D-Alanyl-lipoteichoic acid in *Lactobacillus casei*: secretion of vesicles in response to benzylpenicillin

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Vesicles containing lipoteichoic acid (LTA) have been isolated from *Lactobacillus casei* ATCC 7469 grown in the presence of either benzylpenicillin or D-cycloserine. These cell wall antibiotics enhanced the rate of LTA and lipid secretion 6·7 times, whereas chloramphenicol inhibited their release. The formation of these vesicles from peripheral and septal wall regions did not appear to be the result of bacteriolysis. The vesicle composition of LTA and lipid was similar to that of the cytoplasmic membrane whereas the protein composition was dissimilar. The size of these vesicles ranged from 20 to 40 nm and the length of LTA ranged from 5 to 50 glycerol phosphate residues. The isolation of these vesicles provides a potential *in vitro* acceptor system for studying the D-alanylation of lipoteichoic acid.

Introduction

Lipoteichoic acid (LTA) appears to play a vital role in bacterial growth. It has been postulated that this polymer functions in the regulation of autolytic activity (Cleveland *et al.*, 1975, 1976; Hölte & Tomasz, 1975), binding of Mg²⁺ for enzyme function (Lambert *et al.*, 1977), and assembly of wall polymers (Fiedler & Glaser, 1974; Hancock & Baddiley, 1976). The D-alanine esters of LTA may provide one of the mechanisms for modulating these functions. To study the pathway for the D-alanylation of LTA in *Lactobacillus casei* 7469 (Ntamere *et al.*, 1987), it was proposed that vesicles secreted in the presence of penicillin might provide an *in vitro* acceptor system that would conserve the *in vivo* organization of the LTA and associated proteins.

Lactobacilli, bacilli and streptococci spontaneously secrete LTA into the culture medium during exponential growth (Joseph & Shockman, 1975; Markham *et al.*, 1975; Tomasz & Waks, 1975; Horne *et al.*, 1977; Horne & Tomasz, 1977, 1979; Straus *et al.*, 1977; Alkan & Beachey, 1978; Hakenbeck *et al.*, 1978; Shockman *et al.*, 1978; Waks & Tomasz, 1978; Jacques *et al.*, 1979; Kessler & Shockman, 1979; Kessler & van de Rijn 1981;

Brissette *et al.*, 1982; Wicken *et al.*, 1982; Card & Finn, 1983). This secretion is greatly stimulated during the interruption of cell wall synthesis by penicillin (Horne *et al.*, 1977; Alkan & Beachey, 1978; Waks & Tomasz, 1978; Horne & Tomasz, 1979; Nealon *et al.*, 1986; Al-Obeid *et al.*, 1990; Leon & Panos, 1990). The process of secretion is not the result of bacteriolysis since this response is seen by penicillin-tolerant or lysis-deficient bacteria (Horne & Tomasz, 1977; Brissette *et al.*, 1982). Since LTA may regulate cellular autolytic activity, the secretory process may be involved in the control of autolysis and cell division in these organisms (Horne & Tomasz, 1977; Shockman *et al.*, 1978; Tomasz, 1979).

In addition to the release of LTA, penicillin also stimulates the secretion of lipids (Hebeler *et al.*, 1973; Veerkamp, 1976; Horne *et al.*, 1977; Horne & Tomasz, 1977; Cabacungan & Pieringer, 1980; Brissette *et al.*, 1982; Rogers *et al.*, 1983; Brissette & Pieringer, 1985) and proteins (Hakenbeck *et al.*, 1983). In *Streptococcus mutans* BHT, the major lipids secreted into the culture medium during inhibition of growth by penicillin are diglucosyldiacylglycerol, monoglucosyldiacylglycerol, phosphatidylglycerol and diphosphatidylglycerol (Cabacungan & Pieringer, 1980; Brissette *et al.*, 1982). Rozgonyi *et al.* (1990) observed that penicillin greatly stimulated the release of *anteiso*-fatty-acid-containing lipids in *Staphylococcus aureus* SG 511. In *Streptococcus pneumoniae* R6, a complex set of membrane-bound

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Abbreviation: LTA, lipoteichoic acid.

cellular proteins is co-secreted with LTA and lipid into the medium from this autolysin-deficient strain (Hakenbeck *et al.*, 1983). Thus, inhibition by penicillin of peptidoglycan synthesis in a number of organisms causes enhanced secretion of LTA, lipids and protein.

