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Cell Wall Polymers and Phage Lysis of *Lactobacillus plantarum**

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ABSTRACT: Two bacteriophages virulent for strains of *Lactobacillus plantarum* were isolated. Phage 1 lysed strain 8014 which has a ribitol teichoic acid and a phosphorylated polysaccharide (molecular ratio P:Rha:Glc:Gal:Glc_{NH_2}, 1:6:1:1:1) in its cell wall, but not strain 10241 which has a wall teichoic acid but no polysaccharide. Phage 2 lysed both strains. Mutants of strain 8014 resistant to phage 1 possessed a cell wall polysaccharide of different composition from that found in wild type cells; in most cases rhamnose and galactose were absent or present only in small amounts. Isolated cell walls of one of these mutants (M1) did not inactivate phage 1. A mutant of strain 10241 resistant to phage 2 differed from the parent organism in the glucose content of its ribitol teichoic acid (P:Glc in teichoic acid, mutant = 1:0.04 and parent = 1:1.04). These results indicate that the cell wall polysaccharide is essential for infection by phage 1 whereas the glucose moiety of ribitol teichoic acid is an important determinant of phage 2 infection.

The cell wall of *Lactobacillus plantarum* ATCC 8014 comprises, in addition to peptidoglycan, a ribitol teichoic acid with glucose substituents (Armstrong *et al.*, 1958) and a rhamnose-containing, phosphorylated polysaccharide (Ikawa and Snell, 1960; Anderson *et al.*, 1969). The detailed structure of the teichoic acid is well established (Archibald *et al.*, 1961), and it has been identified as the antigenic determinant of group D *Lactobacillus* (Sharpe *et al.*, 1964). Little information, however, is available on the chemical composition of the wall polysaccharide.

Other strains of *L. plantarum* show considerable variation in wall composition (Adams *et al.*, 1969). The polysaccharide component, for example, may be completely absent, or the ribitol teichoic acid may be replaced by a polyglycerol phosphate polymer, as in *L. plantarum* N.I.R.D. Cα106.

Teichoic acids are known to be involved in the phage receptor sites of certain Gram-positive bacteria. The glucose moiety of ribitol or glycerol teichoic acid is essential for phage adsorption in strains of *Bacillus subtilis* (Glaser *et al.*, 1966; Young, 1967). Similarly the *N*-acetylglucosamine residue of ribitol teichoic acid is necessary for phage fixation in *Staphylococcus aureus* (Coyette and Ghuysen, 1968; Chatterjee, 1969).

The present communication describes an attempt to correlate phage sensitivity with differences in wall composition of various strains of *L. plantarum*. In addition, the wall compositions of several phage-resistant mutants were examined in order to gain more specific information about the nature of the phage receptor sites.

Materials and Methods

Growth of Bacteria. L. plantarum Cα106 was a gift from M. E. Sharpe; all other strains were obtained from the American Type Culture Collection. Stock cultures were maintained on Brewer's thioglycollate agar (Difco). The medium of DeMan *et al.* (1960) was used for large-scale cultivation. Batches (20 l.) of medium were inoculated with an overnight culture (1 l.) of the organism and incubated at 28° for 20 hr. Cells were harvested with a Sharples refrigerated centrifuge and washed twice with 0.6% NaCl.

Bacteriophage Isolation, Propagation, and Assay. Phage 1 was isolated from corn silage and phage 2 from anaerobic sewage sludge after enrichment on *L. plantarum* ATCC 8014. The methods of Adams (1959) were used in enrichment, plaque isolation, and phage assay procedures. The growth, plating, and dilution medium was Brewer's thioglycollate broth (BT) or agar.

Phage propagation was accomplished by inoculating logarithmically growing cultures (10 ml) of *L. plantarum* ATCC 8014 with 0.1 ml of a phage suspension in BT prepared from a single plaque. After overnight incubation at 28°, the lysates were filtered through membrane filters (Millipore type HA, 0.45 μ). The filtrates (10^8–10^9 plaque-forming units (pfu)/ml) were stored at 4°.

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Phage was assayed by adding 0.1 ml of phage suspension to 3 ml of BT soft overlay agar (0.65%) together with 0.3 ml of an overnight culture of *L. plantarum* ATCC 8014 and plating on 30 ml of hard BT agar (1.5%). Plaques were counted after incubation at 28° for 16–24 hr under a gas phase of 95% N_2–5% CO_2.

