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LIPOTEICHOIC ACIDS FROM D-GLUCOSYLATION-DEFECTIVE, CELL-WALL MUTANTS OF *Bacillus subtilis*

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ABSTRACT

Lipoteichoic acids from *Bacillus subtilis* 168 and its *gta* mutants were partially purified and characterized. The teichoic acids contain glycerol residues and phosphorus in equimolar ratios, and fatty acids. Some contaminating protein and nucleic acids were also present in the preparations. Gel electrophoresis of the preparations revealed the presence of common proteins, suggestive of functional protein–lipoteichoic acid complexes in bacterial membranes. All of the lipoteichoic acids were partially D-glucosylated, as evidenced by enzymic analysis. The D-glucose residues were in the α anomeric configuration, as shown by precipitation with concanavalin A. The data suggested that some, but not all, of the pathways leading to the D-glucosylation of the cell-wall teichoic acid are functional in the D-glucosylation of the membrane teichoic acids. The lipoteichoic acids were complexed with methylated bovine serum albumin, and injected into rabbits, in order to elicit a humoral immune-response. Antibodies were directed against poly(glycerol phosphate) and not against the D-glucosyl residues of the lipoteichoic acids. The antibody preparations cross-reacted with all of the membrane teichoic acids. Soluble, cell-wall teichoic acid was a potent inhibitor of passive hemmagglutination in a system containing lipoteichoic acid-sensitized, sheep red-blood cells and anti-lipoteichoic acid antiserum.

INTRODUCTION

Teichoic acids, derived from bacteria, are water-soluble polymers consisting of linear chains of polyol phosphate to which certain substituents, such as D-alanine or sugars, can be covalently attached. Baddiley *et al.*[1] found that the polymers are both bound to the cell wall and "intracellular". The intracellular teichoic acids were later shown to contain fatty acids, and to be bound to constituents of the membranes of Gram-positive bacteria; these are called lipoteichoic acids[2]. In fact, all Gram-positive bacteria appear to possess lipoteichoic acids (LTA)[3].

Lipoteichoic acids are considered to play several functional roles in bacteria. Fiedler and Glaser[4,5] proposed that the polymers may function as acceptors for the synthesis of wall teichoic acids and, possibly, act as carrier molecules for peptido-

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glycan precursors. Bacteriophage particles are known to bind lipoteichoic acids in several Gram-positive bacteria[6-8]. In addition, when membrane teichoic acids are added to certain autolysin preparations, the enzymes fail to degrade cell walls readily[9]. These observations suggested that lipoteichoic acid can serve to regulate autolytic activity in the bacterial-cell cycle. Many membrane-bound, biosynthetic enzymes require bivalent cations for activity, and it has been postulated that lipoteichoic acids, known for their metal-binding properties, could serve as needed ion-scavengers during cellular growth[10]. In this context, the lipoteichoic acids could serve to provide the metals for membrane enzymes. Despite the extensive literature on the subject, an exact physiological need for membrane teichoic acids has not been shown. However, the molecules must possess an important role in cell growth, because mutants lacking the polymers have not been described.

Almost all membrane teichoic acids are D-glucosylated, although the extent of glycosylation appears to vary from organism to organism[11]. The D-glucosylation occurs at O-2 of the glycerol moiety, and involves a direct linkage with a D-glucosyl or, in certain cases, a kojibiosyl group[12]. Little is known about the biosynthetic routes leading to D-glucosylation of lipoteichoic acids. With respect to cell-wall teichoic acids, several enzymes that ultimately lead to the D-glucosylation of the *Bacillus subtilis* polymers are known; these are identified in Table I. Mutants of *B. subtilis* that are defective in their ability to incorporate D-glucosyl groups into wall teichoic acids are called *gta* mutants, and several classes have been identified[7,8]. In *gta*A mutants, the enzyme UDP-glucose:teichoic acid·polyglycerol:teichoic acid transferase (TAG-transferase) is lacking. In *gta*B mutants, the cells behave as though they lacked UDP-glucose pyrophosphorylase, although the enzyme can be isolated from the bacteria. Class C mutants lack phosphoglucomutase. Cell-wall teichoic acids isolated from these *B. subtilis* mutants are not D-glucosylated to the normal extent (as compared with the parent). In one of the mutants, *gta*C, the D-glucosylation of wall teichoic acids is possible, provided that the cells are grown in the presence of D-galactose[8].

The present studies were designed to answer two basic questions concerning the lipoteichoic acids of *B. subtilis*. First, are the pathways of D-glucosylation for

TABLE I

CHARACTERISTICS OF *Bacillus subtilis* 168 *gta* MUTANTS

Class	*Phenotypic expression*
*gta*A	D-Glucosylation of teichoic acids, lacks UDP-glucose.polyglycerol:teichoic acid glucosyltransferase
*gta*B	D-Glucosylation of teichoic acids; behaves as if lacking UDP.glucose pyrophosphorylase, but does not have significant enzymic defect
*gta*C	D-Glucosylation of teichoic acids; lacks phosphoglucomutase; D-glucosylation of cell-wall teichoic acid occurs if cells are grown in the presence of D-galactose

wall teichoic acids required for the D-glucosylation of membrane teichoic acids? Second, what is the probable structure of the membrane teichoic acid of *B. subtilis*? These questions have been addressed by isolating lipoteichoic acids from *gta* mutants, examining their compositions, studying their antigenic characteristics, and examining their interactions with a lectin, concanavalin A.