This communication describes the secretion, isolation and partial characterization of these vesicles from *L. casei* 7469. It is our goal to study the mechanism of D-alanylation in reconstituted systems using these vesicles with recombinant D-alanine-activating enzyme (M. P. Heaton and F. C. Neuhaus, unpublished observations) and the D-alanine:membrane acceptor ligase (Linzer & Neuhaus, 1973).

Methods

Materials. L-[³⁵S]Methionine (Trans ³⁵S-label) (600 Ci mmol⁻¹), L-[³H]methionine (12 Ci mmol⁻¹), [1,3-¹⁴C]glycerol (50 mCi mmol⁻¹) and D-[1-¹⁴C]glucosamine hydrochloride (45 mCi mmol⁻¹) (1 mCi = 37 MBq) were purchased from ICN Biomedicals. D-Cycloserine, chloramphenicol and benzylpenicillin were purchased from Calbiochem-Behring. Tryptone and yeast extract were purchased from Difco. Metrizamide was purchased from Aldrich. Acrylamide solution (30%, w/v) was purchased from 5 Prime → 3 Prime Inc., Boulder, Col., USA. Spurr's embedding medium was from Ladd Research Industries, Burlington, Vt., USA. The monoclonal antibody to LTA was the generous gift of Dr G. D. Shockman.

Preparation of vesicles, membranes and labelled cells. *Lactobacillus casei* ATCC 7469, obtained from the American Type Culture Collection, was grown in LC broth consisting of 2% (w/v) tryptone, 1% (w/v) yeast extract, 2% (w/v) glucose, 150 mM-NaCl, 40 mM-KH₂PO₄ and salts (1.6 mM-MgSO₄, 59 µM-MnSO₄, 9 µM-FeSO₄). The pH was adjusted to 6.4 with NaOH. For the preparation of vesicles, a culture (250 ml) was grown to an optical density of 0.2 (540 nm) at 37 °C and 5.4 µM- (2.0 µg ml⁻¹) benzylpenicillin was added to the culture. After 4 h, the culture was centrifuged at 5000 r.p.m. for 20 min to remove cells. The vesicles were collected by centrifugation at 250 000 g for 1.5 h. They were suspended in 5 mM-piperazine/acetate buffer (pH 6.4) containing 150 mM-NaCl and the suspension was centrifuged at 5000 g to remove any remaining cells. After sedimenting the vesicles a second time they were purified on a step gradient (15, 30, 35 and 45%, w/v) of metrizamide at 250 000 g for 2 h. The vesicles were collected, suspended in the above buffer, and stored at 4 °C.

For the preparation of labelled cells, 10 ml cultures in LC broth were grown with either 5 µCi D-[1-¹⁴C]glucosamine, 10 µCi [1,3-¹⁴C]glycerol or 480 µCi L-[³⁵S]methionine (1 µCi = 37 kBq) for seven generations at 37 °C. The labelled cells were harvested by centrifugation and washed three times in piperazine/acetate buffer (pH 6.4). Membranes from *L. casei* 7469 were prepared according to the procedures described by Reusch & Neuhaus (1971) and Linzer & Neuhaus (1973). For membranes isolated from cells grown in the presence of penicillin, cultures were grown as described above in the presence of 5.4 µM-benzylpenicillin.

Release of labelled cellular components. To measure the kinetics of release or secretion of labelled cellular components in response to antibiotics, samples of labelled cells were suspended in fresh LC medium (10 ml) to an optical density of 0.7 and grown at 37 °C. At 110 min antibiotic was added to the growing culture. Aliquots (1 ml) were removed and centrifuged in a Beckman Microfuge for 5 min, and the amounts of radiolabel in these aliquots were quantified.

Electron microscopy. For transmission electron microscopy, cells and vesicles were fixed with a combination of glutaraldehyde (2.5%, v/v) and osmium tetroxide (1%, w/v) in 50 mM-PIPES buffer (pH 7.5) for 1.5 h at 4 °C. After washing with buffer, the samples were post-fixed in 1% osmium tetroxide overnight. The pellets were washed three times in buffer and were embedded in 2% (w/v) agarose. The agarose pellets were dehydrated in a graded series of acetone concentrations, embedded in Spurr's low viscosity medium and sectioned (silver-gold) on an MT-2 ultramicrotome (Ivan Sorvall). The sections were stained in 2% (w/v) uranyl acetate for 10 min followed by lead citrate (Reynolds, 1963) for 10 min and examined in a JEOL JEM-100 CX 11 transmission electron microscope at 80 kV.

