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Lipoteichoic Acid, a Major Amphiphile of Gram-Positive Bacteria That Is Not Readily Extractable

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Commonly used procedures effected the extraction of only 10% of the lipoteichoic acid of stationary-phase cells of *Staphylococcus aureus* and *Streptococcus faecium*, unless the cells were first disrupted.

Lipoteichoic acid (LTA) is a major membrane-associated amphiphilic molecule found in most gram-positive organisms. In prior studies, LTA was extracted by using acid (5), aqueous phenol (6, 9, 16), enzymatic lysis (11, 14, 15), mechanical disruption (1, 3), hot water (21), or a combination of these (13). Cells at different stages of growth, disrupted and nondisrupted, were used for such extractions. However, there has been little experimental work directed at the yield of LTA as a function of extraction procedure (21), stage of growth (16), or cellular disruption (18). The cellular yield of LTA on a weight basis was reported in only a few instances (2, 14).

The purpose of this study was to determine the total amount of LTA contained in the cell as a function of bacterial growth. In the final method adopted, cells were either disrupted by Braun pretreatment with glass beads (p-BRN) or by lysostaphin lysis (p-LYS). After either pretreatment, LTA concentration was determined by a procedure (method A) which involved hot-phenol extraction, DNase treatment, and fractionation on Sepharose 6B (4). For comparison, whole cells were defatted and dried by pretreatment with solvents (p-PAB); LTA concentration was then determined by method A performed at 4°C for phenol extraction (method B).

For p-BRN, cells were washed and suspended in 0.05 M Tris—0.02 M $MgCl_2$, pH 7.5 (Tris-M), at 25 mg of cells per ml. The cells were shaken with glass beads (0.17-mm diameter) in a Braun disintegrator for 5 min (5,160 rpm; 4 to 10°C). The disrupted cells were lyophilized, extracted with chloroform-methanol-water (2:1:0.03 [vol/vol/vol]) and ether; then the cells were dried. For p-LYS, *Staphylococcus aureus* cells (1 g in 40 ml of Tris-M) were treated with 2 mg of lysostaphin (Schwarz/Mann) and 1,000 U of DNase I (Worthington Diagnostics) for 10 min at 37°C, lyophilized, and extracted as described above. For LTA determination by method A, the dried preparation from the pretreatment (p-BRN or p-LYS) (100 to 500 mg in 10 ml of water; 4°C) was treated in a Sorvall Omnimixer (15 s; 16,000 rpm). Phenol (5 ml; 80% aqueous) was then added, and the preparation was treated again in the Omnimixer, heated (1 h; 80°C), chilled (4°C), and centrifuged (20 min; 1,800 × *g*). The supernatant was removed, and the phenol layer was extracted twice (3 ml of water; 80°C). The pooled supernatants were dialyzed (30 h; 4°C against water), lyophilized, and treated with DNase I; 1-ml fractions were collected upon chromatography on Sepharose 6B (10- by 470-mm column) in 0.2 M ammonium acetate–0.02% sodium azide, pH 6.9 (4). LTA-I (pooled fractions 12 through 24) or LTA-II (pooled fractions 25 through 32) was dialyzed, lyophilized, and weighed (Cahn Microbalance). For p-PAB, whole cells were mixed with 5 volumes of pyridine-acetic acid-butanol (PAB) (22:31:100 [vol/vol/vol]), filtered through Whatman no. 50 filter paper, washed with chloroform-methanol-water, rinsed with ether, and dried.

Since the exact molecular structure of LTA was not known, Sepharose 6B-purified LTA was measured as dry weight, and separate estimates were made of the purity. Assuming a structure of (D-alanine-glycerol phosphate)$_{21}$-diglucoside-dipalmityl glyceride (molecular weight = 5,620) for *Staphylococcus aureus* (Lafferty) LTA, the same structure as for *Staphylococcus aureus* H (9), the calculated glycerol/phosphate ratio and glycerol content were 1.05 and 38%, respectively (observed values were 1.02 ± 0.05 and 18 ± 4% for cold-phenol-extracted LTA and 1.05 and 20% for hot-phenol-extracted LTA). Assuming a structure of (kojibiosyl [60%]-glycerol phosphate)$_{28}$-kojibiosyl-dipalmityl glyceride-dipalmityl glycerol phosphatide (molecular weight = 11,765) for *Streptococcus faecium* (ATCC 9790) LTA (12), the calculated glycerol/phosphate ratio and glycerol content were 1.03 and 24%, respectively (observed values were 1.09 and 13 ± 4% for cold-phenol-extracted LTA and 1.02 and 15% for hot-phenol-extracted LTA). Cold-

and hot-phenol-extracted LTA contained, respectively, 11 and 2% protein. The presence of protein did not explain the low observed glycerol values. The low glycerol values could be a result of structural variation from the assumed structure or of as-yet-undetermined contaminants in the LTA.