MATERIALS AND METHODS

Bacterial strains. — *Bacillus subtilis* 168 (*trp*C2), *Bacillus subtilis gta*B290 (*trp*C2, *gta*B290), and *Bacillus subtilis gta*C10 (*trp*C2, *gta*C10) were obtained from D. C. Birdsell, University of Florida. The cells were maintained on AK sporulation agar (BBL, Baltimore, Md.). All bacterial strains were grown in Spizizen's[13] minimal-salts medium supplemented with 50 μg of L-tryptophan per mL and 0.08% of casein hydrolyzate. After autoclaving, D-glucose or D-galactose (final concentration, 0.5%) was added to the medium, as needed. After inoculation, cultures were incubated overnight on a gyratory platform-shaker for 18 h at 37° and cells were harvested by centrifugation for 10 min at 6,000 *g*. The cells were then washed twice with cold, de-ionized water, and freeze-dried.

Preparation of crude LTA. — Crude LTA was prepared according to a modification of the method of Wicken *et al.*[14]. Chloroform–methanol [2:1 (v/v). 150 mL] was added to dried cells (6 g), and the suspension was stirred for 1 h at room temperature. The suspension was filtered by use of a No. 5 glass filter, and the insoluble residue was re-extracted with the same solvent. Finally, the extracted cells were air-dried overnight at room temperature, and then extracted with hot, aqueous phenol according to Wicken *et al.*[14]. The cells were dispersed in de-ionized water (120 mL), an equal volume of phenol solution was added, and the mixture was incubated for 30 min in a water bath at 64 to 68°. After incubation, samples were centrifuged for 10 min at 12,000 *g*, and the aqueous layer was dialyzed against de-ionized water, and freeze-dried.

Preparation of purified LTA. — Crude LTA (300 mg) was added to 0.02M $MgCl_2$ (20 mL) along with a few crystals of sodium azide, RNAse (bovine pancreas, Calbiochem, San Diego; 1 mg), and DNAse (bovine pancreas, Calbiochem; 1 mg). The samples were then incubated overnight at 37°, and centrifuged at 3,000 *g* for 5 min, and any insoluble materials were discarded. This RNAse,DNAse-digested LTA (20 mL) was added to 0.4M ammonium acetate (20 mL), and 20 mL of the mixture was applied to a column (71 × 2.5 cm) of agarose (Biogel A-0.5 m, 50–100 mesh; Bio-Rad Laboratories, Richmond, CA). The samples were eluted with 0.2M ammonium acetate, and fractions (10 mL) were collected at a flow rate of 15 mL/h. The absorbance (at 230 nm) of fractions was read by use of a Beckman DB spectrophotometer. Teichoic acid fractions were collected, dialyzed against de-ionized water, and freeze-dried.

Preparation of cell walls. — Cell walls were prepared according to Doyle and Birdsell[15]. Briefly, cells were ruptured at 20,000 lb.in.$^{-2}$ in a French press (American

Instrument Co.), and centrifuged for 5 min at 2,000 *g* to remove unbroken cells. The turbid, supernatant liquor was now centrifuged for 15 min at 35,000 *g* and the white, cell-wall layer was scraped off the pellet, resuspended in distilled water, and recentrifuged; this process was repeated twelve times. The cell walls contained active autolysin and a small proportion of nucleic acids. No further attempts at purification were made.

Chemical analyses. — Glucose was determined with the "Glucostat" reagent-kit (Worthington Biochemical, Freehold, NJ), using D-glucose (Sigma Chemical Co., St. Louis, MO) as the standard. Total hexose was quantitated by the anthrone method of Mokrasch[16]. Phosphorus was assayed by the ashing method of Ames[17]. Protein was analyzed as outlined by Lowry *et al.*[18], using bovine plasma albumin (Dickinson & Co., N.Y.C., N.Y.) as the standard. DNA was determined by use of the diphenylamine reagent described by Richards[19]. RNA was determined by the method of Webb[20]. Glycerol was estimated by the method of Dittmer and Wells[21], using "Spectro Grade" glycerol (J. T. Baker Co., Phillipsburgh, N.J.) as the standard.

Fatty acid analysis. — Each LTA was hydrolyzed according to the method of Ganfield and Pieringer[22]. LTA (5 mL) was hydrolyzed with 0.2M HCl (1 mL) in a Teflon-sealed, screw-capped tube for 1 h at 100°, the hydrolyzate was extracted with 6:4:1 (v/v/v) chloroform–methanol–water, and the aqueous layer was twice more extracted with the same solvent. The extracts were combined, and evaporated to dryness by passing a stream of air (filtered through glass wool) over the tubes. Methanolysis of the extracts was conducted according to the method of Kates[23]. To each sample was added 3% methanolic HCl (1.5 mL), and the samples were incubated for 1 h at 100°, and allowed to cool to room temperature; petroleum ether (2 mL) and distilled water (1 mL) were added to each tube, and the contents were then mixed by vortexing. The supernatant liquors were evaporated to dryness by passing a stream of air (filtered through glass wool) over the tubes. Petroleum ether (20 μL) was added to each sample, and mixed (to dissolve the fatty acid methyl ester). One or two μL of each sample was analyzed in a Hewlett–Packard model 402 gas–liquid chromatograph equipped with a flame-ionization detector. A glass column, packed with 15% of succinoylated ethylene glycol polymer (EGSS-X) on Gaschrom P-4' (Applied Science Lab., State College, PA), was used isothermally at 175°. The samples were examined along with two standards, a short-chain standard (BC-mix-L 0627, Applied Science Lab.) and a long-chain standard (BC-mix-L 2843, Applied Science Lab.). The fatty acids from the LTA samples were qualitatively identified by their relative retention-times, compared to those of the authentic standards. The areas under each peak were integrated, and the peaks summed, and the relative contribution of each peak was calculated.