Monoclonal antibody absorption. Monoclonal antibody against tri-glycerol diphosphate (Jackson *et al.*, 1980) was serially diluted in water. Equal volumes of antibody solution and vesicle suspension, labelled with either [1,3-¹⁴C]glycerol or L-[³H]methionine, were allowed to incubate at 37 °C for 2 h. Each sample was then collected on a 0.2 µm filter, washed with water, and the radiolabel on the filter and in the filtrate were quantified.

Analytical methods. The distribution of polymer lengths in LTA was determined by a modification of the methods described by Min & Cowman (1986), Wolters *et al.* (1990), and Maurer & Mattingly (1991). LTA was extracted with 40% (w/v) phenol/water according to the procedure of Fischer *et al.* (1983) and chemically deacylated with 15% NH₄OH at 20 °C for 12 h. The samples were taken to dryness at 60 °C, dissolved in water, and analysed by PAGE on a 20% (w/v, acrylamide) gel (1.5 mm thickness) using an electrode buffer of 0.25 M-Tris/borate (pH 8.2). The samples were electrophoresed at 32 V cm⁻¹ until the phenol red tracking dye was 1 cm from the bottom of the gel. The ladders of deacylated LTA were visualized by the alcian blue-silver stain of Min & Cowman (1986) as described by Wolters *et al.* (1990). Similar results were obtained by chemical deacylation of the LTA samples with 0.1 M-NaOH for 1 h at 60 °C. The average chain length of LTA was determined from the ratio of phosphorus to glycolipid as described by Fischer *et al.* (1980). D-Alanine was determined by the procedure of Diven *et al.* (1964). Membrane proteins were resolved by SDS-PAGE by the method of Laemmli (1970) as described by Garfin (1990) and stained with Coomassie brilliant blue R-250. Glycolipids and phospholipids were identified by thin-layer chromatography in chloroform/methanol/water (65:25:4, by vol.) (Nakano & Fischer, 1977) and butanol/acetic acid/water (12:3:5, by vol.) (Brautigan *et al.*, 1981).

Results

Secretion and isolation of vesicles

The secretion of vesicles from *L. casei* 7469 can be observed in photomicrographs of penicillin-treated cultures (Fig. 1). They were secreted from peripheral wall (a), septum (b) and regions of wall enlargement (c). From these micrographs, it would appear that they are released from the cell surface as vesicular structures. For example, in the grossly deformed septal region the vesicles can be observed to be secreted into the peptidoglycan matrix (Fig. 1b). The vesicles were isolated from the culture medium after growth of the organism in the presence of benzylpenicillin and were partially purified by density gradient centrifugation on a step gradient of metriz-

Fig. 1. Electron micrographs of penicillin-treated cells of *L. casei*. The culture was treated with 5.4 μ M-benzylpenicillin and grown for 4 h. The three panels, (a) peripheral, (b) septal and (c) regions of wall enlargement, illustrate the secretion of vesicular structures. Bars, 200 nm.

Fig. 2. Vesicles isolated from *L. casei* grown in the presence of $5.4\ \mu\text{M}$ -benzylpenicillin for 4 h. The vesicles were isolated from the metrizamide gradient and processed according to the procedures described in Methods. Bar, 50 nm.

amide (see Methods). The size of these vesicles ranges from 20 to 40 nm (Fig. 2).

L. casei as a tolerant organism

Evidence that bacteriolysis is not the cause of vesicle formation is shown in Fig. 3. If gross disruption of the PG matrix and lysis were the cause of vesicle release, cell death would be concurrent with apparent secretion. However, if *L. casei* 7469 is tolerant to $5.4\ \mu\text{M}$ -benzylpenicillin, vesicle formation must occur by a mechanism different from that in cellular lysis. The MIC of this β -lactam is $2.2\ \mu\text{M}$ (Ntamere *et al.*, 1987). In Fig. 3, the no. of c.f.u. are compared with optical density. From these growth experiments, the ratio of c.f.u./optical density was constant from the time of penicillin

application to the time of vesicle isolation (4 h). These results suggest that the formation of vesicles which is described in this paper is not a consequence of bacteriolysis.

