Use of Lectins to Characterize the Receptor Sites for Bacteriophage PL-1 of *Lactobacillus casei*

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The polysaccharide (PS) located outside the peptidoglycan layer in *Lactobacillus casei* ATCC 27092 was found to inhibit the adsorption of PL-1 phage to cell wall preparations without inactivating free phage. Electron microscopic examination of adsorption mixtures showed that the phage were adsorbed to fragments of PS material in a tail-first orientation. Phage did not adsorb to isolated peptidoglycan. The PS was composed of L-rhamnose, D-glucose, D-glucosamine and D-galactosamine, with the hexosamines possibly in N-acetylated form. Prior treatment of *L. casei* with *Streptomyces* haemagglutinin, an anti-human blood group B agglutinin that binds specifically to L-rhamnose, resulted in a concentration-dependent inhibition of phage adsorption. Phage adsorption was inhibited partially by lectins specific for D-glucose and N-acetyl-D-glucosamine, but not by lectins specific for N-acetyl-D-glucosamine or N-acetyl-D-galactosamine. The inhibition of phage adsorption occurred immediately upon the addition of the effective lectins, and was reversed by the addition of the respective lectin inhibitors, α -phenyl galactoside or α -methyl glucoside. The results indicate that L-rhamnosyl residues are the main determinants of the PL-1 phage receptor sites, while D-glucosyl residues may be involved more indirectly.

INTRODUCTION

The chemical structure of the receptor sites of a *Lactobacillus casei* phage PL-1, which belongs to the B-group in the classification of Ackerman & Eisenstark (1974) has been studied. The cell walls of this organism contain a polysaccharide external to the basal peptidoglycan layer; teichoic acids are, however, absent. Therefore, the phage receptor sites are most probably part of the cell surface polysaccharide.

Several lectins bind in a highly specific way to polysaccharide moieties on the surface of various cells (Goldstein & Staub, 1970; Goldstein & Misaki, 1970; Reeder & Ekstedt, 1971; Sharon & Lis, 1972; Doyle & Birdsell, 1972; Nicolson & Baustein, 1972; Osawa, 1975; Liener, 1976; Hamada *et al.*, 1977; Goldstein & Hayes, 1978; Choy *et al.*, 1979). Therefore, lectins whose binding sugars are known can be used to identify constituent sugars in cell surface polysaccharides. Since both PL-1 phage particles and lectins probably bind to polysaccharides on the cell surface, pretreatment of cells with specific lectins might prevent the adsorption of phages to their receptor sites. This approach has been used to determine which sugars in the cell wall polysaccharide are responsible for the binding of PL-1 phage, using a variety of lectins whose main binding sugars are those found in the wall polysaccharide of *Lactobacillus casei* ATCC 27092.

METHODS

Bacteriophage and bacterium. Bacteriophage PL-1 and its host strain, *Lactobacillus casei* ATCC 27092, were used. Bacteria were grown in MR medium (pH 6.0) at 37 °C, harvested by centrifugation in the early-exponential growth phase, washed twice, and resuspended in 50 mM-Tris/maleate buffer (pH 6.0) containing 10 mM-CaCl₂. MR medium (pH 6.0) contained (g per litre deionized water): polypeptone, 10; sodium acetate, 10; yeast extract, 3; beef extract, 3; NaCl, 1; CaCl₂·2H₂O, 1.5; MgSO₄·7H₂O, 0.2; MnSO₄·H₂O, 0.01; FeSO₄·7H₂O, 1; and