Interaction between LTA and concanavalin A. — Con A was prepared according to the method of Agrawal and Goldstein[24], by affinity chromatography on Sephadex G-100. LTA (500 μg) was added to dilutions of con A in the presence of 0.05M Tris hydrochrolide buffer (pH 7.4). The mixture (final volume, 1.5 mL) was vortexed, and incubated overnight at 20°. After incubation, samples were centrifuged for 3 min

at 17,000 *g*, and the layer of precipitate was vortexed, and washed with buffer (5 mL). The suspensions were then centrifuged for 3 min at 17,000 *g*, and the supernatant layers were decanted. Sodium hydroxide (0.1M; 2 mL) was added to dissolve the precipitate, and the amount of protein present was determined by the Lowry method[18], with con A as the standard. Con A solutions were standardized by an ultraviolet-absorbance measurement, assuming that[25] 1 mg of con A per mL gives an absorbance of 1.14 at 280 nm.

Immunological studies. — A methylated bovine serum albumin–LTA complex (mBSA–LTA) was prepared according to Fiedel and Jackson[26]. Briefly, mBSA (4 mg) was added to distilled water (4 mL) as solution A, and LTA (1 mL) was dissolved in distilled water (4 mL) as solution B. Solution A was added dropwise to solution B, followed by vigorous stirring after each drop. When the addition was complete, the mixture was incubated for 15 min at 37°, centrifuged for 10 min at 120 *g*, and the precipitate collected. The precipitate was washed with distilled water (2 mL), 2% Tween 80 (4 mL) was added, and the precipitate was dispersed by vortexing. The suspension was then mixed with Freund's incomplete adjuvant (Difco Lab., Detroit, MI; 2 mL) in a hypodermic syringe until homogeneity was obtained; mixing was repeated prior to each injection. Pre-immune blood was collected, and mBSA–LTA–Freund's incomplete adjuvant (1 mL) was injected intradermally at various sites on the neck and hind legs of a rabbit. After 1 week, blood was collected, and 1 mL of antigen with adjuvant (id) on adjacent sites was given, to provoke an anamnestic response. Weekly injections (1 mL) were administered until the experiment was terminated.

Titers of mBSA–LTA antisera were determined by passive hemagglutination (PHA) of sensitized erythrocytes. Sensitized sheep-erythrocytes were prepared by adding LTA extract (2 mL) and 8 mL of PBS-A (phosphate-buffered saline A containing 1.416 g of Na_2HPO_4, 0.018 g of KH_2PO_4, and 8.418 g of NaCl per liter of solution, pH 7.4) to packed, washed erythrocytes (0.2 mL). Cells were suspended, and allowed to incubate for 30 min at 37°. Sensitized cells were collected by centrifugation, washed twice with PBS-A, and suspended in PBS-A (10 mL). In the assay, 0.1 mL of serial dilutions of serum in PBS was mixed with 0.1 mL of sensitized, sheep red-blood cells, and incubated for 1 h at 37°. The titers were expressed as the reciprocal of the highest serum dilution giving hemagglutination. Agglutination was confirmed by observation of all tubes under the microscope.

Gel electrophoresis. — Purified LTA preparations were dissolved in a buffer containing 10mM sodium phosphate, 1% (w/v) sodium dodecyl sulfate (SDS), and 1% (v/v) 2-mercaptoethanol. The pH was adjusted to 7.0 prior to dissolution of the samples. Fifty μL of each sample was applied to 10% poly(acrylamide) gels (8 × 0.5 cm) containing SDS (see ref. 27). The gels were treated at a constant current of 8 mA/gel for 10 h. Protein standards (bovine serum albumin, mol. wt. 68,000; ovalbumin, mol. wt. 44,000; pepsin, mol. wt. 35,000; trypsin, mol. wt. 23,300; and ribonuclease, mol. wt. 13,700) were used in parallel. The gels were stained with Coomassie Brilliant Blue R-250, and de-stained with acetic acid–methanol–water[27].

Determinations of molecular weight were performed according to Weber and Osborn[27].

RESULTS

Prior to the preparation of lipoteichoic acids, it was necessary to verify the phenotypic characteristics of the mutants. On the basis of known, enzymic defects, it would be predicted that *gta*B and *gta*C mutants would have low levels of hexose in their cell walls. In addition, when *gta*C is grown in the presence of D-galactose, normal D-glucosylation of its cell wall should occur. Accordingly, walls were prepared from *B. subtilis* 168 (wild type) and from the *gta* mutants, and subsequently analyzed for hexose. The data, shown in Table II, clearly demonstrate the predicted behavior of the mutants. The parent, *B. subtilis* 168, contained approximately the same proportions of hexose and phosphorus regardless of the carbohydrate added to the medium. Similarly, the proportions of hexose and phosphorus in the walls of *gta*B290, although much lower, did not vary with the carbon source. In contrast, the proportion of hexose in the cell wall of *gta*C10 was strongly dependent on the carbon source in the medium.

The preparation of pure lipoteichoic acids has presented a difficult problem for several years. Most preparations of LTA have been heavily contaminated with protein and nucleic acids. In this study, initial attempts were made to purify the lipoteichoic acids by conventional means. The first method used was originally described by Wicken *et al.*[14], and involved a hot-water extraction (100°) of whole cells treated with chloroform–methanol. The material obtained by lyophilization of the water extract was highly insoluble in buffers and salt solutions. In addition, ~65% of the product was protein and nucleic acid. The phenol–water extraction method, also described by Wicken *et al.*[14], was then attempted, but, again, the final products contained a large proportion of contaminants. Finally, a modification of the phenol–

TABLE II

CELL-WALL HEXOSE AND PHOSPHORUS CONTENTS OF *Bacillus subtilis gta* MUTANTS

Strain of B. subtilis	*Growth conditions*[a]	*Hexose (μmol/mg)*	*H/P*[b]
168	D-glucose	1.08	0 81
168	D-galactose	1.12	0 80
*gta*C10	D-glucose	0.03	0 05
*gta*C10	D-galactose	0.80	0 62
*gta*B290	D-glucose	0 02	0 03
*gta*B290	D-galactose	0 05	0.05

[a]Cells were grown in Spizizen's minimal medium[13], supplemented with the carbohydrate indicated (0.5%, final concentration), 50 μg of L-tryptophan per mL, and 0 08% casein hydrolyzate. [b]H/P, molar ratio of hexose to phosphorus.

