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Serological Studies on the Teichoic Acids of *Lactobacillus plantarum*

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Both the wall ribitol teichoic acid and the membrane glycerol teichoic acid (lipoteichoic acid) from *Lactobacillus plantarum* NCIB 7220 have α-D-glucosyl substituents. Antisera to the ribitol teichoic acid were obtained by injecting whole or disintegrated organisms, the antigenicity of the wall teichoic acid apparently depending on its association with protein. It was necessary to inject disintegrated organisms or purified lipoteichoic acid to ensure the production of antibodies to the glycerol teichoic acid; these antibodies did not react with ribitol teichoic acid. The specificity of antibodies to the wall ribitol teichoic acid depends primarily on the α-D-glucosyl substituents, as the antibodies cross-react with α-D-glycosyl-substituted glycerol teichoic acids but not with an unsubstituted ribitol teichoic acid. The specificity of antibodies to the membrane glycerol teichoic acid may be directed against either the glucose or glycerol components, depending on the preparation injected.

Investigations by Sharpe, Davison, and Baddiley (21) on the serological properties of the teichoic acids from lactobacilli identified a number of the group antigens. In group D lactobacilli, the specific substance was isolated from the wall of *Lactobacillus plantarum* NCIB 7220 and was shown to be a glucosyl-substituted ribitol teichoic acid. It was concluded that its structure was similar to that reported earlier for strain 17-5 of *L. plantarum* (previously designated *L. arabinosus*), in which there were some unsubstituted ribitol phosphate units and some units with one or two α-D-glucosyl residues attached (2). More recent studies have shown that some strains of *L. plantarum* lack the group-specific ribitol teichoic acid (1), thus accounting for the observation that not all strains classified as *L. plantarum* reacted with group D antiserum (20).

Strains in group D are the only known examples of lactobacilli containing ribitol teichoic acid. Glycerol teichoic acids occur more frequently, being found as group-specific wall components in strains from groups A and E (15, 16, 21), and as membrane components of all strains (3). This report describes comparative studies on the antigenicity of the wall ribitol teichoic acid and the membrane glycerol teichoic acid from *L. plantarum* NCIB 7220, and on the specificity of the resultant antibodies.

MATERIALS AND METHODS

Organisms. *L. plantarum* NCIB 7220 was kindly supplied by M. E. Sharpe, National Institute for Research in Dairying, Reading, England, and the R1 mutant of *L. plantarum* ATCC 10241, by W. J. Wolin, College of Agriculture, University of Illinois, Urbana. Strain NCIB 7220 belongs to serological group D (20); the R1 mutant forms a cell wall teichoic acid virtually lacking the D-glucose substituent of the parent strain (L. J. Douglas and M. J. Wolin, Bacteriol. Proc., p. 72, 1970). Organisms were grown for 18 hr at 37 C in the medium described by Sharpe, Davison, and Baddiley (21).

Preparation of teichoic acids. Wall and membrane teichoic acids were isolated and characterized by the procedures previously used in studies on lactobacilli of groups F and A (15, 22). The method for obtaining membrane teichoic acid employs phenol extraction, and, in contrast to the usual extraction with trichloroacetic acid, yields a high-molecular-weight teichoic acid-lipid complex (lipoteichoic acid).

Preparation of walls. Organisms were disrupted and walls (preparation I) were obtained after trypsin and ribonuclease treatment as described previously (18). Wall preparation IIa was obtained from disintegrated organisms by centrifugation and washing with 0.85% NaCl and distilled water (8). Part of this preparation was treated with hydroxylamine (preparation IIb) to remove D-alanyl ester residues from teichoic acid (8, 13). Further portions of preparation IIa were digested with trypsin and ribonuclease (preparation IIc), treated with 4% sodium dodecyl sulfate at 100 C (preparation IId; 4), heated at 100 C for 1 hr in phosphate buffer, *p*H 6 (preparation IIe; 12), and washed with 0.5 M KCNS (preparation IIf).

Preparation of substituted alditols. 4-*O*-α-D-glucosylribitol and 3:4-di-*O*-α-D-glucosylribitol were obtained from alkali hydrolysates of wall teichoic acid from *L. plantarum* NCIB 7220 (2), and were purified

by repeated preparative paper chromatography on washed Whatman 3 MM paper in butan-1-ol-pyridine-water (6:4:3, v/v). These were identical in their properties with the products isolated from *L. arabinosus* 17–5 (2). 2-*O*-α-D-glucosylglycerol was similarly obtained from 60% hydrofluoric acid hydrolysates of wall teichoic acid from *L. helveticus* NCIB 8025 (15).