The importance of cellular disruption of stationary-phase *Staphylococcus aureus* (Lafferty) either by mechanical breakage (p-BRN) or enzymatic lysis (p-LYS) for successful extraction of LTA-I is shown in Table 1. If undisrupted cells were used, the LTA extracted from stationary-phase cells was greatly reduced. Once the cells were disrupted by p-BRN, no further increase in LTA was obtained by treating the cells with lysostaphin (Table 1). Solvent extraction (p-PAB) of whole cells followed by method B isolation of LTA yielded LTA from exponential-phase cells (Table 2) but not from stationary-phase cells (Table 1), unless the stationary-phase cells were subsequently treated by p-BRN or p-LYS followed by method A isolation (E. Huff, unpublished data). If cells were disrupted by Braun disintegration and then treated with PAB, little LTA was obtained by method A phenol extraction (Table 1). This was found to be due to the passage of LTA through Whatman no. 50 filter paper at cell concentrations of less than 11 mg/ml. This loss was avoided if disrupted cells were lyophilized before PAB treatment or if samples of disrupted cells were filtered through Whatman no. 50 filter paper only if they were in concentrations greater than 11 mg (dry weight)/ml (total volume, 22 ml). For small samples (20 mg or less), it was best to centrifuge (10 min, 100,000 × *g*) the PAB precipitate.

To determine the time course of change in LTA extractability during growth, I measured LTA-I during the growth of *Staphylococcus aureus* (Lafferty) both by p-BRN followed by method A and also by p-PAB followed by method B. The LTA-I level remained unchanged when LTA was extracted from disrupted cells (Fig. 1A). A rapid increase in the amount of LTA-I extractable from whole cells occurred as the cells went into exponential growth. This was followed by a rapid decrease in the extractability of LTA at the end of exponential growth. Similar results were observed for *Streptococcus faecium* (9790) (Fig. 1B). The decreased levels of LTA-I in stationary-phase as compared with exponential-phase cells of *Streptococcus faecium* (9790) and *Streptococcus mutans* reported by Joseph and Shockman (16) was probably due to the use of whole rather than disrupted cells for LTA extraction. Kessler and Shockman (17) observed that the LTA-I level was unchanged during growth of *Streptococcus faecium* (9790). This constant LTA-I level is consistent with the present results, since I extracted LTA-I from cells in balanced (exponential) growth, at which stage LTA-I extraction was complete without cell disruption (Table 3).

TABLE 1. Extraction of LTA-I from stationary-phase *Staphylococcus aureus* (Lafferty)[a]

Expt.	Pretreatment (°C of incubation)	LTA-I extracted (% dry wt of cells)[b]
1. 18-h cells	Control (37)	0.25
	p-LYS (37)	1.60
	p-BRN (4)	1.50
	p-BRN (37)	1.60
	p-BRN + p-LYS (37)	1.48
2. 6-h cells	p-PAB; method B	0.11
	Lyoph; CM; method A	0.19
	Lyoph; CM; method B	0.06
	Braun; p-PAB; method B	0.03
	p-BRN; method A	1.7
	p-BRN; method B	1.5

[a] *Staphylococcus aureus* (Lafferty) was grown in Trypticase soy broth (BBL Microbiology Systems) containing $^{32}PO_4$. Cells grown from a 10% inoculum for 6 or 18 h showed similar low values for extractability of LTA when p-PAB and method B were used. In experiment 1, 431 mg of cells (in 0.1 M Tris–0.03 M NaCl, pH 7.5) was treated with 2 mg of lysostaphin and 955 U of DNase I. In experiment 1, cells were incubated after p-BRN or during enzymatic lysis or both. Incubations (25 min for each pretreatment) were followed by lyophilization (Lyoph) and solvent extraction. In experiment 2, cells were extracted at 16.5 mg/ml, with the exception of Braun-disrupted cells (Braun), which were extracted at 4.4 mg (dry weight)/ml. CM, chloroform-methanol.

[b] After the indicated pretreatment, LTA concentrations were determined by method A in experiment 1 and as indicated in experiment 2. Extracted LTA concentrations were determined as follows: (dry weight of LTA-I × 100)/dry weight of cells extracted. All LTA values are corrected for the presence of any nucleic acid and protein present.

Whenever the comparison was made, undisrupted exponential-phase cells yielded LTA-I in amounts (1 to 1.8% of the dry weight of the cells) equivalent to that yielded by disrupted stationary-phase cells (Table 3), with no increase upon disruption (Table 2). Considerable variation was observed in the amount of LTA-I extracted from different species of undisrupted stationary-phase cells, as compared with undisrupted exponential-phase cells (Table 3). The exponential-phase LTA/stationary-phase LTA ratio varied from 55 for *Staphylococcus aureus* (6538P) to 2 for *Streptococcus mutans* BHT.