Extract 6 g of freeze-dried cells
with 2:1 (v/v) $CHCl_3$-CH_3OH (375 mL)
↓
Extract dry residue with 0.44:1 (v/v)
phenol–water (120 mL) at 66°,
repeat, combine aqueous phases
↓
Dialyze aqueous extract *vs*
water, freeze-dry
↓
Incubate (18 h) with
DNAse and RNAse (pH 5 1)
↓
Chromatography on agarose

Scheme 1. Isolation of the lipoteichoic acid from *Bacillus subtilis* 168 and its *gta* mutants.

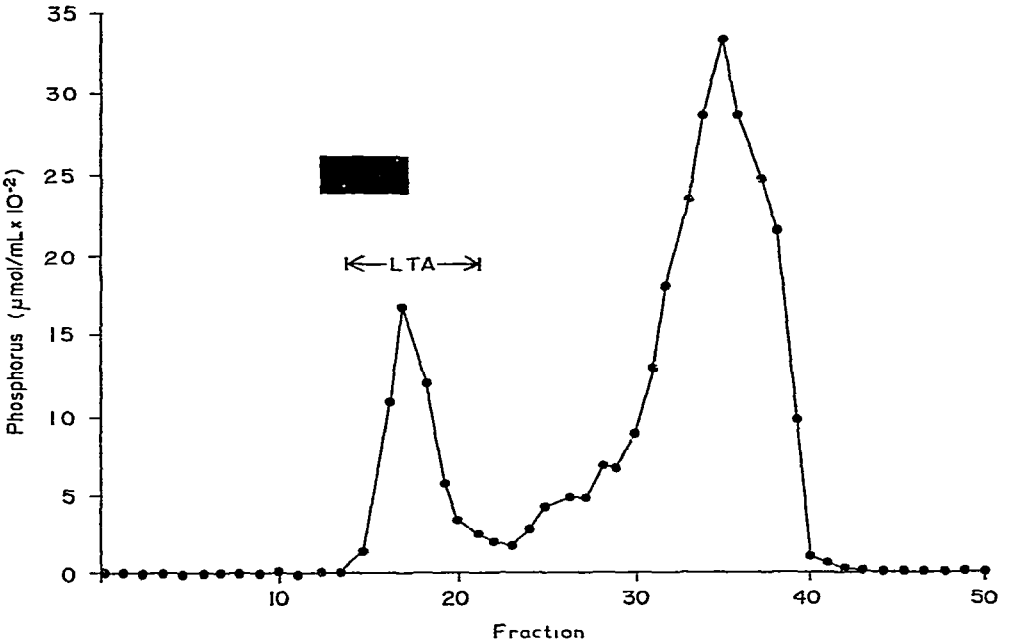

Fig. 1. Agarose elution-profile for the partially purified lipoteichoic acid of *Bacillus subtilis* 168.

water method, outlined in Scheme 1, was devised that gave a product of acceptable purity. Cells were treated with chloroform–methanol to extract lipids not covalently bound to macromolecules. The cells were then extracted with phenol–water at 66°, a temperature at which the solvents form a single phase (after cooling, the phenol and water phases reappear, and most of the protein is extracted into the phenol phase). The aqueous phase was collected, and digested with RNAse and DNAse, to degrade the nucleic acids, and the digest was subjected to chromatography on agarose. A typical elution-profile for the digest from an agarose column is shown in

TABLE III

CHEMICAL COMPOSITION[a] OF LIPOTEICHOIC ACIDS OF *Bacillus subtilis*

Strain	*Carbon source*[b]	*P*	*Glycerol*	*Glucose*	*Protein*	*RNA*	*DNA*
168	D-glucose	2.71	2.50	0.17	73.4	6 0	1.6
*gta*B290	D-glucose	2 90	2.49	0 01	66.1	4.1	1.9
*gta*C10	D-glucose	2 97	3 06	0.15	50 7	5.1	0 8
*gta*C10	D-galactose	2.93	2 16	0 08	51 0	8 3	3.7

[a]Values given for phosphorus, glucose, and glycerol are in μmol/mg Values for protein, RNA, and DNA are in μg/mg. [b]D-Glucose or D-galactose (0 5%) was used to supplement the basic growth-medium.

Fig. 1. The profile, measured by the presence of phosphorus, shows three distinct peaks. The first peak consists of the LTA. The second, small peak (fractions 25–28) is probably a contaminant (wall teichoic acid or deacylated LTA). The last peak, containing phosphorus, consists of degraded RNA and DNA. The LTA emerges near the void volume as shown by the boxed area. The apparently high molecular weight of the LTA is probably due to its tendency to form micelles. The profile shown in Fig. 1 was similar for all lipoteichoic acids examined. In addition, it was found that the contents of the effluent could be monitored by measurement of absorbance at 230 nm; most experiments were subsequently made by using this method for determining the major peak of the phosphorus-containing material.

