Phage Receptor Material in *Lactobacillus casei*

By TERUO YOKOKURA

*Yakult Institute for Microbiological Research,
1796 Yaho, Kunitachi, Tokyo, Japan*

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SUMMARY

In *Lactobacillus casei* S-1, D-galactosamine and L-rhamnose comprised a phage receptor for phage J-1. A mixture of D-galactosamine and L-rhamnose effectively inactivated phage J-1, and a J-1-resistant mutant strain, *L. casei* S-1/J-1, lacked D-galactosamine in its surface component. The phage-inactivating effects of D-galactosamine and L-rhamnose were strongly dependent on the concentration of each substance and on temperature.

It is suggested that the receptor for phage J-1 involves both D-galactosamine in the cytoplasmic membrane and L-rhamnose in the wall of the host bacterium *L. casei* S-1, which lacks teichoic acid in its wall.

INTRODUCTION

In a previous paper (Yokokura, 1971), it was concluded that L-rhamnose in the wall *Lactobacillus casei* was one of the components of receptor material for phage J-1 since L-rhamnose not only inhibited phage adsorption on to the wall but also desorbed from the wall phages which had been adsorbed to it. The effects of D-galactosamine on these phenomena have been studied.

METHODS

Bacteria and bacteriophage. The bacterial strain, *Lactobacillus casei* S-1, and the bacteriophage J-1 were those previously reported (Yokokura, 1971). *Lactobacillus casei* S-1/J-1, which is a mutant strain resistant to phage J-1, was isolated from strain S-1 as described by Chatterjee (1969) using *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. The Y.P. broth and crude wall, purified wall and TCA (trichloroacetic acid)-soluble fraction were prepared as described previously (Yokokura, 1971). Phage J-1 was purified as described by Yamamoto *et al.* (1970) using polyethylene glycol. It was inactivated with hexosamine and sugar as follows: a suspension of purified phage J-1 [0.05 ml, 10^9 plaque-forming units (p.f.u.) ml⁻¹] was added to 5.0 ml 0.1 M-Tris/HCl buffer (pH 7.4) containing various hexosamines and sugars, and the mixture was incubated at 37 °C. From this incubation mixture, samples were withdrawn at intervals and the number of active phages was assayed.

Chemical analyses. Quantitative analysis for sugars and sugar alcohols in the wall or TCA-soluble fraction was performed as described by Sweeley *et al.* (1963) with a GC-5A gas-liquid chromatograph (Shimadzu Seisakusho, Kyoto, Japan) equipped with a hydrogen flame ionization detector on a 3 × 1000 mm glass column with 20 % Silicone SE-52 on Celite 545 (Nihon Kuromato Kogyo Co., Tokyo, Japan). Amino acids and hexosamines were estimated with a KLA-3B amino acid autoanalyser (Hitachi, Tokyo, Japan) after hydrolysis as described by Ikawa & Snell (1960). Hexosamines were also estimated by the method of Stewart-Tull (1968).

Table 1. *Chemical composition of L. casei* S-1 and S-1/J-1 wallsResults are expressed as μmol (mg dry wt material)⁻¹.

Component	Strain S-1		Strain S-1/J-1
	Wall	TCA-soluble fraction from wall	Wall
Rhamnose	1.58	2.92	1.90
Glucose	0.81	1.97	0.83
Galactose	0.70	1.79	—
Glucosamine	0.69	—	0.70
Galactosamine	0.09	—	—
Muramic acid	0.54	—	0.66
Lysine	0.29	—	0.44
Aspartic acid	0.19	—	0.20
Glutamic acid	0.31	—	0.40
Alanine	0.53	—	0.65

—, Indicates a value of less than 0.01.

Electron microscopy. Phage J-1 was treated with a mixture of hexosamine and sugar and dialysed against 0.1 M-ammonium acetate at 5 °C for 6 h. The phages, which were negatively stained with phosphotungstic acid, were examined in a JEM-7A electron microscope (Japan Electron Optics Laboratory, Tokyo, Japan).

Preparation of antisera and antigens. *Lactobacillus casei* S-1 or S-1/J-1 was cultivated in Y.P. broth at 37 °C for 18 h. Cells were collected by centrifugation, washed with saline and suspended in saline. The cell suspension was injected into rabbits intravenously after it had been heated at 60 °C for 30 min. The TCA-soluble fraction was used as antigen in the test for two-dimensional precipitin reaction in gel (Ouchterlony, 1967).

RESULTS

Composition of the walls of L. casei S-1 and S-1/J-1

Chemical compositions of the wall and TCA-soluble fraction isolated from the wall are summarized in Table 1. The peptidoglycan of these strains was composed of muramic acid, glucosamine, lysine, aspartic acid, glutamic acid and alanine. Strains S-1 and S-1/J-1 differed in the composition of their wall polysaccharide, neither galactosamine nor galactose being detected in the latter strain. The number of amino acids decreased during purification of the walls. No glycerol or ribitol was detected.