Glycerol - phosphoryl - glycerol - phosphoryl - glycerol (G_3P_2) was prepared by mild deacylation (24) of beef heart cardiolipin (General Biochemicals, Chagrin Falls, Ohio).

Serological methods. The procedures used for obtaining antisera and testing serological reactivity have been described previously (9, 14). For the preparation of antisera to walls, the different preparations (10 mg/ml in 0.85% NaCl) were injected intravenously into rabbits twice a week for 3 weeks, the doses being 0.1, 0.2, and 0.5 ml, and three doses of 1.0 ml.

Detection and separation of IgM and IgG. Reduction of antisera with 2-mercaptoethanol was carried out as described by Yoshida and Ekstedt (25).

Serum (2-ml portions) was fractionated on a column (40 by 2.6 cm) of Sephadex G-200 (Pharmacia, Uppsala, Sweden) equilibrated with 0.05 M potassium phosphate buffer (*p*H 7.3) containing 0.4 mole of NaCl and 0.2 g of NaN_3 per liter (11). Elution with the same buffer was carried out at a flow rate of 10 to 12 ml/hr and 4-ml fractions were collected. The extinction at 280 nm of the column eluate was recorded continuously with a Uvicord II (LKB, Sweden) with the use of a 3-mm flow cell.

RESULTS

Isolation and characterization of teichoic acids. Wall teichoic acid, extracted with trichloroacetic acid from strain NCIB 7220, was eluted from columns of Sephadex G-75 as a sharp peak with $Kd \simeq 0.5$. The mole ratio of glucose to phosphorus was 0.95:1.00. Degradation with alkali, and alkali followed by phosphomonoesterase, yielded mono- and diglucosyl substituted ribitols as well as products indicative of unsubstituted ribitol residues in the polymer. Acid hydrolysis showed that the only components detectable were those expected of a ribitol teichoic acid. The mole ratio of glucose to phosphorus in walls (preparation I) was 0.82:1.00, lower than that for the isolated material but indicative that the soluble preparation of teichoic acid is similar to the polymer in the wall in its degree of sugar substitution.

Wall teichoic acid from the R1 mutant showed the acid and alkali degradation products expected for a polyribitol phosphate, and quantitative analysis of acid hydrolysates gave a mole ratio of glucose to phosphorus of 0.05:1.00 compared with 0.07:1.00 for whole cell walls.

The membrane teichoic acid from strain NCIB 7220 had similar chromatographic properties on columns of 6% agarose to the lipoteichoic acids from *L. fermenti* and *L. helveticus*, which have been characterized as glycerol teichoic acid in a complex with glycolipid and protein (15, 23). The presence of lipid was indicated by the detection of fatty acids in acid hydrolysates. Alkali hydrolysis followed by phosphomonoesterase treatment gave a 2-substituted glycerol, chromatographically indistinguishable from 2-*O*-α-D-glucosylglycerol, and indicative of a glucosyl-substituted glycerol teichoic acid. Traces of a diglycosyl-1-glycerol containing glucose and galactose were also detected; this is consistent with the presence of a glycolipid. The mole ratios of glucose to galactose to phosphorus were 0.19:0.03:1.00, the minor galactose component probably reflecting the glycolipid component characteristic of other lipoteichoic acids (15, 22).

TABLE 1. *Reactivity of antisera to Lactobacillus plantarum NCIB 7220 with wall and membrane teichoic acids*

Rabbit	Antibody precipitated (mg/ml)		
	Wall teichoic acid	Membrane teichoic acid	
		Whole serum	Absorbed serum[a]
162	0.50	0.65	—[b]
163	1.3	1.1	—
164	1.3	1.0	—
187	0.42	0.93	0.49
188	0.42	0.52	—
214	1.2	1.1	—
215	1.2	1.3	—
216	1.5	2.1	0.48

[a] Serum absorbed with wall teichoic acid so that no further reaction was given with wall teichoic acid.

[b] No significant reaction.