The data in Table III show the chemical compositions of the LTA preparations. The preparations were analyzed for phosphorus, glycerol, and D-glucose, and for nucleic acid and protein contaminants. The approximately equal molar ratios of glycerol and phosphorus suggest that the LTA preparations contained the expected poly(glycerol phosphate). In terms of content of D-glucose, the parent 168 strain contained 0.17 μmol/mg of LTA, whereas the *gta*B strain contained 0.1 μmol/mg of LTA. The low proportion of D-glucose in the 168 strain indicates that the extent of D-glucosylation of the LTA is not so great as that for cell-wall teichoic acids (see Table II). All of the hexose present in the preparations could be accounted for as D-glucose. None of the LTA preparations were completely free from contaminants (see Table III). However, the extent of contamination of these preparations by protein and nucleic acid was generally lower than has been reported for membrane teichoic acids from other bacteria.

When all four purified preparations of lipoteichoic acids (see Table III) were subjected to electrophoresis in SDS–2-mercaptoethanol, several distinct protein-bands appeared (see Fig. 2). The major bands, of molecular weights 64,000, 26,000, 18,000, and 16,000, were common to each sample. Several faint, nonreproducible bands were occasionally found near the polypeptide of mol. wt. 16,000 (see Fig. 2). The common, protein bands may be the result of adventitious binding of proteins by the LTA

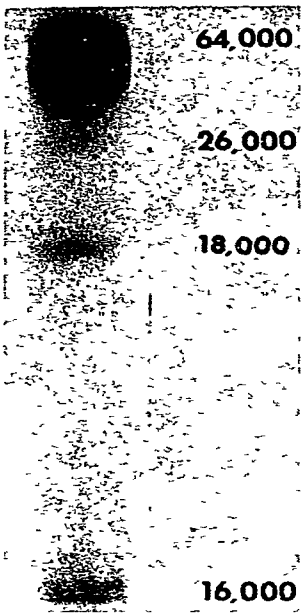

Fig. 2. Gel-electrophoresis pattern of *Bacillus subtilis* 168 LTA [The numbers on the right of the stained gel show the apparent molecular weights (in thousands) of the proteins associated with the lipoteichoic acid.]

TABLE IV

FATTY ACID COMPOSITION[a] OF LIPOTEICHOIC ACIDS OF *Bacillus subtilis*

Fatty acid	*Strain*			
	168	gta*B290*	gta*C10Glc*[b]	gta*C10Gal*[b]
i-C_{14}	0.16	0.12	0 44	0 42
n-C_{14}	0 44	1.39	0 26	0 58
i-C_{15}	14 12	18 03	21 27	15.16
a-i-C_{15}	19 80	26 52	38.25	19 86
i-C_{16}	3.39	2.46	2.54	2 50
n-C_{16}	9 64	7 22	5 03	4 55
i-C_{17}	16 45	13.36	12.34	20.21
a-i-C_{17}	25 00	23 40	18 89	22 17
n-C_{18}	7.50	4 07	0.95	10.69
a-i-C_{19}	3.54	3 22	trace	3.86

[a]Values are percentages of the total fatty acid composition. [b]Glc and Gal refer to D-glucose and D-galactose as the carbon source for growth.

molecules, or may reflect the presence of LTA–protein complexes in the *B. subtilis* membrane. Because of the extensive, preparative procedures, it is unlikely that non-specific proteins could have remained associated with the lipoteichoic acids.

In addition to determination of the content of D-glucose, phosphorus, glycerol,

protein, and nucleic acids, the LTA preparations were also analyzed for fatty acids. The LTA samples were hydrolyzed with acid, and the fatty acids liberated were converted into their methyl esters. The volatile derivatives were then subjected to gas–liquid chromatography; the data are tabulated in Table IV. A total of ten different kinds of fatty acid was found. The majority were* *i*-C_{15}, *a-i*-C_{15}, *i*-C_{17}, and *a-i*-C_{17} acids. However, when grown in the presence of D-galactose, the *gta*C10 strain showed a relatively large proportion of *n*-C_{18} acids. This result was due to a property of the strain, because, when the wild type (168 strain) of the organism was grown in D-galactose solution, the proportion of *n*-C_{18} acids present in the LTA was approximately equal to that present when the cells were grown in D-glucose solution. In general, the fatty acid composition appears to reflect the known fatty acid composition for the protoplast membranes of *B. subtilis*[28].

It has been shown by Doyle and Birdsell[15] that con A reacts specifically, and reversibly, with α-D-glucosylated, cell-wall teichoic acids. Thus, if the LTA preparations contain α-D-glucosyl residues having unsubstituted hydroxyl groups at C-3, C-4, and C-6, precipitation with con A should occur. Lipoteichoic acid (500 μg) was mixed with various concentrations of con A, incubated overnight, and the resulting precipitates analyzed for protein. The results, shown in Fig. 3, reveal that all of the