Isolation of Phage-Resistant Mutants. Spontaneous phage-resistant mutants were isolated by plating phage and host in BT overlays at a multiplicity of about 1. Resistant colonies appeared after 40-hr incubation at 28°. The resistance of selected colonies was confirmed by streaking on phage-coated BT agar or by adding a loopful of phage suspension to a plate inoculated with a small patch of the suspected mutant. Confirmed mutant colonies were freed of phage particles by streaking twice on BT plates, then transferred to BT slants.

Cell Wall Preparation. Cell walls were prepared essentially as described by Davison and Baddiley (1963). Washed cells (7.5 g wet wt), suspended in 0.6% NaCl (10 ml), were disrupted by shaking with Ballotini glass beads (30 ml) for 2.5 min in a Braun homogenizer operating at 4000 rpm. Beads were removed by filtration and the filtrate was centrifuged at 15,000*g* for 20 min in a Sorvall refrigerated centrifuge. The supernatant fluid was decanted and the sedimented wall fraction, after being separated from a small pellet of intact cells, was washed seven times with 0.6% NaCl and four times with water. The walls were finally suspended in water and freeze-dried. Cell walls were examined by electron microscopy, after shadowing with palladium and were almost entirely free of non-cell-wall contaminants.

Bacteriophage Inactivation by Cell Walls. Phage inactivation by cell walls was measured by following the decrease in phage titer after incubation of phage particles with walls in BT at 28° or 37°. In a typical experiment, phage particles (1.2×10^3 pfu) and walls (1.0 mg) suspended in BT (final volume 2 ml) were incubated at 37° for 30 min. Walls were removed by centrifugation at 15,000*g* for 5 min, and duplicate samples (0.1 ml) of the supernatant fluid were assayed for free phage.

Isolation of Teichoic Acid and Polysaccharide from L. plantarum ATCC 8014. Walls (500 mg) were stirred in 10% trichloroacetic acid (20 ml) at 4° for 24 hr. The wall residues were removed by centrifugation at 27,000*g* for 20 min and cold ethanol (two volumes) was added to the supernatant fluid. The resulting precipitate, which was pure ribitol teichoic acid, was collected by centrifugation, washed with acetone and ether, and dried. Acetone (two volumes) was added to the supernatant fluid and the resulting precipitate, consisting of a mixture of teichoic acid and polysaccharide, was collected, washed with acetone and ether, and dried.

Hydrolysis of Walls and Wall Polymers. Teichoic acid was hydrolyzed in 2 N HCl or 1 N NaOH in sealed tubes for 3 hr at 100°. Alkaline hydrolysates were passed through a small column of Dowex 50 (NH_4^+ form) resin. The eluates were evaporated to dryness *in vacuo*, and the residues were dissolved in 0.75 ml of 50 mM ammonium carbonate buffer, pH 9.4, containing calf intestinal phosphomonoesterase (0.5 mg/ml). After incubation at 37° for 4 hr, samples were examined chromatographically.

For quantitative determination of glucose and galactose in the wall polysaccharide of *L. plantarum* ATCC 8014, samples were hydrolyzed in 0.5 N HCl at 100° for 4 hr. Glucosamine was determined following hydrolysis in 2 N HCl for 3 hr at 100°.

Paper Chromatography. Descending chromatography was carried out on Whatman No. 1 paper in the following solvent systems: A, 1-propanol–ammonia (sp gr 0.88)–water (6:3:1) (Hanes and Isherwood, 1949); B, 1-butanol–pyridine–water (6:4:3) (Jeanes *et al.*, 1951); C, 1-propanol–ethyl acetate–water (7:1:2) (Baar and Bull, 1953); D, 1-butanol–acetic acid–water (4:1:1) (Partridge, 1948).

Products were detected by the periodate–Schiff reagents for glycols (Baddiley *et al.*, 1956), the molybdate reagent for phosphate esters (Hanes and Isherwood, 1949), the alkaline $AgNO_3$ reagent for reducing sugars (Trevelyan *et al.*, 1950), and the ninhydrin reagent for amino compounds (Consden and Gordon, 1948).