Reaction of teichoic acids from strain NCIB 7220 with homologous antisera. Intravenous injection of whole organisms into rabbits yielded antisera reacting with wall teichoic acid from strain NCIB 7220. By the quantitative precipitin method, the antibody content of sera from eight rabbits varied from 0.4 to 1.5 mg/ml (Table 1). Analysis of precipitates formed on adding various amounts of teichoic acid to 10 ml of antiserum 162 suggested the presence of ribitol teichoic acid molecules differing in the extent of glucose substitution. Adding 1.25 mg of teichoic acid, the amount calculated to give maximal antibody precipitation, yielded a precipitate in which the mole ratio of glucose to phosphorus was 0.42:1.00; with less teichoic acid (0.5 mg) the ratio was 1.12:1.00, and with excess teichoic acid (2.5 mg) the ratio was 1.60:1.00. The maximal recovery of

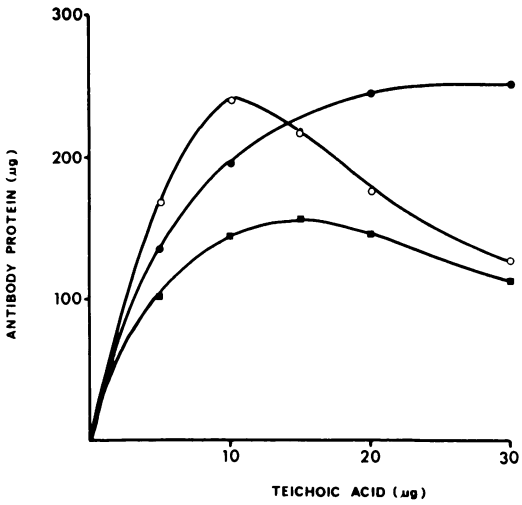

FIG. 1. *Precipitation of teichoic acid from Lactobacillus plantarum NCIB 7220 by antiserum 215 (0.2 ml); ○, wall teichoic acid; ●, membrane teichoic acid; ■, ammonia-degraded membrane teichoic acid.*

teichoic acid in a precipitate was achieved by adding 0.5 mg, but even then represented only 32% of added phosphorus and 36% of added glucose.

Membrane glycerol teichoic acid reacted with the antisera obtained by injecting suspensions of organisms (Table 1). For only two of the eight rabbits tested (187 and 216) did the amount of antibody reacting with membrane teichoic acid significantly exceed that reacting with wall teichoic acid.

Cross-reaction of membrane teichoic acid with antibodies to wall teichoic acid. Each of the eight antisera was tested for antibodies reacting with both wall and membrane teichoic acids. Addition of sufficient membrane teichoic acid to precipitate all of the antibodies reacting with this component also removed all of the antibodies reacting with wall teichoic acid. The addition of sufficient wall teichoic acid to precipitate all of the antibody reacting with wall teichoic acid generally removed 80 to 90% of the antibody reacting with membrane teichoic acid; the exceptions were sera 189 and 216, which still reacted strongly with membrane teichoic acid (Table 1).

In the cross-reaction of membrane teichoic acid with antibodies to wall teichoic acid, the results (Table 1) suggest that there is very little difference in the degree of reactivity of the antibodies with each of the teichoic acid preparations. The precipitin curves, as illustrated in Fig. 1 for antiserum 215, did, however, indicate differences in the amounts of each teichoic acid required for maximal antibody precipitation; the curve for wall teichoic acid is that expected for a low-molecular-weight product, whereas that for the membrane fraction is typical for a high-molecular-weight lipoteichoic acid (14). Because it seemed likely that the reactivity of membrane teichoic acid was being enhanced by its high molecular weight, membrane teichoic acid was deacylated with aqueous ammonia, a procedure which causes a dissaggregation of the molecules by decreasing hydrophobic interaction without affecting serological specificity (14). The deacylated membrane teichoic acid (Fig. 1) precipitated 65% of the amount of antibody precipitated by wall teichoic acid, compared with 104% by the untreated teichoic acid. Wall teichoic acid treated with ammonia retained approximately 95% of its serological reactivity.

TABLE 2. *Reactivity of antisera to cell wall preparations with wall and membrane teichoic acids*

Prepn	Rabbit	Antibody precipitated (mg/ml)		
		Wall teichoic acid	Membrane teichoic acid	
			Whole serum	Absorbed serum[a]
I (trypsin)	181	—[b]	0.87	
	182	—	0.69	
	183	—	2.3	
IIa	223	0.45	1.5	0.87
	224	0.78	2.2	1.6
	225	3.0	4.8	2.1
IIb (hydroxylamine)	226	2.7	4.8	2.0
	227	1.5	3.5	2.1
	228	0.47	2.1	1.3
IIc (trypsin)	229	—	0.29	
	230	—	0.13	
	231	—	0.22	
IId (sodium dodecyl sulfate)	268	3.1	3.8	0.9
	269	2.3	3.1	0.7
	270	0.95	1.3	0.3

[a] Serum absorbed with wall teichoic acid so that no further reaction was given with wall teichoic acid.