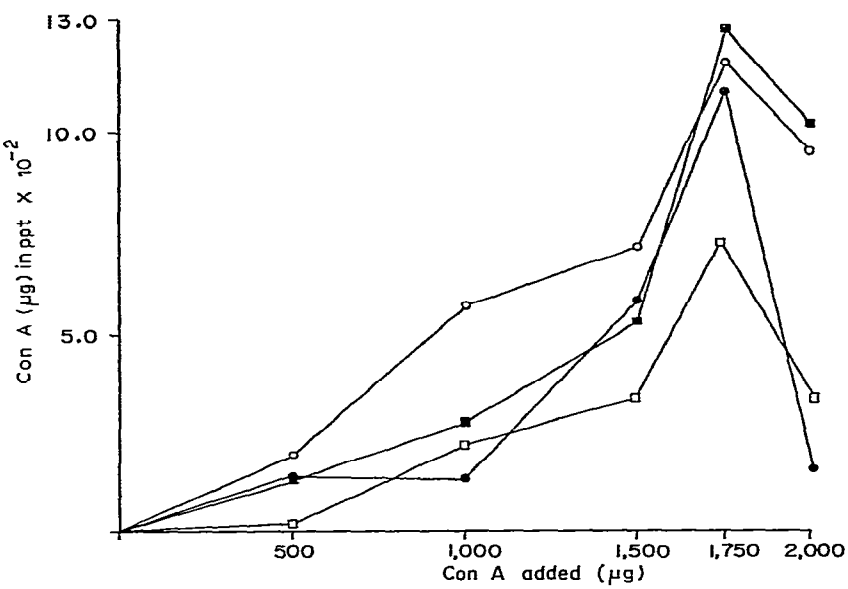

Fig. 3. Precipitate formation between concanavalin A and *Bacillus subtilis* lipoteichoic acids [Lipoteichoic acid (0.5 mL, 500 μg) was added to 1.0 mL of the indicated concentration of con A. Following an overnight incubation, precipitates were collected by centrifugation (17,000 *g*, 4 min), and washed in 5 0 mL of Tris hydrochloride (0 05M, pH 7.4). After being washed, the precipitates were dissolved in 0.1M NaOH, and analyzed for protein by the procedure described by Lowry *et al*[18] *B. subtilis* 168, ○——○; *B. subtilis gta*B290, ■——■; *B subtilis gta*C10Glc, ●——●; and *B. subtilis gta*C10-Gal, □——□.]

*The symbol *i* denotes iso; *a-i*, ante-iso; and *n*, normal.

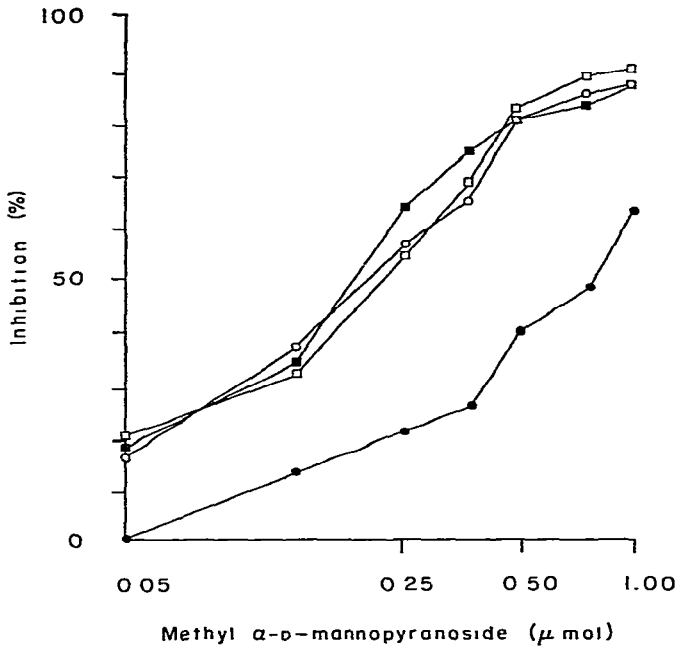

Fig. 4. Inhibition of the reaction between concanavalin A and the lipoteichoic acids of *Bacillus subtilis* 168 and its *gta* mutants by methyl α-D-mannopyranoside [Concanavalin A (1.750 mg, 1 0 mL) and lipoteichoic acids (90.5 mL, 500 μg) were incubated in the indicated concentrations of methyl α-D-mannopyranoside. After an overnight incubation, the precipitates were collected by centrifugation (17,000 *g*, 4 min), washed in 5 mL of Tris hydrochloride (0.05M, pH 7 4), and subsequently dissolved in 0.1M NaOH. Samples were removed for protein analysis *B subtilis* 168, ●——●, *B subtilis gta*B290, □——□; *B subtilis gta*C10Glc, ■——■; and *B. subtilis gta*C10Gal, ○——○.]

LTA preparations gave precipitin-like complexes with con A The amount of con A precipitated was proportional to the amount of D-glucose in the LTA preparation (see Table III and Fig. 3). The *gta*B lipoteichoic acid, although low in D-glucosyl residues, reacted with con A. The reactions were readily reversed by methyl α-D-mannopyranoside (MeαMan*p*) (see Fig. 4). The amount of MeαMan*p* required for 50% inhibition was ~0.24 μmol for lipoteichoic acids from *gta*B290 and *gta*C10. In contrast, ~0.9 μmol of MeαMan*p* was required for 50% inhibition of the wild type of lipoteichoic acid. These results confirm that the D-glucose is attached to the lipoteichoic acid molecules in the α-anomeric configuration.

Should the lipoteichoic acids from the parent strain and the mutants be similar in structure, they would probably exhibit similar antigenic specificities. Antibodies were raised in rabbits against lipoteichoic acids from *B. subtilis* 168 and from strain *gta*B290. [Strain 168 contains the most extensively D-glucosylated lipoteichoic acid, whereas the *gta*B290 strain contains very little D-glucose in its membrane teichoic acid (see Table III).] Pre-immune serum was obtained from each rabbit. The rabbits were then given weekly injections of an mBSA–LTA complex emulsified in Freund's incomplete adjuvant. Sera were collected each week prior to the administration of booster antigen. Sheep red-blood cells were coated with LTA, washed, and resus-

TABLE V

SERUM ANTILIPOTEICHOIC ACID TITER AFTER INJECTIONS OF AN mBSA–LTA COMPLEX[a]

Rabbit	*LTA*	*Week*				
		Preimmune	*2*	*3*	*4*	*5*
1	168	16	16	32	32	64
2	*gta*B290	64	128	256	1024	1024
3	*gta*B290	32	32	128	1024	1024

[a]Following adsorption with sheep red-cells, the serum titers were determined by passive hemagglutination.

