands which were not associated with Coomassie stain (lane 2). The products of this reaction have been previously characterized as D-alanyl-LTA of different chain lengths (8, 38). The separation of this labeled material into bands at the origin of the polyacrylamide stacking and resolving gels is not understood. D-[^{14}C]alanyl-LTA was not apparent in the corresponding lane containing *E. coli* ACP (lane 4). Furthermore, the addition of *L. casei* membranes appeared to inhibit the formation of D-[^{14}C]alanyl~ACP.

The identification of D-alanyl~Dcp as an intermediate in the system for incorporating D-alanine into mLTA allows a more detailed description of the requirements. Since the labeled products of the incorporation system, D-alanyl~Dcp and D-alanyl-mLTA, are quantitatively recovered by filtration of TCA-precipitated material, the assay requires correction for the amount of D-alanyl~ACP present (Fig. 6). The maximal capacity of 100 μg of membrane protein to incorporate D-alanine into mLTA was 90 pmol. In a reconstituted system which is designed to measure the transfer of D-alanine to mLTA, and not the formation of D-alanyl~Dcp, a high ratio (>10:1) of membrane capacity to the amount of Dcp is an essential feature. This ratio ensures the turnover of D-alanyl~Dcp in the incubation mixture. This system was used to characterize the requirements of D-alanyl-mLTA biosynthesis. Table 2 shows that ATP, Dcl, and Dcp are required for the formation of D-alanyl-mLTA. The addition of either 1 mM DTT or 10 mM Mg^{2+} caused a 1.4-fold stimulation of activity. These observations were similar to those for D-alanyl~Dcp

TABLE 4. Requirements for transfer of D-[^{14}C]alanine from D-[^{14}C]alanyl~Dcp to LTA

Addition[a]	D-[^{14}C]alanyl-mLTA formed (pmol)[b]
None	13
Mg^{2+} (10 mM)	17
Dcl (1.35 U)	14
EDTA (10 mM)	11
D-Alanine (1 M)	11
ATP (10 mM)	8.7
DTT (1 mM)	3.0
None (heat-treated membranes)	<1.0

[a] The reaction mixture contained 30 mM bis-Tris buffer (pH 6.5), *L. casei* membranes (100 μg of membrane protein), and 55 pmol of D-[^{14}C]alanyl~Dcp in a total reaction volume of 50 μl.

[b] See Fig. 7.

formation (Table 2). However, unlike the ligation reaction, the addition of 10 mM EDTA to the incorporation system did not inhibit the formation of D-alanyl-mLTA. This feature of the membrane incorporation system distinguishes it from the ligation reaction. The results presented in this section describe the reconstitution of the cytosolic components with the membrane system for the synthesis of D-alanyl-mLTA.

Specificity of carrier protein function in the biosynthesis of D-alanyl-mLTA. Since Dcl was observed to ligate D-alanine to *E. coli* ACP, the specificities of the ligation reaction and membrane incorporation system were investigated with carrier proteins from other organisms. Results of the ligation reaction showed that *E. coli* ACP is not exceptional and that Dcl also ligated the ACPs from *Vibrio harveyi* and *Saccharopolyspora erythraea* with D-alanine. D-Alanyl~ACP was not detected with either *B. subtilis* ACP or spinach ACP-I. Since *E. coli*, *V. harveyi*, and *S. erythraea* ACPs are ligated with D-alanine, these ACPs were also tested in the D-alanyl-mLTA incorporation system containing *L. casei* membranes. None of these ACPs were able to function, and thus, the incorporation system appears to show a higher specificity for the carrier protein than Dcl does in the ligation reaction.