Analytical Methods. Total phosphorus was determined by the method of Chen *et al.* (1956) and total hexose by the phenol–sulfuric acid method (Dubois *et al.*, 1956). Rhamnose was determined by the method of Dische and Shettles (1948). Glucose and galactose were determined enzymically using glucose oxidase and galactose oxidase, respectively (Worthington Biochemical Corp.). Glucosamine was determined either with the ninhydrin reagent (Moore and Stein, 1948) or by the method of Rondle and Morgan (1955). The ester-linked alanine content of teichoic acid preparations was determined with the ninhydrin reagent after hydrolysis in 0.2 N NaOH at 37° for 1 hr.

Results

Isolation and Chemical Composition of the Cell Wall Polysaccharide of L. plantarum ATCC 8014. Extraction of walls with cold 10% trichloroacetic acid followed by the addition of two volumes of ethanol precipitated pure teichoic acid, identified by its characteristic products on hydrolysis in acid and alkali (Archibald *et al.*, 1961). The further addition of two volumes of acetone to the ethanol supernatant fluid precipitated material containing both teichoic acid and polysaccharide. The two polymers were separated by column chromatography on DEAE-cellulose (Figure 1). A similar fractionation procedure was described recently by Anderson *et al.* (1969). Fraction 1 contained polysaccharide free of ribitol teichoic acid; paper chromatographic analyses of acid hydrolysates showed that rhamnose, glucose, glucosamine, and galactose were the component sugars. Fraction 2 was identified as ribitol teichoic acid.

A quantitative analysis of fraction 1 is shown in Table I. The rhamnose content of the polymer was determined on unhydrolyzed material; hydrolysis in 2 N HCl for 3 hr at 100° destroyed approximately 0.4 of the rhamnose. Glucose was similarly destroyed on acid hydrolysis; graded hydrolysis of the polysaccharide in 0.5 N HCl at 100° indicated maximal release after 4 hr, corresponding to 1 mole of glucose per mole of phosphorus in the polysaccharide.

Cell Wall Composition of Other Strains of L. plantarum. Trichloroacetic acid extraction, followed by ethanol and acetone precipitation, was carried out with walls of three other strains of *L. plantarum* by procedures that were identical with those described above for strain 8014. The ethanol precipitate and the acetone precipitate of the trichloroacetic acid soluble fractions were hydrolyzed in acid and examined by paper chromatography. Table II lists some of the products identified. Acid hydrolysis of ethanol-precipitable material of *L. plantarum* ATCC 10241 and hydrolysis in alkali followed by enzymic dephosphorylation of products (Archibald *et al.*, 1961) indicated that the walls of this strain contained ribitol teichoic acid possessing glucose and alanine substituents. Ribitol teichoic acid was also found in walls of strains 8041, as were glucose, glucosamine, and galactose; whether these sugars were present as teichoic acid substituents or as compo-