TABLE VI

CROSS-REACTIONS BETWEEN ANTI-LTA ANTISERA AND MEMBRANE TEICHOIC ACID PREPARATIONS OF *Bacillus subtilis* MUTANTS[a]

Anti-serum directed against	*LTA used to sensitize red cells*	*Titer*
*gta*B290 LTA	*gta*B290	64
*gta*B290 LTA	168	64
*gta*B290 LTA	*gta*C10 (grown in the presence of D-glucose)	64
*gta*B290 LTA	*gta*C10 (grown in the presence of D-galactose)	64
*gta*B290 LTA	*gta*B290	1064
*gta*B290 LTA	168	1064
168 LTA	*gta*B290	64
168 LTA	168	64

[a]Anti-sera were adsorbed with sheep red-cells prior to use. Wells contained anti-serum (50 uL) and sensitized, sheep red-cells (50 uL) Anti-*gta*B290 sera shown were obtained at different times during the immunization of a rabbit, and reflect changes in titer against the homologous antigen.

TABLE VII

INHIBITION OF PASSIVE HEMAGGLUTINATION BY CELL-WALL AND LIPOTEICHOIC ACIDS[a]

Teichoic acid[b]	*Amount of TA required for hemagglutination inhibition (μg)*
*gta*B290 membrane	3.1
168 membrane	6.3
*gta*C10 (D-glucose-grown) membrane	3.1
*gta*C10 (grown in the presence of D-galactose) membrane	6.3
168 cell wall	0 80

[a]Rabbit anti-*gta*B290 LTA antiserum was used at a titer of 32 in all of the experiments. Prior to use, anti-sera preparations were adsorbed with sheep red-blood cells. [b]The test system contained 50 μL of antiserum (titer = 32), 100 μL of sensitized, sheep red-blood cells, and 50 μL of LTA (or wall teichoic acid) or 50 μL of buffer. The red cells were sensitized with *gta*B290 LTA.

pended in buffered saline. The sensitized cells were then mixed with dilutions of serum and the agglutination was measured visually. The results are given in Table V, from which it may be seen that the animals injected with *gta*B290 antigen responded with increasing titers of antibody following the weekly challenges. At the end of five weeks (four weekly injections), the titer for both animals was 1024. In contrast, the animal injected with the 168 lipoteichoic acid produced a much lower titer over the same period of time. All of the animals showed a pre-immune titer of 16 to 32. When the antisera were tested with all of the antigens (lipoteichoic acids from strain 168, *gta*B290, *gta*C10Gal, and *gta*C10Glc), the respective antisera titers remained constant (see Table VI). For example, an anti *gta*B290 LTA serum gave a titer of 64 against 168 LTA as well as against the other antigens.

Attempts to inhibit passive hemagglutination with glycerol, D-glucose, glycerol 1-phosphate, or glycerol 2-phosphate (up to 50 mmol of each inhibitor) were unsuccessful. When soluble LTA or a cell-wall teichoic acid was used as inhibitor, it was observed that only a small proportion was necessary in order to prevent passive hemagglutination (see Table VII). The cell-wall teichoic acid was a much more potent inhibitor of the agglutination than the preparations of membrane teichoic acid. The inhibition data and the observed cross-reactivities of the anti-sera with the LTA preparations (see Table VI) suggest that the antigenic determinant is a unit of a poly(glycerol phosphate).

DISCUSSION

The partial purification of the lipoteichoic acids was made possible by successively extracting whole cells with chloroform–methanol, and with phenol–water at 66°. The crude, water-soluble lipoteichoic acids were then digested with nucleases, and the products chromatographed on agarose. The lipoteichoic acids emerged near the void volume. Analysis showed that most of the preparations contained relatively small proportions of protein and nucleic acids. Wicken *et al.*[14] had pointed out the difficulty of preparing lipoteichoic acids that are completely free from contaminants, but the extent of contamination by protein and nucleic acids for the lipoteichoic acids reported in this study is generally lower than that reported by others.

In terms of chemical composition, all of the lipoteichoic acids contain approximately equimolar proportions of phosphorus and glycerol, which would be expected if the lipoteichoic acid molecules contain alternating glycerol residues and phosphate groups. The observation that glycerol phosphates did not inhibit the agglutination of lipoteichoic acid-sensitized erythrocytes by antilipoteichoic acid antisera further suggests that the polymers contain long-chain glycerol phosphate residues. Decker *et al.*[29] found that short-chain polymers of glycerol phosphate inhibit the reaction between a cell-wall teichoic acid of *B. subtilis* and its specific antiserum.

The presence of acid-labile, fatty acids in the lipoteichoic acids confirms the designation of the preparations as lipoteichoic acids. In *B. subtilis*, all of the fatty acids are found in the membrane[28]. The fatty acid composition of the lipoteichoic

acids as determined in this study parallels the known fatty acid composition of *B. subtilis* membranes[28]. These results are consistent with the findings of others, who have shown that the fatty acid composition of lipoteichoic acids generally conforms to the spectrum of fatty acids found in chloroform–methanol extracts of whole cells or cell membranes[11].