Release of cellular constituents from labelled cells

To study the kinetics of release of cellular constituents from *L. casei*, separate cultures were labelled with either [^{14}C]glycerol, [^{14}C]glucosamine or [^{35}S]methionine. Growth of these radiolabelled cells in fresh culture media provided a system for monitoring the release of labelled constituents in the presence of three antibiotics. Inhibition of growth by either penicillin or D-cycloserine enhanced the secretion of glycerol-labelled material (Fig. 4a). This enhancement was characterized with a $t_{1/2}$ of

Fig. 3. Tolerance of *L. casei* to benzylpenicillin. The no. of c.f.u. and the optical density of the cultures are presented in (a) and (b), respectively. At 3 h (↓), 5.4 μ M-benzylpenicillin was applied to the exponential-phase culture (▲). For comparison, the control culture (●) is shown.

Fig. 4. Effects of benzylpenicillin, D-cycloserine and chloramphenicol on the release of glycerol-labelled (a), L-methionine-labelled (b), and D-glucosamine-labelled (c) cellular components. Pre-labelled cells were washed and grown in fresh culture medium as described in Methods. The release of radiolabel (c.p.m. ml⁻¹) was measured in (a) with [¹⁴C]glycerol-labelled (2.02×10^4 c.p.m.), in (b) with L-[³⁵S]methionine-labelled (2.07×10^6 c.p.m.) and in (c) with D-[¹⁴C]glucosamine-labelled (1.12×10^6 c.p.m.) cells. The radiolabelled cells were added to 10 ml of LC medium (Methods). At the arrow, either 5.4 μ M-benzylpenicillin (○), 5.4 μ M-D-cycloserine (●) or 50 μ M-chloramphenicol (□) was added to labelled exponential phase cultures of *L. casei* (control, Δ). The amount of released radiolabel was measured according to the procedure described in Methods.

88 min whereas the release of this material from control cells was characterized with a $t_{1/2}$ of 590 min. The turnover of this material in the control culture was 20.8% per generation (Ntamere *et al.*, 1987). In contrast to penicillin and D-cycloserine, chloramphenicol inhibited the release of glycerol-labelled material when compared with that in the control culture (Fig. 4a). Thus, the two inhibitors of cell wall synthesis, D-cycloserine and

benzylpenicillin, promote the secretion of glycerol-labelled constituents from inhibited cells.

Penicillin and D-cycloserine also stimulated the release of [³⁵S]methionine-labelled proteins by 10 and 20%, respectively (Fig. 4b). The kinetics for release of glycerol-labelled material in the presence of D-cycloserine and penicillin are distinctly different from those observed with methionine-labelled cells. Each of the cell

Fig. 5. Profile of chemically deacylated LTA. After deacylation of the LTA, the sample was resolved as described in Methods. LTA from either vesicles (lane 3), membranes from cells grown in the absence of penicillin (lane 1), or membranes from cells grown in the presence of 5.4 μ M-penicillin (lane 2) is shown. For comparison wall teichoic acid extracted with 5% trichloroacetic acid from *B. subtilis* is shown in lane 4. The amount of LTA phosphorus in lanes 1, 2 and 3 is 0.4 μ g. Oligomers with less than 5 repeat units are not visualized by the alcian blue method (Wolters *et al.*, 1990).

wall antibiotics as well as chloramphenicol inhibited the turnover of pre-labelled peptidoglycan and hence its release into the medium (Fig. 4c). The kinetics of [14 C]glucosamine-labelled peptidoglycan release in the control reflected the exponential growth of the culture. The fact that D-cycloserine and penicillin inhibit release of pre-existing peptidoglycan further supports the conclusion that bacteriolysis is not occurring in the growth arrested culture. Thus, in addition to stimulating the release of glycerol-labelled material, these cell wall inhibitors also stimulated a small release of methionine-labelled protein.