Table 1. *Sugar binding specificity of lectins used*

Lectin	Binding sugar*	Reference
<i>Canavalia ensiformis</i> : jack bean (Con A)	α -D-Man > α -D-Glc > α -D-GlcNAc	Sumner & Howell (1936)
<i>Lens culinaris</i> : lentil (LcH)	α -D-Man > α -D-Glc > α -D-GlcNAc	Howard & Sage (1969)
<i>Pisum sativum</i> : garden pea (PEA)	α -D-Man > α -D-Glc > α -D-GlcNAc	Entlicher <i>et al.</i> (1970)
<i>Glycine max</i> : soybean (SL)	α -D-GalNAc >> β -D-GalNAc	Liener (1958)
<i>Bauhinia purpurea</i> : mountain ebony (BPA)	α -D-GalNAc >> α -D-Gal	Irimura & Osawa (1972)
<i>Phaseolus lunatus</i> : lima bean (LBA)	α -D-GalNAc > α -D-Gal	Gould & Scheinberg (1970)
<i>Phaseolus vulgaris</i> : red kidney bean (PHA)	α -D-GalNAc > α -D-Gal	Robbins (1964)
<i>Helix pomatia</i> : edible snail (HPA)	α -D-GalNAc >> α -D-GlcNAc	Hammarström & Kabat (1969)
<i>Sophora japonica</i> : Japanese pagoda tree (SJA)	β -D-GalNAc > β -D-Gal > α -D-Gal	Osawa & Akiya (1961)
<i>Wisteria floribunda</i> : Japanese wisteria (WFA)	D-GalNAc	Toyoshima <i>et al.</i> (1971)
<i>Triticum vulgaris</i> : wheat germ (WGA)	β -D-GlcNAc-(1→4)- β -D-GlcNAc	Nagata & Burger (1972)
<i>Ricinus communis</i> : castor bean (RCA)	β -D-Gal-(1→4)-D-GlcNAc	Nicolson & Blaustein (1972)
<i>Streptomyces</i> sp. 27S5 (SHA)	L-Rha > α -D-Gal	Fujita <i>et al.</i> (1973)

* Glc, glucose; Gal, galactose; Man, mannose; Rha, rhamnose; NAc, *N*-acetyl; >, more strongly than; >> much more strongly than.

glucose, 10. Propagation of phage, their assay as p.f.u. ml⁻¹, and other related methods have been described (Watanabe *et al.*, 1970; Watanabe & Takesue, 1975; Watanabe *et al.*, 1977).

Chemicals. The origin of the lectins used in this study and their sugar-binding specificities are summarized in Table 1. The lectins Con A, SL and WGA were from Pharmacia and LcH, PEA, BPA, LBA, PHA, HPA, SJA and WFA from E. Y. Laboratories. SHA, an anti-human blood group B haemagglutinin produced by *Streptomyces* sp. 27S5, was prepared by the method of Fujita *et al.* (1973, 1975). RCA was kindly supplied by Dr G. Funatsu, Kyushu University. Phenyl α -D-galactoside, methyl α -D-glucoside, crude beef pancreas DNAase I and bovine pancreas RNAase A were from Sigma, and trypsin was from Merck.

Preparation of cell wall polysaccharide (PS) and peptidoglycan (PG) fractions. Exponential phase *L. casei* cells suspended in an appropriate amount of deionized water were disrupted in a Dyno-Mill (type KDL, 150 ml capacity) with 120 ml glass beads (0.1 mm diam.) for 2 min in the cold. The lysates were centrifuged at 1300 *g* for 10 min to remove the unbroken cells. The supernatants were again centrifuged, at 7500 *g* for 15 min, to sediment the cell walls. The crude cell wall preparation was then suspended in 50 mM-phosphate buffer (pH 7.6) and digested with DNAase (5 μ g ml⁻¹), RNAase (0.5 mg ml⁻¹) and trypsin (0.5 mg ml⁻¹) for 1 h at 37 °C. Finally, they were washed with water and lyophilized. Electron microscopic examination showed the cell wall preparations to be free from unbroken cells. The cell walls (300 mg) were treated with 50 ml 5% (w/v) TCA at 90 °C for 15 min, and, after being cooled, the mixture was centrifuged at 11000 *g* for 15 min to obtain the TCA-insoluble fraction enriched for peptidoglycan. The precipitate was washed first with 5% TCA and then thoroughly with deionized water; it was lyophilized and used as the PG preparation. Then, a ninefold volume of ethyl alcohol was added to the supernatant (TCA-soluble fraction), and left overnight at 4 °C. The precipitate containing the polysaccharide (PS) was recovered by centrifugation, washed with ethyl alcohol and ether, and lyophilized.