[b] No significant reaction.

Antigenicity of wall teichoic acid. None of the sera from rabbits (181, 182, and 183) injected with wall preparation I reacted with wall teichoic acid, though each did react with membrane teichoic acid (Table 2). These results suggested

that the wall preparation, which was obtained by tryptic digestion, contained membrane teichoic acid, whereas the wall teichoic acid was no longer capable of eliciting the production of antibodies detectable by the precipitin method. Analyses of the wall preparation confirmed that ribitol teichoic acid was still the major component, whereas glycerol teichoic acid was only present in trace amounts.

The antigenicity of wall teichoic acid was investigated further by injecting wall preparation II subjected to different treatments (Table 2). Preparation IIa, obtained by washing the wall fraction from disintegrated cells, and preparation IIb, treated with hydroxylamine, each yielded antibodies specific for both wall and membrane teichoic acids. Injection of preparation IIc, treated with trypsin, gave antisera which did not react with wall teichoic acid, thus confirming the earlier observation; these antisera reacted weakly with membrane teichoic acid. Treatment of wall with sodium dodecyl sulfate (preparation IId) did not affect the antigenicity of wall teichoic acid but did apparently remove most of the associated membrane teichoic acid. Washing wall with phosphate buffer (preparation IIe) or KCNS (preparation IIf) did not influence the antigenicity of wall or membrane teichoic acid, and the results have therefore not been recorded.

Detection of IgM and IgG antibodies. Reduction of antisera 163, 164, and 214 with 2-mercaptoethanol decreased their ability to precipitate wall teichoic acid by 80, 90, and 50%, respectively, suggesting the presence of variable amounts of immunoglobulin M and G (IgM and IgG) components.

The IgM and IgG components in serum 214 were separated on Sephadex G-200. The resulting dilution meant that the fractions were not suitable for the quantitative precipitin method but were suitable for the hemagglutination reaction with teichoic acid-sensitized erythrocytes. Wall teichoic acid, being lipid-free, does not absorb to erythrocytes; membrane lipoteichoic acid does absorb, and the titer of serum 214 with the use of these sensitized erythrocytes was 1,600. Allowing for the dilution of the serum components on chromatography, the titers of the IgM and IgG fractions were each estimated to be 1,600. Prior incubation of serum dilutions with wall teichoic acid (10 μg) decreased the titer of both fractions to 200. Within the limitations of the method, the results support those obtained by mercaptoethanol reduction, namely, that IgM and IgG antibodies to wall teichoic acid contribute equally to the serological reactivity of serum 214.

Antisera containing antibodies specific for membrane teichoic acid (Table 2) were reduced with 2-mercaptoethanol after removal of antibodies specific for wall teichoic acid. As determined by the quantitative precipitin method, the reduction in serological activity varied from approximately 20 to 50%, suggesting the presence of a variable proportion of IgM antibodies to the membrane teichoic acid.

Specificity of antibodies to wall teichoic acid from strain NCIB 7220. Several of the sera obtained by injecting whole organisms of *L. plantarum* NCIB 7220 were tested for the specificity of their reaction with wall ribitol and membrane glycerol teichoic acids. In each case, methyl-α-D-glucoside was a more effective inhibitor of the precipitin reaction than methyl-β-D-glucoside. Table 3 provides typical results (antiserum 163), and also shows the effectiveness as inhibitors of glucosyl derivatives of ribitol and glycerol; ribitol (50 μmoles) did not give a detectable inhibition. Further details of the inhibitions for wall teichoic acid are given in Fig. 2.