Characterization of vesicles

LTA was isolated from vesicles by phenol/water extraction and the distribution of chain lengths was examined by the electrophoretic procedure described in Methods. The heterogeneous profile of chemically deacylated LTA from vesicles resembled that derived from membranes (Fig. 5). Although the average chain length determined from the phosphorus/glycolipid ratio was 38 ± 3 , no predominant length was observed. The polyglycerol phosphate moieties were estimated to range from 5 to 50 residues in length. For comparison, wall teichoic acid

Fig. 6. Co-sedimentation of [¹⁴C]glycerol-labelled LTA and [³H]methionine-labelled protein in vesicles (a) and agglutination of [³H]methionine-labelled vesicles with monoclonal anti-polyglycerol phosphate (7B11) antibody (b). In (a), [³H]methionine-labelled vesicles (1.7×10^4 c.p.m.) (●) were mixed with [¹⁴C]glycerol-labelled vesicles (3.6×10^3 c.p.m.) (▲) and sedimented on a step gradient of metrizamide at 250 000 g for 6 h. Fraction 33 is the bottom of the gradient. In (b), increasing concentrations of monoclonal antibody were used to agglutinate the [³H]methionine-labelled (1200 c.p.m.) vesicles containing LTA. The radiolabel (●) on a 0.45 μ m filter represents the LTA-antibody complex and the radiolabel in the filtrate (▲) represents the uncomplexed LTA.

from *Bacillus subtilis* (Fig. 5, lane 4), which is also heterogeneous in size, was determined to have an average of 60 glycerol phosphate residues per chain (J. H. Pollack and F. C. Neuhaus, unpublished observation). These results confirm similar reports by Maurer & Mattingly (1991) and Leopold & Fischer (1991) that LTA from a number of bacteria is polydisperse. In *L. casei* chain lengths between 30 and 65 residues were reported. The D-alanine/phosphorus ratio of the LTA was 0.26 ± 0.06 for LTA from both vesicles and membranes. In a separate series of experiments, the profile of lipids from the vesicles was identical to that isolated from membranes (data not shown).

The co-secretion of glycerol-labelled constituents and methionine-labelled protein suggested that the isolated vesicles may contain protein as well as LTA (Fig. 4a, b). Since both radiolabels co-migrated at the same density when the vesicles were purified by density gradient centrifugation (Fig. 6a), it was inferred that the vesicles contain both protein and LTA. This conclusion was supported by agglutinating methionine-labelled vesicles with monoclonal antibodies directed to polyglycerol phosphate. As shown in Fig. 6(b), the anti-polyglycerol-phosphate antibody precipitated the labelled vesicles. Similar results were found with [¹⁴C]glycerol-labelled vesicles (data not shown). Thus, the vesicles described in this paper contain both LTA and protein.

In contrast to the LTA and lipid profiles, the protein profile of vesicles was distinctly different from that found in membranes. Although the vesicles were collected from cells treated with penicillin and could be expected to reflect the composition of the membranes derived from these cells, this result was not observed.

Instead, the protein composition of vesicles consisted of a subset of those found in the membranes with a number of major proteins (four examples of which are indicated by the ►) not being found in the vesicles (Fig. 7). Of the five major proteins in the vesicles shown by open arrowheads, one (<1) is present in membranes from both penicillin-treated and untreated cells. Several (<2, <3 and <4) appear to reflect the amounts found in membranes from untreated cells, whereas only one (<5) reflects the amount in membranes from penicillin-treated cells. Thus, the protein composition of the vesicles does not reflect that of membranes either from cells grown in the presence or in the absence of penicillin. These results also underscore the observation that significant differences result in the protein profiles of membranes when the bacteria are grown in the presence of penicillin (Fig. 7, lane 1 versus lane 2). Thus, the vesicles retain an LTA and lipid composition similar to that of the cytoplasmic membrane; however, their protein composition differs significantly from that of the membrane.

Discussion

The isolation of vesicles containing LTA from *L. casei* provides a potential acceptor system for studying the *in vitro* D-alanylation of this polymer (Baddiley & Neuhaus, 1960; Reusch & Neuhaus, 1971; Linzer & Neuhaus, 1973; Taron *et al.*, 1983). *L. casei* was chosen for our studies because of its well-defined LTA (Fischer, 1988, 1990), lack of wall teichoic acid, and high level of D-alanine-activating enzyme. Mutants of this organism that are deficient in D-alanine ester residues show

Fig. 7. Protein profile of the vesicles and cytoplasmic membranes from *L. casei*. Membrane proteins, visualized with Coomassie blue, from cells grown in the absence of benzylpenicillin (lane 1) and in the presence of 5.4 μ M-benzylpenicillin (lane 2) are compared with proteins from the vesicles (lane 3). The amount of protein is 16 μ g in lanes 1 and 2. Proteins that are not secreted into vesicles are designated $\blacktriangleright$. Major proteins secreted into vesicles, designated $\blacktriangleleft$, are described in the text.

aberrant cell shape (Ntamere *et al.*, 1987). Thus, it was concluded that the D-alanylation system may play a role in defining some of the postulated regulatory properties of this macromolecule (Archibald *et al.*, 1973; Lambert *et al.*, 1975; Fischer *et al.*, 1980, 1981; Koch *et al.*, 1982; Fischer, 1988). With the vesicle preparation described in this paper, it is our goal to address additional features which determine the ability of LTA to accept activated D-alanine. These features may be a function of topology, organization or other membrane constituents.