Adsorption of bacteriophage by intact *B. subtilis* cells depends on the presence of D-glucosylated teichoic acids[30]. The mutants described in this study were derived from cells demonstrating spontaneous resistance to the lytic bacteriophage Ø25. When cell walls were examined for hexose, it was found that *B. subtilis gta*B290 and *B. subtilis gta*C10 (grown in the presence of D-glucose) contain low levels of carbohydrate (see Table II). A central question of the present studies was whether mutations in the pathway for D-glucosylation of cell-wall teichoic acids were also expressed in D-glucosylation of lipoteichoic acids. The data show that D-glucosylation of membrane lipoteichoic acid is much lower than for cell-wall teichoic acid (see Tables II and III). For example, the content of hexose in cell-wall preparations of *B. subtilis* 168 was 1.08 μmol/mg, whereas the membrane teichoic acid from the same organism contained only 0.17 μmol/mg. The hexose:phosphorus ratio for the parent 168 organism was 0.81:1 for cell-wall and 0.06:1 for membrane teichoic acids. The *gta*B290 strain had a hexose:phosphorus ratio of 0.02:1 for cell-wall and 0.03:1 for membrane teichoic acids. These data clearly show that the mutation in class B mutants (UDP-glucose: pyrophosphorylase) is expressed in both the cell-wall and membrane teichoic acids. The class C mutant, in contrast, does not appear to express the mutation in its lipoteichoic acid. For example, when *gta*C10 was grown in the presence of D-glucose, the cell wall contained 0.03 μmol of hexose per mg of wall, and a hexose:phosphorus ratio of 0.05:1 (see Table V). The lipoteichoic acid from the same strain contained 0.15 μmol of D-glucose per mg, with a D-glucose:phosphorus ratio of 0.62:1. The lipoteichoic acid from the D-galactose-grown cells contained 0.08 μmol of D-glucose per mg, and had a D-glucose:phosphorus ratio of 0.27:1. The latter value is the direct opposite of that predicted on the basis of analysis of the cell walls. These results imply that the D-glucosylation of lipoteichoic acids of *B. subtilis* does not require phosphoglucomutase, and suggest an alternative pathway, as yet obscure, that is independent of phosphoglucomutase in the D-glucosylation of membrane teichoic acids. Knox and Wicken[31] had shown that the cell-wall teichoic acid of *Lactobacillus helveticus* contains more of an α-D-glucosyl component than the LTA from the same bacterium.

The immunochemical studies of the lipoteichoic acids (see Table V) showed that the polymers are antigenic when complexed with mBSA. Because the antibody titer from any one of the animals injected was the same for the different lipoteichoic acid preparations, it is inferred that the antigenic determinant-groups for the preparations are identical. Evidently, the antigenic determinants are segments of the poly-(glycerol phosphate) chains. Monomeric glycerol 2- or 3-phosphate, glycerol, or D-glucose did not inhibit passive hemagglutination, although both the cell-wall teichoic acid and the LTA were potent inhibitors. Kabat *et al.*[32] reported that the

antigenic determinant-groups of *Staphylococcus aureus* cell-wall teichoic acid were either 2-acetamido-2-deoxy-α- or -β-D-glucose. However, the teichoic acid preparations used by these authors were almost fully substituted on the ribitol residue by the amino sugars. In the preparations used in the present study, the extent of D-glucosylation of the teichoic acid chain was probably too low for the hexose to act as an antigenic determinant-group (see also, ref. 33).

In all of the immunological studies, it must be borne in mind that the antigens were not pure lipoteichoic acids. In Table II, it was shown that the LTA preparations were contaminated with small proportions of protein and nucleic acids, and it is possible that the precipitating antibodies were directed against these contaminants; there is, at present, no definitive method for precluding the foregoing possibility. The failure of RNA, DNA, or soluble protein from *B. subtilis* to inhibit passive hemagglutination strongly suggests that these macromolecular constituents were not responsible for eliciting the immune response observed. In addition, the observation that the cell-wall teichoic acid was a potent inhibitor of passive hemagglutination must be taken as evidence for a poly(glycerol phosphate) determinant.

The observation that con A precipitated all of the lipoteichoic acid preparations (see Fig. 3) indicates that the polymers were substituted with (terminal) α-glucopyranosyl groups (see ref. 34). It is surprising that the lipoteichoic acid of strain *gta*B290 precipitated with the lectin, especially in view of the fact that the teichoic acid contained only 0.01 μmol of hexose per mg. Presumably, the extent of D-glucosylation was high enough for con A to recognize the D-glucosyl groups, and, in turn, to form an insoluble complex with the lipoteichoic acid. The complex did not result from electrostatic or nonspecific interactions, because the con A inhibitor, methyl α-D-mannopyranoside, readily prevented the formation of an insoluble matrix (see Fig. 4). Although no reports have appeared that take advantage of the affinity of con A for lipoteichoic acids, it is suggested that the lectin could be used to aid in the purification of the polymers. In this regard, Doyle *et al.*[35] showed that the cell-wall teichoic acid of *B. subtilis* 168 could be purified by one-step affinity chromatography on an agarose–con A conjugate.

It is impossible completely to assess the structures of the lipoteichoic acids from the results in this study. However, certain features are apparent that may provide insight regarding the probable structures. The preparations were nondialyzable, and emerged near the void volume on an agarose column, indicating that the lipoteichoic acids were polymers of high molecular weight, or existed as micelles. All of the preparations contained approximately equimolar proportions of phosphorus and glycerol. It is likely, therefore, that the lipoteichoic acids are polymers of a glycerol phosphate, in agreement with the expected, polymeric nature of membrane teichoic acids.

When D-glucosylation occurred, the anomeric linkages were in the α-D configuration as evidenced by precipitability with con A. In this context, D-glucosylation of the lipoteichoic acids of *B. subtilis* 168 appears to parallel the D-glucosylation of cell-wall teichoic acids. The pathways leading to the D-glucosylation of lipoteichoic

acid may not be totally common with respect to cell-wall teichoic acids. The *gta*B mutation led to depressed levels of D-glucose in both the wall and membrane teichoic acids. However, the *gta*C mutation did not result in a depressed level of D-glucose in the membrane teichoic acid, regardless of the presence of D-glucose or D-galactose in the growth medium.

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