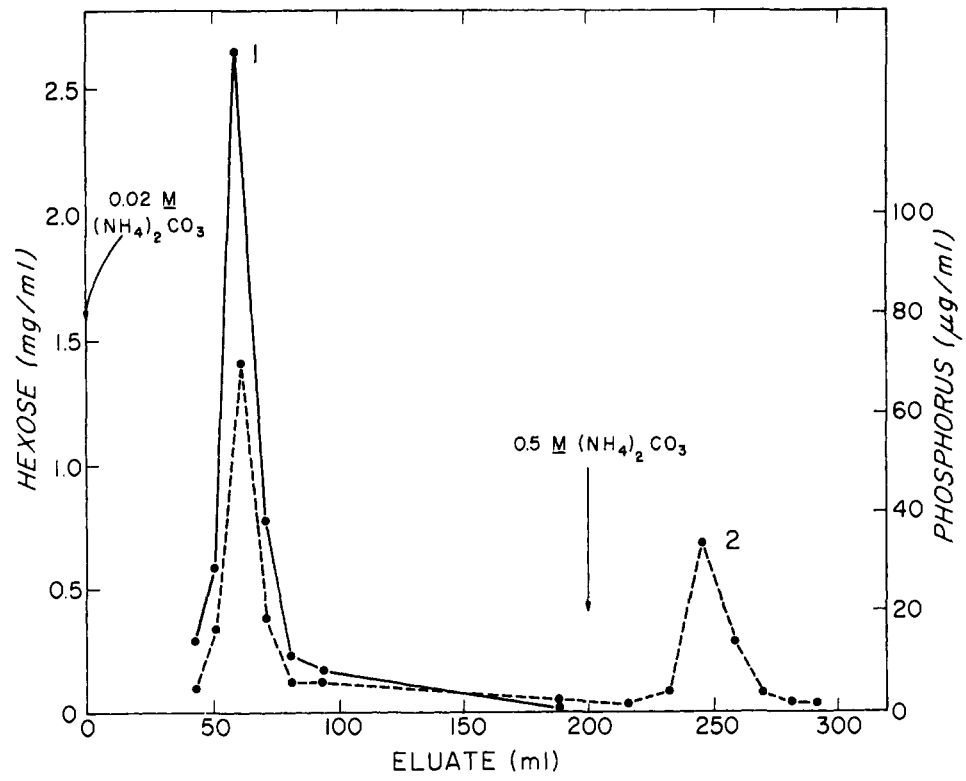

FIGURE 1: DEAE-cellulose (CO_3^{2-} form) chromatography of the acetone-precipitable fraction of trichloroacetic acid extracts of *L. plantarum* ATCC 8014 walls. Acetone-precipitated material (100 mg) was dissolved in 2 ml of 0.02 M $(NH_4)_2CO_3$ and applied to a column (24 cm × 1.5 cm) of DEAE-cellulose (CO_3^{2-} form) at a flow rate of 20–25 ml per hr. Elution was with $(NH_4)_2CO_3$ solutions as indicated. Fractions (5 ml) were collected automatically and samples (0.1 ml) were analyzed for phosphorus and hexose. Fractions corresponding to peaks 1 and 2 were concentrated by rotary evaporation and then freeze-dried. Hexose is the solid line and phosphorus is the dashed line.

nents of a distinct wall polysaccharide was not determined. Neither ribitol, anhydroribitol, nor glycerol was ever detected following hydrolysis of the precipitates obtained from trichloroacetic acid extracts of *L. plantarum* ATCC 8292 walls (Table II).

Host Specificity of L. plantarum Bacteriophages. Two virulent bacteriophages were isolated for *L. plantarum* ATCC 8014. Phage 1 was obtained from corn silage and phage 2 from anaerobic sewage sludge. The host specificity of these phages is shown in Table III. Although strain 8014 is lysed by both phages, strain 10241 is lysed only by phage 2. Both strains contain ribitol teichoic acids but only strain 8014 possesses a rhamnose polysaccharide. None of the other strains tested was attacked by either phage.

Mutants of L. plantarum ATCC 8014 Resistant to Phage 1. Twelve spontaneous phage 1 resistant mutants of strain 8014 were isolated. Teichoic acid was prepared from the cell walls of one of the mutants (M1). Hydrolysis in acid gave products identical with those obtained from teichoic acid prepared from the parent strain, *i.e.*, ribitol, ribitol mono- and diphosphates, inorganic phosphate, anhydroribitol, anhydroribitol phosphate, glucose, and alanine. A comparison of the phosphorus, alanine, and glucose contents of parent and M1 teichoic acid is shown in Table IV; in each case the overall composition is the same, the ratio P:glucose:alanine being 1:1:0.6.

TABLE I: Quantitative Composition of the Cell Wall Polysaccharide of *L. plantarum* ATCC 8014.

	mg/100 mg of Polysaccharide	moles/mole of P
Phosphorus	1.98	1.00
Rhamnose	63.5	6.05
Glucose	11.3	0.98
Galactose	11.3	0.98
Glucosamine	10.8	0.94

TABLE II: Hydrolysis Products of Trichloroacetic Acid Extracted Material from Walls of Strains of *L. plantarum*.

	ATCC 8041		ATCC 10241		ATCC 8292	
	Ppt 1[a] (36 mg)[c]	Ppt 2[b] (38 mg)	Ppt 1[a] (41 mg)	Ppt 2[b] (5 mg)	Ppt 1[a] (61 mg)	Ppt 2[b] (60 mg)
Glucose	+	+	+	+	+	+
Glucosamine	+	+	−	−	+	+
Galactose	+	+	−	−	+	+
Rhamnose	−	−	−	−	+	+
Anhydroribitol	+	+	+	+	−	−

[a] Material precipitated by 2 volumes of ethanol. [b] Material precipitated by 2 volumes of acetone after removing ppt 1 by centrifugation. [c] Values in parenthesis indicate the dry weight of the precipitate obtained from 500 mg of cell walls.

