A Phage-resistant Mutant of *Lactobacillus casei* which Permits Phage Adsorption but Not Genome Injection

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SUMMARY

A phage-resistant mutant of *Lactobacillus casei* (strain YIT 9021) was capable of adsorbing both PL-1 and J-1 phages, but did not yield phage-infected cells. The mutant and wild-type strains were identical in morphology and sugar composition of the cell walls. Attempts to induce prophages from strain YIT 9021 were unsuccessful. Electron microscopical examination of negatively stained mixtures of phage PL-1 and YIT 9021 bacteria revealed that the phages were adsorbed to the cells in a tail-first orientation. All the adsorbed phages had DNA-filled heads. It was concluded that PL-1 adsorbed normally but was blocked in the injection of the phage genome into the cell.

INTRODUCTION

The early steps of phage infection, i.e. adsorption and penetration, are not as well understood as are the later steps. We have been investigating the mechanism of infection by phages that have a long non-contractile tail and belong to the B-group in the classification of Bradley (1967), using phages active against *Lactobacillus casei* as our model. One approach to the understanding of the mechanism of the early steps of phage infection is to use host mutants affecting the interaction between phages and cells. Therefore, we have isolated and characterized a phage-resistant mutant to which the phages adsorbed successfully, but failed to inject their DNA.

METHODS

Phages and bacteria. Phages PL-1 (Watanabe *et al.*, 1970, 1982) and J-1 (Hino & Ikebe, 1965; Kohsaka, 1977), and their host strain *L. casei* ATCC 27092 (YIT 9002) were from our stock cultures. The phage-resistant mutant YIT 9021, which allows the adsorption of PL-1 and J-1, but is not lysed by these phages, was isolated originally from colonies which appeared on plates of confluent lysis by J-1. The relative efficiencies of plating of PL-1 and J-1 on strain YIT 9021 as compared to strain YIT 9002 were about $5 \times 10^{-5}$ and $3 \times 10^{-3}$, respectively. The lysogenic *L. casei* IAM 1043 (Yokokura *et al.*, 1974) was kindly supplied by Dr T. Mitsuoka (University of Tokyo). General phage methods have been previously described (Watanabe *et al.*, 1970; Watanabe & Takesue, 1972, 1975).

Cell wall preparations and analytical methods. Bacterial cell walls were prepared as described by Ishibashi *et al.* (1982). For quantitative determination of the constituent neutral sugars, 5 mg of cell walls were hydrolysed at 100 °C for 12 h with 0.5 ml 0.5 M-H$_2$SO$_4$ in a sealed glass tube. After centrifugation of the hydrolysates at 3000 rev/min for 15 min, 0.3 ml quantities of the supernatants were passed through a small column of Amberlite IRA-400 resin followed by elution with deionized water for 30 min. The eluates were evaporated in a water-bath at 50 °C, and the residues were dissolved in 15 ml 0.15 M-citrate buffer pH 2.2. Analysis of these sugars was carried out in a Hitachi 834 liquid chromatograph. Phosphorus was determined by the method of Chen *et al.* (1956).

Electron microscopy

Bacterial surfaces. Washed cells from an exponentially growing culture were fixed with 1% glutaraldehyde in 0.1 M-phosphate buffer pH 7.2 for 1 h at room temperature. After washing with deionized water, the fixed specimens were dehydrated with a graded series of ethanol–water mixtures and finally with absolute ethanol. They were
dried in amylacetate by the critical point method, coated with a thin layer of gold–palladium while rotating in a vacuum evaporator, and examined in a Hitachi HFS-2 field emission source scanning electron microscope at 25 kV.

Negatively stained samples. A drop of specimen in 0.1% ammonium acetate was placed on a 400-mesh grid coated with Formvar–carbon film, and stained with 2% uranyl acetate for 0.5 to 2 min. Electron micrographs were taken with a JEOL 100-C transmission electron microscope at 80 kV.

RESULTS

General bacterial properties

A scanning electron micrograph of YIT 9021 cells showed that the cell surface was somewhat rough and wrinkled in the trunk area, was rounded and smooth at the ends. No appreciable morphological differences were apparent between strains YIT 9021 and YIT 9002. Similarly, they were indistinguishable in terms of their biological and culturing characteristics.

Interaction with phages

To compare the rate of adsorption of phage PL-1 to strains YIT 9021 and YIT 9002, an exponential phase culture of each bacterial strain was incubated at a low multiplicity of infection, in MR medium (pH 6-0) at 37 °C. At intervals, unadsorbed phage were assayed in the cell-free supernatants of these adsorption mixtures. The rates of phage adsorption to the two strains in terms of the decrease in the number of unadsorbed phages were very similar. Very similar results were obtained for phage J-1. The formation of PL-1-infected cells with strain YIT 9021 was then compared with that of strain YIT 9002 by an anti-phage serum method. Fig. 1 shows the time course of the formation of the phage-infected cells with each strain. When PL-1 was adsorbed to YIT 9002 cells, almost all input phages were recovered as infective centres. However, in the case of strain YIT 9021 the yield of infective centres was less than 0.5% under the same conditions, even after 40 min of incubation.

To determine whether adsorption of PL-1 to YIT 9021 cells was lethal, the number of surviving colony formers was determined after incubating the bacterial cells (B) with phage (P), at P/B ratios varying from 1 to 50, and compared with the corresponding number of surviving YIT 9002 cells. For these control cells, the number of colony formers decreased as expected with increasing P/B ratios. On the other hand, with YIT 9021, almost all the cells that had adsorbed PL-1 multiplied to form colonies regardless of the P/B ratio. Thus, there was no detectable killing of the cells by phage particles.