Analytical methods. For quantitative determination of the constituent sugars, PS was hydrolysed at 100 °C for 3 h with 3 M-HCl in a sealed glass tube. After evaporation in a rotary evaporator at 30 °C, the residue was dissolved in 0.15 M-borate buffer (pH 8.0) for neutral sugars and in 0.2 M-citrate buffer (pH 2.2) for amino sugars. Analysis of these sugars was carried out in a Hitachi 034 liquid chromatograph.

Assay of phage adsorption to *L. casei* cells and the effect of lectins. The exponential phase *L. casei* cells (3 × 10⁸ cells ml⁻¹), suspended in 50 mM-Tris/maleate buffer (pH 6.0) containing 10 mM-CaCl₂, were mixed with PL-1 phage at a multiplicity of infection (m.o.i.) of 5 × 10⁻⁴ and incubated at 37 °C. At intervals, aliquots of the mixtures were withdrawn and centrifuged at 5000 *g* for 10 min, and the cell-free supernatants were assayed for

unadsorbed phage. For testing the effect of lectins on phage adsorption, the cells were preincubated with lectins at 37 °C for 5 min before being mixed with phage, and the percentage of unadsorbed phage was compared with that of the control without lectins. The lectins used did not inactivate PL-1 phage.

Electron microscopy. A drop of the phage/PS or phage/PG mixture was placed on a copper grid coated with collodion/carbon film, and negatively stained with 2% (w/v) uranyl acetate. After removal of excess stain on the grid with filter paper, the specimens were examined in a JEOL 100-C electron microscope at 80 kV.

RESULTS

Effect of polysaccharide on the adsorption of PL-1 phage to the host cell walls

PL-1 phage have been shown to adsorb specifically to isolated cell walls as well as to intact cells of *L. casei* (Watanabe *et al.*, 1977, 1980). Because the outermost layer of the cell walls is polysaccharide (PS), this is the most likely phage receptor site. To obtain direct evidence for this, we examined whether the phage adsorption to the cell walls would be inhibited by preparations of PS free from peptidoglycan (PG). Cell walls were mixed with various concentrations of PS and then incubated with the phage at 37 °C for 40 min. As Table 2 shows, phage were rapidly adsorbed in the absence of added PS, so that the proportion of unadsorbed phage was 13.6% of input phage after incubation. In the presence of PS, however, the proportion of unadsorbed phage increased with increasing concentrations of PS, showing that the phage adsorption was inhibited by PS and that PS therefore contains receptor sites for PL-1 phage. There was no adsorption of phage to the PG preparation.

Electron microscopic appearance of PL-1 phage mixed with polysaccharide or peptidoglycan

PS material (200 µg ml⁻¹) was incubated with approximately 6×10^{10} PL-1 phage ml⁻¹ at room temperature for 5 min in Tris/maleate buffer, and negatively stained with uranyl acetate. As Fig. 1(a, b) shows, phage particles appear to be aggregated when compared with the control (Fig. 1d) without PS. The phage were seen associated by their tail tip to fragments of PS material, which could not be characterized morphologically even by electron microscopy. The adsorbed phage appeared to be intact, with DNA present in the heads. In another control (Fig. 1c), where the phage were mixed with PG, the phage were uniformly dispersed in the same manner as without PG or PS.

Chemical composition of polysaccharide

Acid-hydrolysis of PS material yielded L-rhamnose, D-glucose, D-glucosamine and D-galactosamine in the approximate molar ratio of 4:2:1:1 (Table 3). Teichoic acid was not detected. The presence of *N*-acetyl residues in the PS was suggested by a strong absorption at 1650 cm⁻¹, typical of the carboxamide group of hexosamines (detailed data not shown). On this basis it is possible that both the D-glucosamine and D-galactosamine of the native PS are *N*-acetylated.