Inactivation of phage J-1

The number of active phages was assayed after incubation with D-galactosamine, D-glucosamine or their *N*-acetyl derivatives (0.5 M). Although D-galactosamine completely inactivated phage J-1 and D-glucosamine slightly inactivated it, their *N*-acetyl derivatives had no effect (Fig. 1). Neither 0.5 M-hydrochloric acid nor any of the amino acids found in the crude wall had any effect. The rate of phage inactivation by D-galactosamine in the presence of L-rhamnose was more than 100 times faster than that with D-galactosamine alone (Fig. 2). L-Mannose and L-fucose slightly enhanced the inactivating effect of D-galactosamine. The longer the period of exposure to D-galactosamine, the more rapidly the phages were inactivated on subsequent addition of L-rhamnose (Fig. 3*a*). The period of exposure to

Fig. 1

Fig. 2

Fig. 1. Inactivation of phage J-1 with 0.5 M-hexosamine. Phage J-1 suspension (10^8 p.f.u. ml $^{-1}$, 0.05 ml) was incubated in 5.0 ml 0.1 M-Tris/HCl buffer (pH 7.4) containing D-galactosamine (○), D-glucosamine (●), or *N*-acetylhexosamine (*N*-acetylgalactosamine or *N*-acetylglucosamine) (□). Samples were withdrawn at intervals and the number of active phages was counted, after dilution, by the agar layer method.

Fig. 2. Inactivation of phage J-1 with 0.2 M-D-galactosamine plus 0.2 M-sugar. (○) L-Rhamnose; (●) L-mannose; (□) L-fucose; (■) D-galactose; (△) D-mannose or D-glucose; (▲) D-galactosamine alone. The mixture was incubated at 37 °C and the number of active phages was assayed at 30 min intervals.

L-rhamnose, however, did not affect the rate of phage inactivation on addition of D-galactosamine (Fig. 3*b*). The survival rate of phage J-1 was assayed after incubation with various concentrations of D-galactosamine and L-rhamnose for 90 min. Although L-rhamnose (0.5 M) did not inactivate phage J-1, it enhanced the inactivating effect of D-galactosamine at all concentrations tested (Table 2). The inactivating effect was observed at temperatures above 20 °C when the concentrations of D-galactosamine and L-rhamnose were 0.2 M (Table 3). Cooling (Fig. 4*a, b*) or dilution (Fig. 4*b*) of the mixture prevented inactivation.

The serological properties of *L. casei* s-1/J-1 and its parent strain s-1 were compared. Two precipitin bands, *a* and *b* (Fig. 5), were strain specific and *L. casei* group B specific, respectively (Shimohashi *et al.*, 1971). *Lactobacillus casei* s-1/J-1 lacked the latter band. When phage J-1 was treated with D-galactosamine and L-rhamnose at 37 °C for 90 min, the shapes of the phages were normal but their heads appeared to be empty (Fig. 6).

Fig. 3. Inactivation of phage J-1 with D-galactosamine and L-rhamnose. Phage J-1 was first incubated at 37 °C with (a) 0.2 M-D-galactosamine (○---○) or (b) 0.2 M-L-rhamnose (○---○) and then (a) L-rhamnose or (b) D-galactosamine was added, as indicated by the arrows, to the incubation mixture to a final concentration of 0.2 M. After the addition, the number of active phages was assayed at 30 min intervals (○—○).

Table 2. *Effect of concentration of D-galactosamine and L-rhamnose on phage J-1 inactivation*

Phage J-1 was incubated with various concentrations of D-galactosamine and/or L-rhamnose in Tris/HCl buffer (pH 7.4) for 90 min at 37 °C. After incubation, the number of surviving phages was counted and the survival rate was calculated as follows:

Survival rate = $\frac{\text{p.f.u. of phage J-1 ml}^{-1} \text{ after incubation}}{\text{p.f.u. of phage J-1 ml}^{-1} \text{ before incubation}}$

D-Galactosamine concn (M)	L-Rhamnose concn (M)	10 ³ × Survival rate
0	0	1000
0.1	0	980
0.2	0	800
0.5	0	190
0	0.5	1000
0.1	0.1	25
0.1	0.2	23
0.1	0.5	17
0.2	0.1	6.5
0.2	0.2	2.7
0.2	0.5	1.2
0.5	0.1	6.6
0.5	0.2	1.6
0.5	0.5	0.88

Table 3. *Effect of temperature on phage J-1 inactivation by D-galactosamine and L-rhamnose*

Phage J-1 was incubated with 0.2 M-D-galactosamine and 0.2 M-L-rhamnose in Tris/HCl buffer (pH 7.4) for 90 min at various temperatures. After incubation, the survival rate was calculated as in Table 2.