Properties of the cell walls

Isolated cell wall fractions from YIT 9021 cells were incubated in various concentrations with phage PL-1, and the numbers of unadsorbed and total (unadsorbed plus cell wall-adsorbed) phages were determined. As Fig. 2 shows, the numbers of unadsorbed phage decreased with increasing concentration of cell walls, but total phage remained at the level of the input, regardless of the cell wall concentration. These results show that the phage were adsorbed successfully to the cell walls, but were not inactivated; i.e. the phage adsorption was reversible. A comparison was then made of the sugar compositions of the cell walls of strains YIT 9021 and YIT 9002. Both contained rhamnose, glucose, galactosamine, glucosamine and muramic acid at the concentrations of about 1.25, 0.51, 0.35, 0.59 and 0.66 μmol/mg, respectively. Furthermore, both cell wall preparations contained less than 0.3% phosphorus. Thus, the phage sensitivity could not be correlated with differences in the cell wall sugar composition of the two strains.

Lysogeny test

In order to determine whether YIT 9021 was lysogenic, attempts were made to induce prophages from the cells by treatment with mitomycin C according to the method of Otsuji et al. (1959). Exponential phase cells of strains YIT 9021, YIT 9002 and IAM 1043 were incubated in MR medium (pH 6-0) in the presence of various concentrations of mitomycin C, and at intervals the absorbance of the cell cultures was measured at 660 nm. The growth of YIT 9021 was
inhibited by mitomycin C, but neither lysis nor prophage induction was observed. The same result was obtained with various concentrations of mitomycin C, and under various conditions of incubation (temperature, pH, age of the cultures). On the other hand, IAM 1043 and YIT 9002 lysed in the presence of mitomycin C and the presence of phage particles in the lysates was confirmed electron microscopically (micrographs not shown here). Induction of prophages from the cells of YIT 9021 by the u.v. light irradiation method of Lwoff et al. (1950) was also unsuccessful.

**Electron microscopic observation of phage–cell interaction**

PL-1 phages, at a high multiplicity of infection, were mixed with cells of strains YIT 9021 or YIT 9002 and incubated at 37 °C in MR medium (pH 6-0). Samples were taken periodically, fixed with glutaraldehyde, negatively stained with uranyl acetate and examined electron microscopically. When incubated with YIT 9021 cells, most of the phage particles were found to be adsorbed to the cells in a tail-first orientation, and all adsorbed particles appeared to be intact, i.e. the DNA was retained in the phage head (Fig. 3). The proportion of 'ghost' phages, (which had ejected their DNA) did not increase during the incubation period (Fig. 4). On the other hand, when the phages were incubated with YIT 9002 control cells, the number of empty coats ('ghosts') among the adsorbed particles increased with incubation time as expected, and more than 80% of the adsorbed phage had ejected their DNA during a 120 min incubation (Fig. 4). These results show that the blocked step in YIT 9021 was the injection of the phage genome into the cell.

**DISCUSSION**

In the early steps of phage infection, phages are adsorbed to a specific receptor site on the host cell surface, and subsequently a specific interaction between phage and cell wall components
Fig. 3. Negative staining of the mixture of PL-1 and strain YIT 9021. Glutaraldehyde fixation. Arrows show 'ghost' particles. Bar marker represents 0.45 μm.

Fig. 4. Time course of the formation of 'ghost' particles on PL-1 adsorption to cells. Each cell suspension (A660 = 0.35) was infected with PL-1 at a m.o.i. of about 300 and incubated at 37 °C in MR medium (pH 6.0). At intervals, aliquots were fixed with 1% glutaraldehyde for 40 min at room temperature and centrifuged. The material from the pellet was negatively stained with 2% uranyl acetate for electron microscope observation. Then, both intact and ghost particles were counted on 9 to 17 separate cells. Vertical bars show the standard deviation of the mean. ●, YIT 9021; ○, YIT 9002.
triggers the ejection of phage genomes from the head into the host cytoplasm. When a host component is altered by mutation, these events may not occur. We described in this paper the isolation of a phage-resistant mutant (strain YIT 9021) of *Lactobacillus* PL-1 that phage PL-1 was adsorbed without subsequent infection and cell killing. Direct examination in the electron microscope showed that most of the phage particles adsorbed to the mutant still contained intact DNA cores. These results clearly indicate that the mechanism of resistance is not based on a failure to adsorb phage because of the absence of receptors, but on a block in the ejection mechanism for phage DNA.

Similar kinds of phage-resistant mutants have been isolated from the *Escherichia coli-λ* (Scandella & Arber, 1974) and *Salmonella anatum-λ* (Kanegasaki & Tomita, 1976) systems. In the former case, it was suggested that particles remained in a reversible state for only a very short time before they became irreversibly adsorbed to the cell surface. However, in the case of the mutant *S. anatum*, a significant portion of active phages could be eluted from the phage–cell complexes on dilution, and the lipopolysaccharide isolated from the mutant had lost its capacity to inactivate $\lambda$ phage. Kanegasaki & Tomita (1976) have suggested that more than just the receptor lipopolysaccharide of the cell surface is involved in the early stages of phage infection. In the case of YIT 9021, the binding of phages to cells was almost as stable as that in the phage-sensitive strain YIT 9002, as shown by the technique of desorption with rhamnose described by Watanabe & Takesue (1975) (data not shown here). Watanabe & Takesue (1973) and Watanabe et al. (1979) found that PL-1 phages were adsorbed to the host cells at 0 °C, but the blender-resistant phage–cell complexes were formed only upon raising the incubation temperature to 37 °C. Furthermore, there was a good relationship between the ATP content of the cells and the extent of formation of blender-resistant phage–cell complexes. These results suggest that for PL-1 phage genome penetration the energy metabolism of host cells is required in addition to the receptor polysaccharide on the cell surface.

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