Effect of lectins on PL-1 phage adsorption to cells

PL-1 phage were incubated at 37 °C for 15 min with host cells pretreated with various lectins, each at various concentrations, and the amounts of unadsorbed phage were assayed (Fig. 2). In a control without lectins, over 90% of the input phage were adsorbed. However, when the cells had been pretreated with SHA, which binds specifically to non-reducing terminal L-rhamnosyl residues, phage adsorption was much reduced, and the proportion of unadsorbed phage increased with increasing concentration of SHA. Con A, LcH and PEA, which are specific for D-mannose, D-glucose and *N*-acetyl-D-glucosamine, inhibited phage adsorption to some extent. However, the lectins SL, BPA, LBA, PHA, HPA, SJA, RCA and WGA, which bind specifically to *N*-acetyl-D-galactosamine or *N*-acetyl-D-glucosamine, had no effect on phage adsorption even at a concentration of 5 mg ml⁻¹.

At the concentrations of SHA and Con A used here, no bacterial agglutination could be seen by the naked eye, although slight agglutination could be seen by phase-contrast microscopy. In order to test whether bacterial agglutination itself could reduce the probability of collision between the phage and their receptors, the following experiment was done. Host cells harvested and washed as described in Methods were resuspended in water and lyophilized. When the

Fig. 1. Negatively stained phage PL-1 adsorbing to polysaccharide (PS) and peptidoglycan (PG). (a, b) Phage/PS mixture: (a) low magnification, bar marker represents 1 μm ; (b) higher magnification, bar marker represents 200 nm. (c) Phage/PG mixture, bar marker represents 1 μm . (d) Phage without either PS or PG, bar marker represents 1 μm . Approximate concentrations: phage, $6 \times 10^{10} \text{ ml}^{-1}$; PS, $200 \mu\text{g ml}^{-1}$; PG, $500 \mu\text{g ml}^{-1}$.

Fig. 2. Effect of various concentrations of different lectins on PL-1 phage adsorption to *L. casei* cells. Cells (3×10^8 ml $^{-1}$) were preincubated with various concentrations of lectins at 37 °C for 5 min. PL-1 phage were added at a m.o.i. of 5×10^{-4} , and incubation was continued for another 15 min. The mixtures were centrifuged, and the proportion of unadsorbed phage in the supernatants was assayed. Lectins: ●, SHA; ○, Con A; ●, WGA; ●, BPA. Results are from three experiments.

Table 2. Effect of cell wall polysaccharide (PS) on PL-1 phage adsorption to *L. casei* cell walls

Cell walls (CW) ($10 \mu\text{g ml}^{-1}$) or peptidoglycan fraction (PG) ($500 \mu\text{g ml}^{-1}$) were incubated with PL-1 phage (1.9×10^5 ml $^{-1}$) in a Tris/maleate buffer (pH 6.0) at 37 °C for 40 min, and the proportion of unadsorbed phage was determined. The cell walls were mixed with various concentrations of PS for 5 min before the phage were added. Results are from three experiments.

Components*	Concn of PS (mg ml $^{-1}$)	Unadsorbed phage (%)
PL-1 phage	0.0	100.0
CW + PL-1 phage	0.0	13.6
(CW + PS) + PL-1 phage	10.0	60.5
	5.0	28.2
	1.0	22.3
	0.1	15.5
	0.01	13.7
PG + PL-1 phage	0.0	101.7

Table 3. Chemical composition of TCA-soluble fractions from *L. casei* ATCC 27092 cell walls

Sugar	Composition	
	$\mu\text{mol mg}^{-1}$	Molar ratio
L-Rhamnose	2.32	4.03
D-Glucose	1.14	1.98
D-Glucosamine*	0.58	1.00
D-Galactosamine*	0.68	1.17

* Presumably *N*-acetylated form.

lyophilized cells were suspended in the Tris/maleate buffer, slight agglutination was seen by microscopy. However, PL-1 phage adsorption proceeded in the agglutinated cell suspensions in exactly the same manner as in unagglutinated ones. Therefore, it is most unlikely that cell agglutination played a role in the inhibition of phage adsorption by lectins.