The transfer of D-alanine from D-alanyl~Dcp to mLTA. Since Dcp is required for the membrane incorporation system, it was hypothesized that D-alanyl~Dcp donates activated D-alanine to a membrane acceptor for incorporation into D-alanyl-mLTA. To test this hypothesis, D-[^{14}C]alanyl~Dcp was prepared. Before the reactivity of this substrate with the membrane incorporation system was tested, it was essential to establish the stability of D-alanyl~Dcp in the reaction buffer. The half-life of D-alanyl~Dcp at pH 6.5 in 30 mM bis-Tris was 6.0 h. The stability increased at a lower pH (9.5 h at pH 4.5) and significantly decreased at a higher pH (1.2 h at pH 8.5). In a reaction mixture which contained only membranes and D-alanyl~Dcp, the amount of D-alanine incorporated into mLTA was proportional to the amount of D-alanyl~Dcp added to the reaction (Fig. 7). The reactivity of this substrate with *L. casei* membranes under a variety of conditions is summarized in Table 4. The transfer of D-alanine from D-alanyl~Dcp required only active membranes and was not significantly affected by the addition of either Mg^{2+}, ATP, Dcl, or D-alanine. The reaction was inhibited fourfold by the addition of DTT and stimulated only slightly by the addition of Mg^{2+} or Dcl (1.4- and 1.1-fold, respectively). Since EDTA did not affect the incorporation of D-alanine, it was concluded that the transfer of D-alanine from D-alanyl~Dcp to mLTA did not appear to require Mg^{2+}. This is in contrast to the ligation of D-alanine to Dcp. These results identify D-alanyl~Dcp as the donor of activated D-alanine to the membrane acceptor in D-alanyl-mLTA biosynthesis.

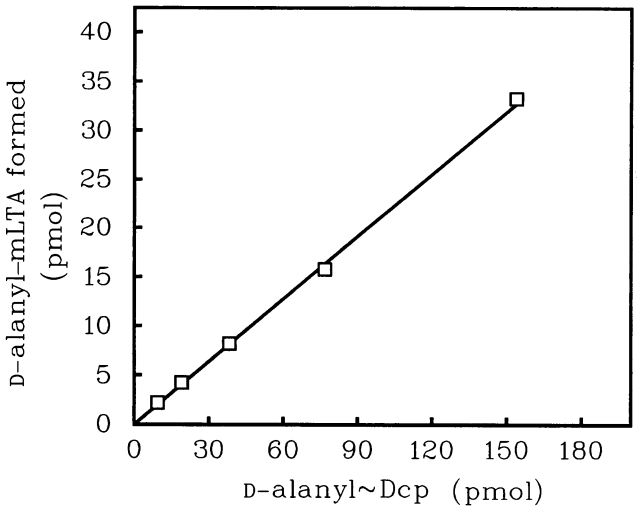

FIG. 7. Effect of the D-[^{14}C]alanyl~Dcp concentration of the formation of D-[^{14}C]alanyl-mLTA. D-[^{14}C]alanyl~Dcp (46 mCi/mmol) was prepared by the procedure described in Materials and Methods and incubated in a reaction mixture (50 μl) containing 30 mM bis-Tris (pH 6.5) and 100 μg of *L. casei* membranes. The D-[^{14}C]alanine-labeled membranes were collected on GN-6 Metricel filters and washed with three 1-ml and one 10-ml portions of cold 30 mM bis-Tris (pH 6.5). The filters were dissolved in ethyl acetate and counted in a scintillation cocktail. D-[^{14}C]alaninyl~Dcp was not retained on the filter with these washing conditions.

DISCUSSION

The isolation of Dcp provides two major insights into the biosynthesis of D-alanyl-LTA. First, the previously named D-alanine-activating enzyme (5) catalyzes not only the activation of D-alanine but also the ligation of the activated amino acid to the carrier protein. Second, these studies identify Dcp as the carrier of activated D-alanine to the membrane acceptor. These insights also allow for a more accurate naming of the proteins that are responsible for the cytosolic phase of D-alanyl-mLTA biosynthesis. Since the activating enzyme catalyzes both the activation and ligation of the amino acid to a carrier protein, it is more properly named the D-alanine–D-alanyl carrier protein ligase (Dcl). Because of the similarity of function and sequence between Dcl and the acid thiol ligases (25), it is also appropriate to change the classification of this enzyme from the amino acid-tRNA ligase subclass (EC 6.1.1) to the acid thiol ligase subclass (EC 6.2.1). In addition, the previously named D-alanine–membrane acceptor ligase (EC 6.3.2.16) (31, 40) may be removed from the ligase division, since it is the Dcp. The proposed two-step mechanism (reactions 1 and 2) for the Dcl-catalyzed formation of D-alanyl~Dcp is