In previous work, membrane fragments provided the source of endogenous LTA acceptor for D-alanylation (Reusch & Neuhaus, 1971; Linzer & Neuhaus, 1973). Attempts to fractionate and reconstitute the acceptor system were unsuccessful (Neuhaus *et al.*, 1974). The only reaction that has been demonstrated with partially purified LTA is the ATP-independent transacylation of D-alanine ester residues in micelles (Childs *et al.*, 1985). The vesicles which are derived from the penicillin-triggered secretory process may conserve the organization of the endogenous LTA, and thus provide an *in vitro* acceptor for the D-alanylation system.

The secretion of glycerol-labelled constituents in response to penicillin has been described in a variety of Gram-positive organisms. For example, in the case of *Streptococcus sanguis* (Horne & Tomasz, 1977), cell wall inhibitors caused the release of substantial amounts of LTA and phospholipids into the growth medium under non-lytic, non-bacteriocidal conditions. In a similar manner, inhibition of peptidoglycan synthesis in *L. casei* also promotes the release of glycerol-labelled polymers from this penicillin-tolerant organism. In addressing the mechanism of protein and lipid secretion, it was concluded that the release of LTA in *S. sanguis* and *S. mutans* occurred as vesicular structures in the absence of cell wall disintegration (Horne *et al.*, 1977; Hakenbeck *et al.*, 1983). In the present work, vesicles were also observed at the outer surface of penicillin and D-cycloserine-treated *L. casei*. Thus, the enhancement of LTA secretion as vesicles at the wall surface is triggered by the inhibition of wall synthesis.

The secretion of LTA has also been reported in other lactobacilli (Markham *et al.*, 1975; Wicken *et al.*, 1982). In these reports it was suggested that LTA is released from the cell membrane as a transient, non-vesicular component into the culture fluid where it would aggregate to form high molecular mass micelles. Since the critical micelle concentration of LTA is sufficiently low, micelle formation could occur from monomolecular, amphipathic LTA (Courtney *et al.*, 1986). However, in the present work LTA appears to be released as vesicular structures in response to penicillin and D-cycloserine as has been proposed for *S. pneumoniae* and *S. sanguis* (Horne *et al.*, 1977; Hakenbeck *et al.*, 1983). The

explanation for this difference may be the result of two mechanisms of secretion: (1) release as monomolecular LTA in the absence of penicillin; (2) release as vesicular structures containing LTA in the presence of penicillin. In *L. casei* it is proposed that in the absence of cell wall inhibitors there is a continual turnover and secretion of monomolecular LTA whereas in the presence of penicillin and D-cycloserine vesicular structures are released. Thus, interruption of peptidoglycan synthesis by cell wall antibiotics generates a signal that promotes the secretion of vesicles of LTA and lipid. The nature of this signal is not understood.

In *S. pneumoniae* penicillin stimulates the secretion of a complex set of proteins in vesicles containing LTA (Hakenbeck *et al.*, 1983). It was concluded that these vesicles were derived from the cytoplasmic membrane. In contrast, the protein profile of vesicles from *L. casei* showed only a selected subset of membrane proteins. These results would appear to support the conclusion that the protein composition in the vesicles from this organism may be the result of a selective secretory mechanism and that the vesicles are not the consequence of an extension or continuum of the cytoplasmic membrane. In addition, the limited protein composition further demonstrates that bacteriolysis is not responsible for the formation of these vesicles.

D-Alanyl ester residues play a major role in determining the net charge of lipoteichoic acid (Fischer, 1988, 1990). Under *in vitro* conditions, a change in the net charge has a major effect on autolytic enzymes. Whether D-alanylation represents an *in vivo* regulatory system that controls these enzymes has not been established. The mechanism and regulation of the D-alanylation system remains one of the unanswered questions for LTA function. The ability to reconstitute the D-alanylation system with purified, defined vesicles containing LTA will greatly facilitate our understanding of this complex system.

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