Kinetics of lectin inhibition of PL-1 phage adsorption to cells

To examine more closely the inhibition of PL-1 phage adsorption to cells by SHA and Con A, a Lineweaver-Burk plot was constructed by plotting the reciprocal of the initial velocity of phage adsorption versus the reciprocal of the cell concentration in the presence of various con-

Fig. 3. Lineweaver-Burk plot showing competitive inhibition of the phage/cell interaction by SHA (a) and Con A (b). Details of the assay are as described in the legend to Fig. 1. Concentrations of SHA (mg ml^{-1}): $\bullet$, 0.2; $\circ$, 0.1; $\circ$, 0.05; $\circ$, 0.0 (control). Concentrations of Con A (mg ml^{-1}): $\bullet$, 2.0; $\circ$, 1.0; $\circ$, 0.5; $\circ$, 0.0 (control). Results are from three experiments.

centrations of lectins. The adsorption velocity (v) was taken as the reduction in percentage of unadsorbed phages after 15 min incubation. As Figs 3 (a) and (b) show, the inhibition of phage adsorption to cells by these lectins was affected by the concentrations of both cells and lectin. As the cell concentration was increased, there was a decrease in the ability of lectins to inhibit phage adsorption. In the plot, the slope of the line became progressively steeper as the concentration of lectins was increased. However, all the intercept values on the ordinate ($1/v$) were almost the same as that of the control without lectins, in both the cases. Thus, the inhibitory pattern of these lectins displayed competitive characteristics. The K_m at each concentration of lectins was calculated from the negative intercept values on the abscissa. The K_m value at SHA of 0.2 mg ml^{-1} was $8.5 \times 10^8 \text{ cells ml}^{-1}$, and that at Con A of 0.5 mg ml^{-1} was $1.5 \times 10^7 \text{ cells ml}^{-1}$, indicating that SHA binds more strongly to cells than Con A.

Effect of the time of addition of lectins on PL-1 phage adsorption

Figure 4 shows the effect of SHA on the kinetics of PL-1 phage adsorption to cells in Tris/maleate buffer. In a control without SHA, the proportion of unadsorbed phage decreased rapidly, and after 10 min incubation it was below 30% under the conditions used. The addition of SHA after 15 min incubation resulted in an immediate increase to approximately 60% in the proportion of free phage, showing that SHA is able to cause an immediate desorption of the previously adsorbed phage. However, the amount of phage that could be desorbed upon SHA treatment gradually declined as incubation continued (data not shown).

Figure 5 shows the results of a similar experiment using Con A instead of SHA. When Con A was added after 15 min incubation, again an immediate desorption of infectious phage occurred, while after a prolonged exposure to phage, Con A treatment was unable to release infectious phage. These results indicate that, as the incubation continues, the forces of phage adsorption, which are initially reversible, gradually become irreversible.

Reversibility of lectin inhibition

With the same experimental design, it was also possible to test whether the inhibitory action of these lectins on phage adsorption was reversible. Phenyl galactoside, one of the competitive inhibitors of SHA, was added to the mixture of cells, SHA and phage after 5 min incubation. The addition of phenyl galactoside (Fig. 4) resulted in partial reversion of phage adsorption, although such a quick and complete phage adsorption as seen without lectins was not observed under the conditions used. Phenyl galactoside itself had no effect on phage adsorption. These results indicate that the binding of SHA to cells is partially reversible and that the inhibition of phage adsorption by SHA is due to the specific interaction of SHA with sugar residues on the cell surface. However, after prolonged exposure to SHA, the cells became gradually unable to desorb

Fig. 4

Fig. 5

Fig. 4. Effect of SHA on the kinetics of PL-1 phage adsorption. The reaction mixtures contained (ml^{-1}): 1.5×10^8 cells, 0.2 mg SHA, 7.5×10^5 p.f.u. phage and 2 mg phenyl galactoside. At the times indicated, samples were taken to determine the proportion of unadsorbed phage. ○, Phage adsorption to untreated cells (control); ●, SHA added to adsorption mixture at 0 and 15 min (filled arrows); ●, SHA added to adsorption mixture at zero time, and phenyl galactoside added 5 and 30 min later (open arrows). Results are from four experiments.