$$\text{Dcl} + \text{D-alanine} + \text{ATP} \rightleftharpoons \text{Dcl} \cdot \text{AMP–D-alanine} + \text{PP}_i \quad (1)$$

$$\text{Dcl} \cdot \text{AMP–D-alanine} + \text{HS-Dcp} \rightarrow \text{D-alanyl~S-Dcp} + \text{Dcl} + \text{AMP} \quad (2)$$

where HS- represents the sulfhydryl group of the 4′-phosphopantetheine moiety of Dcp. In D-alanyl–mLTA biosynthesis, Dcp represents the link between the activated D-alanine (reaction 1) and the membrane acceptor (reaction 3).

$$\text{D-alanyl~S-Dcp} + \text{mLTA} \rightarrow \text{D-alanyl–mLTA} + \text{HS-Dcp} \quad (3)$$

The identity of the membrane acceptor is unknown, since it is not clear whether this acceptor is mLTA or another intermediate in the pathway for D-alanyl-mLTA biosynthesis. Since purified LTA will not function in reaction 3 in the absence of membranes, it was proposed that an unidentified membrane-bound enzyme is required (40). This hypothetical enzyme which utilizes D-alanyl~Dcp may be referred to as the membrane acceptor D-alanyl transferase.

The identification of Dcl as a ligase which interacts with D-alanine and a carrier protein allows the further clarification of two biochemical features of this enzyme. One of these features is the poor affinity of Dcl for D-alanine. Dcl has a K_m for D-alanine of 70 mM (5), a value that is 3,000-fold higher

than that when Dcp is present during the incorporation of D-alanine into mLTA (18 μM) (31). It is proposed that the interaction of Dcp with Dcl increases the affinity of Dcl for D-alanine and, thus, reduces the K_m. The positive effect of this interaction is consistent with the enhanced thermostability of Dcl in the presence of Dcp. A second feature of Dcl is the specificity of this enzyme for its carrier protein substrate. Dcl has a relaxed specificity for the carrier protein, since it will also ligate the ACPs from *E. coli*, *V. harveyi*, and *S. erythraea* with D-alanine. The ability of Dcl to ligate ACPs from both gram-negative and gram-positive bacteria with D-alanine was unexpected. The specificity of Dcl in the ligation reaction is in contrast to the strict requirement for Dcp in the incorporation reaction. These results imply that Dcp contains at least two determinants for specificity, one which is recognized by Dcl and one which is recognized by the putative membrane acceptor D-alanyl transferase.

Whether Dcp is used for the biosynthesis of essential cell lipids and D-alanyl-LTA or whether it functions solely for the synthesis of LTA remains to be determined. The isolation of the *L. casei* ACP for fatty acid synthesis has not been reported. The only lactic acid bacterium for which the isolation of an ACP has been reported is *L. plantarum* (42). The ACP in this organism is not constitutively expressed, since its production is repressed by the addition of exogenous oleate. The issue of multiple ACPs has been addressed for *Rhizobium meliloti* (37). In this organism, a constitutive ACP functions in the biosynthesis of cell lipids, whereas an inducible ACP derived from the *nodF* gene is involved in the synthesis of cell-signaling polysaccharides. An attractive hypothesis is that *L. casei* and related gram-positive organisms have multiple ACPs, each of which has a specific function. These functions may be determined by structural motifs found in the ACPs and their cognate partners. It has been recognized that the definition of these determinants represents an important area for further investigation of carrier protein function (16).

Baddiley and Neuhaus (5) first demonstrated the Mg^{2+} requirement for the activation of D-alanine by Dcl. Ligation of the *E. coli* ACP with D-alanine also shows a strict requirement for Mg^{2+}. In contrast, the ligation of Dcp did not show an absolute requirement for Mg^{2+}. Only after the addition of 10 mM EDTA was this requirement observed. Since ACP from *E. coli* contains two divalent metal ion binding sites, each capable of binding Mg^{2+} or Ca^{2+} (20), it is suggested that analogous sites may exist in Dcp. These results may indicate that the isolated Dcp contains tightly bound metal ions that are made available to Dcl through the interaction of Dcl with Dcp. A detailed study of these sites will be necessary to establish their existence.