Additional evidence that antibodies to wall teichoic acid are primarily specific for the α-D-glucosyl substituents was obtained by studying their cross-reaction with other teichoic acids. The antisera reacted very weakly (less than 10% cross-reaction) with wall teichoic acid from the R1 mutant. However, there was a strong cross-reaction with membrane teichoic acids from *L. helveticus* and *L. fermenti*. The glycerol teichoic

TABLE 3. *Inhibition by glucosides of the precipitin reaction between antiserum to strain NCIB 7220 and teichoic acids from the wall and membrane*

Inhibitor	Amt (μmoles)	Per cent inhibition with teichoic acid	
		Wall (5 μg)	Membrane (20 μg)
Methyl-α-D-glucoside	25	46	65
Methyl-β-D-glucoside	25	23	35
2-*O*-α-D-glucosylglycerol	5	28	58
Mono-D-glucosylribitol	5	44	60
Di-D-glucosylribitol	5	57	Not determined

acid from *L. helveticus* has α-D-glucosyl substituents (15), and in its reaction with antiserum 164 precipitated 120% of the amount of antibody precipitated in the homologous reaction. The corresponding value for the glycerol teichoic acid from *L. fermenti*, which has galactosyl-glucose and galactose substituents (22), was 55%. The reaction between *L. plantarum* NCIB 7220 wall teichoic acid (10 μg) and antiserum 164 (0.1 ml) was inhibited 64% by 100 μmoles of D-glucose and 50% by 100 μmoles of D-galactose, indicating that galactose can also combine effectively with the α-D-glucosyl combining sites of the antibody.

Specificity of antibodies to membrane teichoic acid from strain NCIB 7220. Antisera to wall preparation IIa contained antibodies specific for both wall and membrane teichoic acid. Antibodies to wall teichoic acid were precipitated from antiserum 225 (5 ml) by adding teichoic acid (500 μg/5 ml), and the supernatant solution was tested for the specificity of its reaction with membrane teichoic acid (20 μg), by use of methyl-α-D-glucoside and G_3P_2 as potential inhibitors of the precipitin reaction. The results are compared in Table 4 with those for the reaction between serum 183 (0.08 ml) and membrane teichoic acid (5 μg). Antisera 181 and 182 gave results similar to those for 183. The values in Table 4 show that, if the wall teichoic acid is antigenic (antiserum 225), then antibodies to the membrane teichoic acid are specific for the glucose substituents; if, however, the wall teichoic acid is not antigenic (antiserum 183), then antibodies are specific for the glycerol phosphate "backbone" of the membrane teichoic acid.

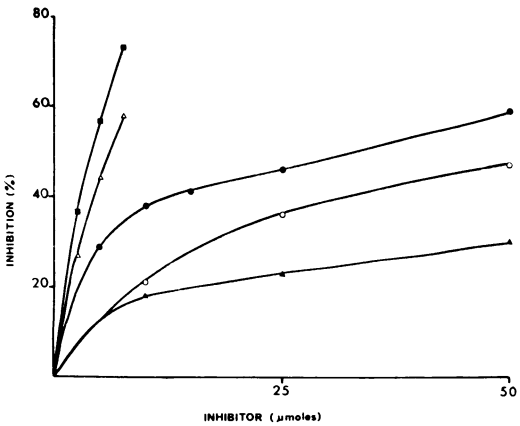

FIG. 2. *Inhibition of the precipitin reaction between wall teichoic acid (5 μg) and antiserum 163 (0.2 ml) by* D-*glucose* (○), *methyl-α-*D-*glucoside* (●), *methyl-β-*D-*glucoside* (▲), *monoglucosylribitol* (△), *and diglucosylribitol* (■).

TABLE 4. *Specificity of antibodies to membrane lipoteichoic acid from Lactobacillus plantarum NCIB 7220*[a]

Prepn injected	Rabbit	Per cent inhibition		
		G_3P_2 (2 μmoles)	Methyl-α-D-glucoside	
			50 μmoles	100 μmoles
Cell wall IIa	225	0	40	—
Trypsinized cell wall I	183	39	—	0
Lipoteichoic acid	193	54	—	33

[a] Values are expressed as percentage inhibition of the precipitation of antibody in each of the sera by purified lipoteichoic acid.

Antisera showing both glucose and glycerol phosphate specificity were obtained by the injection into rabbits of *L. plantarum* membrane teichoic acid in Freund's complete adjuvant (14). The results for the reaction between antiserum 193 (0.07 ml) and lipoteichoic acid (10 μg) are also given in Table 4. The cross-reaction of lipoteichoic acid preparations from other strains of lactobacilli with this antiserum has been described elsewhere (23).

Specificity of antibodies to wall teichoic acid from R1 mutant. Intravenous injection of the R1 strain into three rabbits gave antisera that reacted with wall teichoic acid from both the R1 mutant and strain NCIB 7220, the extent of cross-reaction being approximately 75%. The homologous reaction was not inhibited by 100 μmoles of D-glucose, D-alanine, or ribitol, nor did removal of alanine ester residues with ammonia cause a significant decrease in serological reaction. It seems probable that the cross-reaction depends on a sequence of ribitol phosphate units common to each teichoic acid.