Temperature (°C)	$10^3 \times$ Survival rate
0	1000
10	980
20	810
25	2.0
30	2.4
35	2.6
37	2.7

Fig. 4. Effect of temperature or concentration of D-galactosamine and L-rhamnose on inactivation of phage J-1. (a) Phage J-1 was added to 0.1 M-Tris/HCl buffer (pH 7.4) containing 0.2 M-D-galactosamine and 0.2 M-L-rhamnose chilled on ice and the number of active phages was measured at intervals (○---○). Samples were withdrawn at 30 min intervals, and assayed for active phages after further incubation at 37 °C (○—○).

(b) Phage J-1 was incubated with 0.2 M-D-galactosamine and 0.2 M-L-rhamnose at 37 °C (○—○). The number of active phages was assayed after chilling in an ice bath or diluting 100-fold with 0.1 M-Tris/HCl buffer (pH 7.4) at 37 °C (○---○).

Fig. 5. Double immunodiffusion reaction between antisera and antigens of *L. casei* s-1 and s-1/J-1. A, Anti-s-1 serum; B, anti-s-1/J-1 serum; C, s-1 antigen; D, s-1/J-1 antigen.

Fig. 6. Electron micrographs of phage J-1 (a) treated with a mixture of 0.2 M-D-galactosamine and 0.2 M-L-rhamnose for 90 min at 37 °C; (b) untreated control. Bar markers represent 0.1 nm.

DISCUSSION

The chemical composition of *L. casei* wall is well established (see, for example, Ikawa & Snell, 1960). It contains no teichoic acid and can be classified serologically into two groups, B and C (Knox, 1963; Sharpe *et al.*, 1973). It has now been confirmed that *L. casei* s-1 belongs to group B and that it has no teichoic acid in its wall.

Morse (1965) first suggested that teichoic acid is involved in the phage receptor and supporting evidence was first reported by Wolin, Archibald & Baddiley (1966). Since then, the sugar moiety in wall teichoic acid has been implicated in the phage receptor in many Gram-positive bacteria such as *Staphylococcus aureus* (Coyette & Ghuysen, 1968), *Bacillus subtilis* (Glaser, Ionesco & Schaeffer, 1966) and *Streptococcus faecium* (Vidaver & Brook, 1966). Douglas & Wolin (1971) have also found that polysaccharide is essential for phage infection in *Lactobacillus plantarum*. Phage receptor material in *L. casei* is interesting because this species does not contain teichoic acid in its wall.

Phage J-1 was inactivated by the host's surface substances, D-galactosamine and L-rhamnose; the latter was the most effective sugar of those tested in enhancing the phage-inactivating effect of D-galactosamine. L-Mannose and L-fucose were also effective in the phage inactivation. The same trend was observed in the phage adsorption-inhibiting effects of sugars (Yokokura, 1971).

The group B specific antigen of *L. casei* is determined by the L-rhamnosyl-N-acetylgalactosamine residue (Knox, 1963). Therefore, the acquisition of resistance to phage J-1 and deficiency of group-specific antigen in *L. casei* s-1/J-1 might result from the loss of D-galactosamine from the surface polysaccharide. Phages are inactivated by treatment with reagents such as sodium citrate which causes separation of head from tail and release of nucleic acid (Kuo *et al.*, 1971). Such structural disorders were not observed in phage J-1 inactivated with D-galactosamine and L-rhamnose, but the heads appeared to be empty. D-Galactosamine and L-rhamnose did not inactivate phages unrelated serologically to phage J-1, for example phages FYc and ϕ 219 which are virulent to *L. casei* group C and *L. plantarum*, respectively (Sakurai *et al.*, 1969). The inactivation of phage J-1 with D-galactosamine and L-rhamnose is thus a specific biological effect. In coli phage T4, the process of adsorption is dependent on temperature but the process of inactivation is not (Stent, 1963). In phage J-1, inactivation is dependent on temperature but adsorption is not.

The structure of phage J-1 is simpler than those of T-even phages, having neither tail-sheath nor tail-fibre. Therefore, infection by phage J-1 may be more dependent on the host

bacteria than are T-even phages. Since protease treatment removes the cytoplasmic membrane from crude wall (Sharpe *et al.*, 1973) and the heads of phages adsorbed on to crude wall seem to be empty like those in Fig. 6(a), the irreversible adsorption of phage J-1 on to crude wall described previously (Yokokura, 1971) may result from inactivation of the phage by a component of the cytoplasmic membrane in the crude wall. These findings suggest that phage J-1 needs both L-rhamnose in the wall and D-galactosamine in the cytoplasmic membrane for receptor material in *L. casei* s-1.

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