Fig. 5. Effect of Con A on the kinetics of PL-1 phage adsorption. The reaction mixtures contained (ml^{-1}): 1.4×10^8 cells, 1 mg Con A, 7.5×10^5 p.f.u. phage, and 1 mg methyl glucoside. At the times indicated, samples were taken to determine the proportion of unadsorbed phage. ○, phage adsorption to untreated cells (control); ●, Con A added to adsorption mixture at 0 and 15 min (filled arrows); ●, Con A added to adsorption mixture at zero time, and methyl glucoside added 15 min later (open arrow). Results are from seven experiments.

SHA on addition of phenyl galactoside (compare desorption values when phenyl galactoside was added after 30 min and 5 min incubation, Fig. 4). The inhibition of phage adsorption by Con A was also reversed by the addition of a competitive inhibitor, methyl glucoside (Fig. 5).

DISCUSSION

Several lectins have been shown to interact in a highly specific way with defined polysaccharides on the surface of a variety of cells. Therefore, they have been successfully used as a tool for obtaining structural information on the presence of specific sugars, the configuration of anomeric linkages and the position of sugar residues, e.g. in phage receptor sites (Archibald & Coapes, 1972; Birdsell & Doyle, 1973; Watanabe & Shiomi, 1976; Picken & Beacham, 1977). We have studied the interaction of phage PL-1 with its host, *L. casei*, which has a cell wall polysaccharide consisting of only L-rhamnose, D-glucose, D-glucosamine and D-galactosamine (these hexosamines may be present in *N*-acetylated form). The availability of lectins specific for these compounds prompted us to examine the effect of such lectins on the adsorption of PL-1 phage to the cells in order to obtain indirect evidence of the role of these sugars in the binding of phage. We found that both SHA, which binds specifically to L-rhamnose, and Con A, LcH and PEA, which bind to D-glucose and *N*-acetyl-D-glucosamine, inhibited phage adsorption. However, lectins specific to *N*-acetyl-D-glucosamine or *N*-acetyl-D-galactosamine only were not effective, suggesting that these hexosamines might not be critical determinants in the phage receptor sites.

The inhibition of phage adsorption by SHA and Con A was rapid, occurring almost immediately upon their addition to the mixtures of cells and phage. The interaction between these lectins and cells effected no permanent changes in the cell surface; almost immediately after the addition of the lectin inhibitor phenyl galactoside to SHA/cell mixtures or methyl glucoside to Con A/cell mixtures, phage adsorption resumed.

Among the lectins tested, the most effective inhibitor was SHA. It has a specificity for non-reducing terminal L-rhamnose and the configuration of the hydroxy groups at C-2 and C-4 is

essential for binding (Fujita *et al.*, 1973; 1975). Therefore, it is concluded that an L-rhamnosyl residue is a major determinant in the PL-1 phage receptor site. Con A inhibited the phage adsorption, but to a lesser extent than SHA. Con A binds specifically to α -linked, non-reducing D-glucose and N-acetyl-D-glucosamine residues and is considered to interact with unmodified hydroxyl groups at the C-3, C-4 and C-6 positions of the pyranose ring (Goldstein & Iyer, 1966). Methylation analysis of the isolated polysaccharide yielded 2,3,4-tri-O-methyl-L-rhamnose and 2,3,4,6-tetra-O-methyl-D-glucose in addition to other, unidentified, sugars (unpublished data), suggesting that both these sugar residues occupy non-reducing terminal positions in the cell wall polysaccharide. However, the phage PL-1 appears to bind primarily to L-rhamnose. The lower degree of inhibition by Con A may indicate that the glucosyl residue also participates in the binding; alternatively, the binding of the lectin close enough to the phage binding site may prevent its binding by its steric hindrance.

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