The identification of D-alanyl~Dcp as the donor of activated D-alanine residues to a membrane acceptor allows one to address the membrane stage of the D-alanine incorporation system. Our goal is to establish whether mLTA is the membrane acceptor or whether additional intermediates are involved in the incorporation system. This stage may involve additional steps prior to the incorporation of D-alanine into D-alanyl-mLTA. It has been hypothesized that a membrane acceptor D-alanyl transferase, possibly derived from an ORF (ORF2) contiguous with the *dcl* gene, may play a role in transferring D-alanine ester residues to mLTA via a putative undecaprenol phosphate carrier (26) (reaction 3). This hypothesis is supported by (i) the sensitivity of the incorporation system to amphomycin and tunicamycin and (ii) the similarity of the deduced amino acid sequence of ORF2 with undecaprenol phosphate transferases (26a). A model that illustrates the proposed roles of Dcp, Dcl, and the hypothetical protein derived from ORF2 in the biosynthesis of D-alanyl-LTA is summarized in Fig. 8.

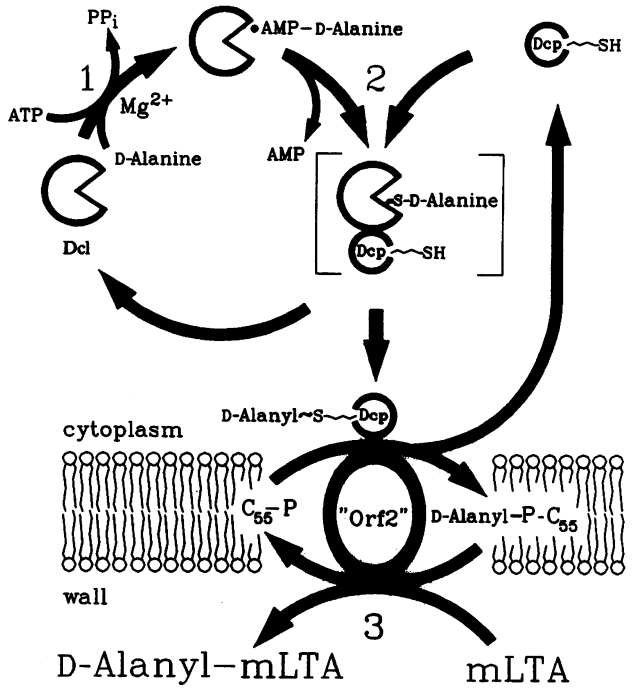

FIG. 8. A model for the biosynthesis of D-alanyl-LTA in *L. casei*. Reactions 1, 2, and 3 are described in the text. It was proposed that Dcl may catalyze the transfer of activated D-alanine via a thiolester intermediate (in brackets) (25). ~SH, 4′-phosphopantetheine prosthetic group of Dcp.

The *dlt* (D-alanyl-LTA) operon which encodes the components depicted in Fig. 8 has been proposed to exist in *L. casei*. (25). *dcl* and ORF2 are immediately preceded by a putative promoter and ribosome binding site. The widespread occurrence of D-alanyl-LTA suggests the presence of similar operons in other gram-positive organisms containing this macroamphiphile. For example, in *B. subtilis* a homologous operon containing five ORFs has been identified at map position 333° as part of the genome project for this organism. Glaser et al. (22) identified ORF1 (*ipa-5r*) as *dcl*, ORF2 (*ipa-4r*) as homologous to ORF2 of *L. casei*, ORF3 (*ipa-3r*) as an ACP, ORF4 (*ipa-2r*) as unknown, and ORF5 (*ipa-1r*) as a protein homologous to 3-ketoacyl-ACP reductase of *E. coli*. Interestingly, the *N*-terminal sequence of Dcp showed greater homology to the deduced sequence for *ipa-3r* than all other ACP-like sequences used in our comparison. This observation together with the high homologies of the first two ORFs of the *B. subtilis* operon (44 and 43% identities, respectively) suggests that the gene for Dcp may be located 3′ of ORF2. Completion of the DNA sequence of the *L. casei* operon will provide insights into the location of *dcp* and its relationship to other putative ACPs in this organism.

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