DISCUSSION

The present study, in conjunction with the earlier work of Baddiley and colleagues (2, 5, 21), indicates that both the wall and membrane teichoic acids of strains of *L. plantarum* in serological group D contain α-D-glucosyl substituents. The wall teichoic acid apparently contains molecules differing in the degree of sugar substitution, as analysis of the precipitate formed on adding antiserum to the preparation gave ratios of glucose to phosphorus varying over a fourfold range—from 0.42:1.00 to 1.60:1.00 compared with 0.95:1.00 for the starting material.

Injection of whole organisms of *L. plantarum* NCIB 7220 yielded antisera which reacted with

both wall and membrane teichoic acids. However, in most cases absorption with wall teichoic acid removed virtually all of the reactivity with membrane teichoic acid, suggesting that antibodies to wall teichoic acid were cross-reacting with membrane teichoic acid. An examination of serum fractions separated on Sephadex G-200 indicated that both IgM and IgG antibodies to wall teichoic acid were cross-reacting with membrane teichoic acid. Reduction with 2-mercaptoethanol provided confirmatory evidence that antibodies to the wall teichoic acid contained a high proportion of IgM, a conclusion which was also reached for antibodies to the ribitol teichoic acid of *Staphylococcus aureus* (25).

Cell wall teichoic acid from *L. plantarum* NCIB 7220 retains its antigenicity on disintegration of cells, whereas similar wall preparations from *S. aureus* strains failed to show the production of antibodies reacting with the ribitol teichoic acid component(s) (6, 10). Injection of cell wall treated with sodium dodecyl sulfate proved the most effective means for obtaining antibodies directed primarily against the cell-wall teichoic acid, with minimal antibody response to membrane teichoic acid. However, the antigenicity of the wall teichoic acid apparently depends on its association with protein that is removed by trypsin but not by aqueous extraction procedures. Analysis of cell wall preparations from gram-positive organisms frequently shows the presence of trace amounts of a variety of amino acids, and Rogers and Perkins have pointed out that "it seems very likely that proteins may be common constituents of the cell walls of intact Gram-positive bacteria" (19).

Antibodies to the cell wall ribitol teichoic acid cross-react with membrane glycerol teichoic acid, owing to the common α-D-glucosyl substituents. Preparations of glucosyl-substituted ribitol and glycerol were more effective inhibitors of the precipitin reactions than the methyl glucoside. However, the very weak cross-reaction of unsubstituted ribitol teichoic acid from the R1 mutant with antiserum to strain NCIB 7220 indicates that the ribitol units make only a minor contribution to the specificity of the group antigen; the strong cross-reaction with glycerol teichoic acids from *L. helveticus* and *L. fermenti* supports this conclusion.

Although membrane teichoic acid cross-reacted with antibodies to wall teichoic acid, antibodies specific for membrane teichoic acid were detected in antisera obtained by injecting the cell wall fraction of disrupted cells. These results contrasted to those obtained on injection of whole organisms and could be partly due to the different concentrations of the injected suspensions, namely 0.2 mg of whole organisms/ml compared with 10 mg of cell wall/ml. The specificity of antibodies to the membrane teichoic acid differed, depending on whether or not antibodies were also formed against the wall ribitol teichoic acid. The reason for this difference is not apparent, though it may be relevant that a recent study on the effect of injecting two unrelated antigens into guinea pigs led to the conclusion "that the cell producing antibody to a given antigen can be affected by an unrelated antigen" (7).

The presence of a chemically and serologically distinct membrane teichoic acid enabled the detection of membrane teichoic acid associated with cell wall preparations, even after treatment with trypsin or sodium dodecyl sulfate. Hofstad (10) had also observed that antibodies to the cell wall preparation from a strain of *S. aureus* reacted with a wall-associated glycerol teichoic acid indistinguishable from membrane teichoic acid, and a recent report provided chemical evidence for preparations of purified cell wall from *S. aureus* being contaminated with membrane components including glycerol teichoic acid (17). These observations, in conjunction with earlier studies showing that antibodies to the membrane teichoic acid of *L. fermenti* are absorbed by intact organisms (14), indicate that caution is required in defining a glycerol teichoic acid as a cell wall component on the basis of serological reactions.

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