TABLE III: Host Specificity of *L. plantarum* Phages.

L. plantarum Strain	Wall P (%)	Teichoic Acid Type	Wall Polysaccharide	Attack by Phage 1	Attack by Phage 2
ATCC 8014	2.25	Ribitol	+	+	+
ATCC 10241	2.4	Ribitol	−	−	+
ATCC 8041	2.4	Ribitol	+	−	−
ATCC 8292	2.05		+	−	−
Cα106	2.5[a]	Glycerol[a]	?	−	−

[a] Data of Adams *et al.* (1969).

TABLE IV: Analysis of *L. plantarum* ATCC 8014 and M1 Mutant Teichoic Acids.

	% Phosphorus	% Glucose	% Alanine	P:Glucose:Alanine	% P in Wall
ATCC 8014	6.7	42.2	11.6	1:1.09:0.61	2.25
M1 mutant	7.1	42.4	11.8	1:1.03:0.58	1.94

When intact cell walls of the M1 mutant and parent strains were hydrolyzed in 2 N HCl and the hydrolysates were examined by paper chromatography, the most striking difference was an apparent absence of rhamnose and galactose in the M1 hydrolysate. Similar examination of the remaining 11 mutants indicated little or no rhamnose and galactose in every case but one (that of the M10 mutant). A quantitative determination of these sugars (Table V) revealed that walls of M1 contain considerably less rhamnose than those of the parent strain, and no galactose. M10 walls, on the other hand, contain a greater proportion of rhamnose and less galactose so that the ratio rhamnose:galactose is twice that of the parent walls.

Inactivation of Phage 1 by Cell Walls. Binding experiments with phage 1 indicated that 96–99% of the phage particles were inactivated after incubation with *L. plantarum* ATCC 8014 walls at 37° for 30 min; incubation for the same time period at 28° was equally effective. According to the criteria of Garen and Puck (1951) inactivation was irreversible, less than 3% of the bound phage being released following dilution and incubation at 0°. The results of binding experiments with walls of phage-resistant mutants are shown in Table VI. Phage 1 was inactivated by walls of the M10 mutant but not by M1 walls. The phage was also irreversibly bound by walls of strain 8041, which contain galactose but no rhamnose (Table V), although it would not lyse this organism. No inactivation was observed with preparations of the purified polysaccharide from strain 8014.

TABLE V: Rhamnose and Galactose Content of Walls of *L. plantarum* Strains.

	Rhamnose (% dry wt)	Galactose (% dry wt)	Rha:Gal
L. plantarum ATCC 8014	8.2	1.6	5.6:1
M1 mutant	0.87	0.0	
M10 mutant	10.7	0.99	11.9:1
L. plantarum ATCC 8041	0.0	4.3	
L. plantarum ATCC 10241	0.0	0.0	
L. plantarum ATCC 8292	10.8		

TABLE VI: Adsorption of Phage 1 to *L. plantarum* Walls.

	Unbound Phage (10^2 pfu[a]/ml)
Control	65
+ 8014 walls	2.4
+ M1 walls	57
+ M10 walls	0.9
+ 8014 polysaccharide	60
+ 8041 walls	1.1

[a] Plaque-forming units.

Mutant of L. plantarum ATCC 10241 Resistant to Phage 2. Phage 2 attacked strain 8014 and the M1 and M10 mutants. It also lysed strain 10241 which contains no polysaccharide. Thus, a cell wall lesion resulting in resistance to phage 2 must lie somewhere other than in the polysaccharide.

Examination of the wall composition of a spontaneous phage 2 resistant mutant of strain 10241 revealed that the teichoic acid of the mutant, R1, contained a much lower proportion of glucose than that of the parent strain (Table VII), giving a P:glucose ratio of 1:0.04 in the mutant compared with that of 1:1 in the parent strain. The alanine content of the mutant teichoic acid is normal.

Discussion

Lactobacillus phages isolated thus far appear to have very limited host ranges. Four *L. casei* phages and 11 *L. fermenti* phages isolated by De Klerk *et al.* (1963) showed considerable species specificity. Moreover, the phages had only limited intraspecies host ranges and, in the case of the *L. fermenti* phages, the host range was different for each phage. The two *L. plantarum* phages isolated in the present study also exhibit intraspecies specificity and phage sensitivity can be related to cell wall composition in some of the strains examined.