The observed difference between the extent of extraction of LTA-I from whole stationary-phase

TABLE 2. Extraction of LTA-I from exponential-phase *Staphylococcus aureus* cells[a]

Cell concn (mg [dry wt]/ml)	Pretreatment	LTA-I extracted (% dry wt of cells)[b]
23	p-BRN; p-PAB	1.22
11	p-BRN; p-PAB	1.21
6	p-BRN; p-PAB	0.08
18	Braun-MC	0.74
23	Boiling; p-PAB	1.10
23	p-PAB	1.22
23	None	1.26[c]
23	Lyoph; p-PAB	1.07

[a] *Staphylococcus aureus* 6538P was grown in Trypticase soy broth from an optical density at 660 nm of 0.16 to an optical density at 660 nm of 0.62 (exponential-phase cells) and harvested. These cells were suspended in water to give the indicated cell concentration in a final volume of 22 ml. In the Braun-MC procedure, 5 volumes of methanol was added to the Braun-treated cell suspension followed by 10 volumes of chloroform; the mixture was then filtered. Boiling was for 10 min. Lyoph, lyophilization.

[b] LTA-I concentrations were determined by method B (except as indicated) on defatted dried cells from the pretreatment step.

[c] Determined by method A.

TABLE 3. Comparison of concentrations of LTA-I extracted from stationary-phase and exponential-phase cells

Organism	LTA (% dry wt of cells)		
	p-PAB extraction of:		p-BRN extraction of stat cells
	Stat cells[a]	Log cells[a]	
Staphylococcus aureus (6538P)	0.02	1.1	
Staphyloccus aureus (Lafferty)	0.07	1.1	1.2
Streptococcus faecium (9790)	0.15	1.2	1.01
Bacillus licheniformis (9945)	0.21	1.6	1.75
Streptococcus pyogenes (56 × 188)	0.12	0.6	
Bacillus subtilis (6051)	0.22	0.6	
Streptococcus mutans (BHT)	0.24	0.4	

[a] Stat, Stationary-phase; Log, exponential-phase.

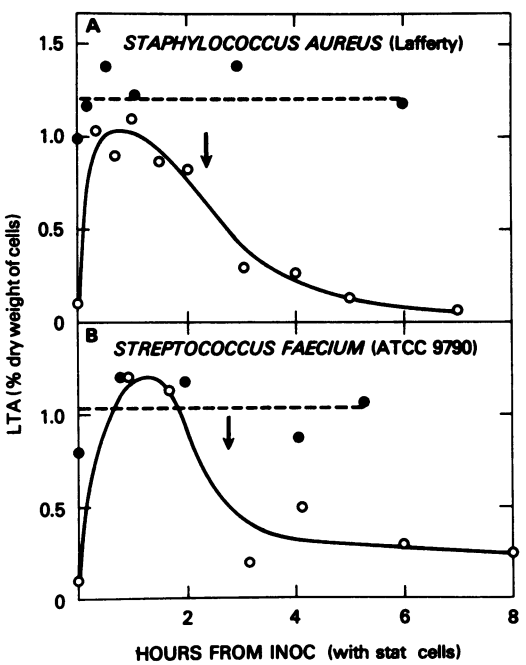

FIG. 1. LTA content of *Staphylococcus aureus* (Lafferty) (A) and *Streptococcus faecium* (ATCC 9790) (B) during growth in Trypticase soy broth at 37°C with shaking. ○, p-PAB and method A; ●, p-BRN and method A. Arrows indicate the end of exponential growth. Stat, Stationary phase; Inoc, inoculation.

cells and from exponential-phase cells remains unexplained. Some change in the cell wall or membrane must occur which makes the cell more permeable to LTA or to the extracting phenol during exponential-phase growth. These changes do not seem to be due to changes in the membrane, since treatment of cells with phenol at 80°C (after p-PAB), a procedure which effects extraction of LTA from exponential- (14) and stationary-phase mesosomes and protoplasts (Huff, unpublished data), failed to effect extraction of LTA from stationary-phase cells (Table 1). Disruption of stationary-phase cells with glass beads in the Braun disintegrator or treatment of cells with lytic enzymes (e.g., treating *Staphylococcus aureus* with lysostaphin) enabled LTA to be extracted with phenol. Since both of these procedures disrupt the cell wall, it would appear that the observed changes in permeability occur in the cell wall. Perhaps during growth, the wall thins as a result of autolysin action or the formation of a newly synthesized cell wall, allowing penetration of phenol and extraction of LTA. In support of this theory are the observations that autolysins are found in *Staphylococcus aureus* (22), *Streptococcus faecium* (20), and *Bacillus licheniformis* (10), in which rapid changes in LTA extractability occur, and that autolysins are lacking from *Streptococcus pyogenes* (19), from which little LTA was extracted at any stage of growth. Cellular disruption has been shown to be necessary for extraction of cardiolipin but not phosphatidyl

glycerol or phosphatidyl ethanolamine from several *Bacillus* species (7).

In summary, although lipoteichoic acid is a major amphiphilic molecule of gram-positive bacteria, accounting for 1 to 2% of the dry weight of the cells, it is not readily extractable. The extraction method described above involves cellular disruption. Whole stationary-phase cells yielded only 10% of the amount of lipoteichoic acid extracted from disrupted stationary-phase cells. For some organisms, the yield of LTA from whole exponential-phase cells was equivalent to the yield from disrupted cells.

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