Of those tested, only strain 8014 of *L. plantarum* was

TABLE VII: Analysis of *L. plantarum* ATCC 10241 and R1 Mutant Teichoic Acids.

	% Phosphorus	% Glucose	% Alanine	P:Glucose:Alanine	% P in Wall
ATCC 10241	4.7	28.3	16.1	1:1.04:1.19	2.4
R1 mutant	6.2	1.5	22.0	1:0.04:1.23	2.7

lysed by phage 1. Most of the isolated phage-resistant mutants of this organism, *e.g.*, M1, have little or no rhamnose and galactose in their walls, suggesting that the wall polysaccharide is involved in phage adsorption. The phage would not bind to isolated walls of M1. However, phage binding occurred with walls of strain 8041 which contain galactose but no rhamnose, even though the phage is not lytic for this organism. Thus galactose rather than rhamnose may be the component of the polysaccharide which is essential for phage binding. It is surprising that the most common phage 1 resistant mutants simultaneously lose the capacity to incorporate two different sugars into their cell walls. It is possible that a single mutational loss of the phage-binding sugar could lead to the loss of ability to attach a second sugar. This situation is well established in the case of phage-resistant *Salmonella* mutants (Wilkinson and Stocker, 1968).

The reason for the resistance of the M10 mutant is not clear; phage inactivation takes place in experiments with isolated walls of this organism but cell lysis does not occur. It is possible that the alteration in the amounts of rhamnose and galactose present in the wall of this mutant in some way precludes further penetration by the phage once adsorption has taken place.

Phage 2 lysed both strains 8014 and 10241 in addition to the M1 and M10 mutants. Clearly, therefore, the wall polysaccharide is not required for adsorption by this phage. Rather, examination of the walls of a mutant of strain 10241 resistant to phage 2 suggested that it is the teichoic acid, and more especially the glucose substituents, which are determinants of adsorption by this phage. Similar results have been obtained with *B. subtillis* W23, in which the teichoic acid is a glucosylated polyribitol phosphate (Glaser *et al.*, 1966) and *B. subtilis* 168 which possesses a glycosylated polyglycerol phosphate (Young, 1967) in its walls: in both cases the glucose moiety is essential for phage fixation. The ribitol teichoic acid of strains of *S. aureus* also appears to be involved in phage adsorption; again it was shown both by isolation of phage-resistant mutants (Chatterjee, 1969) and by adsorption experiments following enzymic treatment of walls (Coyette and Ghuysen, 1968), that the *N*-acetylglucosamine residues of the polymer were specifically required for phage binding. The latter approach was not possible in the present case since separate experiments (L. J. Douglas and M. J. Wolin, unpublished) have shown that *L. plantarum* ribitol teichoic acid, isolated from walls by trichloroacetic acid extraction, is resistant to the action of α-glucosidase, presumably because of steric effects.

Under experimental conditions which resulted in the binding of phage 1 to walls of strain 8014 at an efficiency of over 96%, phage 2 bound poorly, if at all, to walls of 10241. Variations in incubation conditions, including an increased divalent cation concentration, alteration of pH, and the use of sonicated wall suspensions, were all unsuccessful at promoting phage adsorption. Thus, a comparison of the binding efficiency of phage 2 to mutant and wild-type walls, similar to that undertaken with phage 1, was not possible.

Phage 1 was not inactivated by preparations of the purified polysaccharide from strain 8014. Young (1967), using *B. subtilis*, and Morse (1962) and Matthew and Rosenblum (1967), using *S. aureus*, have similarly failed to demonstrate phage inactivation by teichoic acid preparations. With both these organisms, modifications in wall peptidoglycan structure, accomplished either chemically or enzymically, result in a decreased efficiency of phage binding (Young, 1967; Coyette and Ghuysen, 1968; Chatterjee, 1969). Thus, the peptidoglycan component is important, if not as a primary binding site, then as a rigid, supporting framework. This may be even more important in the case of *L. plantarum* ATCC 8014 where, presumably, both teichoic acid and polysaccharide are attached to the peptidoglycan in a highly specific configuration.

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