Reversibility of the Adsorption of Bacteriophage PL–1 to the Cell Walls Isolated from *Lactobacillus casei*

*(Accepted 31 August 1976)*

**SUMMARY**

Bacteriophage PL–1 adsorbed specifically to fragments of the isolated cell walls of its host *Lactobacillus casei* ATCC 27092 and failed to adsorb to cell wall fragments of resistant strains. Soon after mixing, an equilibrium situation of phage adsorption was attained. The equilibrium position was dependent on the cell wall concentration, but was not affected by the incubation temperature. The adsorbed phages were not inactivated by the cell wall fragments, but formed phage-cell wall complexes maintaining original phage infectivity. The infectivity of phage-cell wall complexes was neutralized by antiphage sera in the same manner as free phages. When the phage-cell wall complexes were repeatedly washed by centrifuging and resuspending in a fresh medium, the adsorbed phages were eluted as infective virions, confirming that phage adsorption was reversible. When the reactants concerned were allowed to approach equilibrium from the opposite direction, the same equilibrium state was achieved. The value of the equilibrium constant (*K*<sub>eq</sub>) with respect to reversible adsorption was constant with various phage concentrations under the conditions used here. When a mixture of phages and cell walls at an equilibrium state was diluted, the unadsorbed phages increased in accordance with the decrease in the concentration of the reactants.

The initial event in phage infection involves adsorption of the phage to a specific receptor site on the host cell surface (Garen & Kozloff, 1959). Therefore, to locate the site of receptors and to understand the molecular interactions involved in phage adsorption, the direct reaction of phages with cell wall fractions isolated from their host cells has been examined. Previous studies (Fischetti & Sabriskie, 1968; Chatterjee, 1969; Bartell *et al.* 1971; Brown, 1971; Furuchi & Tokunaga, 1972; Incardona & Selvidge, 1973; Takeda & Uetake, 1973; Zarybnicky, Zarybnicka & Frank, 1973; Schwartz & Minor, 1975; Watanabe & Shiomi, 1975) with different phage-cell wall systems indicated that most phages were irreversibly adsorbed and inactivated by the cell walls isolated from their host cells. The structure of the receptors has been found to be highly specific, differing widely from one phage to another.

We have been investigating the infection mechanism of PL–1 phage active against a strain of *Lactobacillus casei*. Since the phage possessed a long, flexible and non-contractile tail (Watanabe *et al.* 1970), the infection mechanism was supposed to be different from that of T-even phages which possess a contractile tail sheath playing an important role in the penetration of phage genomes into the host cells. In one attempt to study the mechanism of PL–1 phage adsorption and to gain information regarding the specific phage receptor materials, we examined the interaction between phages and cell wall preparations isolated by sonication of the host cells. It was found that PL–1 phages were adsorbed only reversibly to cell walls and that there was an equilibrium state between free phages and the cell wall-adsorbed phages.

As the host cells of PL–1 phage, *Lactobacillus casei* ATCC 27092 (sensitive strain), a phage-resistant mutant derived from the sensitive strain according to the method of Adelberg,
Mandel & Chen (1965), and Lactobacillus casei ATCC 7469, commonly used for the bioassay of vitamin B2, were used. The methods for preparing purified phage stocks and for assaying phage titres (p.f.u./ml) have been previously described (Watanabe et al. 1970). For preparing the cells in logarithmic growth phase, 10 % (v/v) of the overnight cell cultures was incubated at 37 °C in MR medium (Murata, Soeda & Saruno, 1969) for about 3 h until the optical density of the culture in a Hitachi photoelectric colorimeter (model, EPO-B) at 660 nm was about 0.35. The cells in logarithmic growth phase were harvested by continuous centrifugation at 8000 rev/min, washed twice with deionized water and finally suspended in water at an optical density of about 0.8. The cell suspensions in 80 ml vol. were sonicated in a Kubota 20 KC-oscillator (model, Insonator 200-M) for 10 to 15 min at 4 °C until the optical density of suspensions decreased to about 0.3. These lysates were stored at 4 °C overnight, and then centrifuged at 4000 rev/min for 10 min at 2 °C to sediment any remaining intact cells and debris. The supernatant suspension containing the cell wall fractions was again centrifuged at 10000 rev/min for 15 min at 2 °C to sediment the cell walls, which were then suspended in 1 M-NaCl for 5 min to elute viscous nucleic acids, and again centrifuged. After washing with water, crude cell walls were suspended in 50 mM-phosphate buffer, pH 7.0, and treated with 0.5 mg/ml trypsin and RNase for 2 h as described by Cummins & Harris (1956). Finally, they were washed twice with deionized water, and lyophilized for storage. Examination in a JEOL-100 C electron microscope revealed that the cell wall preparations were freed from intact cells but contained some of the cell wall fragments. PL-I phage antisera were prepared in male rabbits intraperitoneally injected with the high titred phage suspensions. The antiphage sera freed from host cell antibodies were heated at 56 °C for 30 min and stored at -20 °C without preservatives until use.

For assaying the phage adsorption, a 2.7 ml portion of the cell walls were incubated at 37 °C with 0.3 ml of phages of 10⁶ p.f.u./ml in MR medium, pH 6.0, or in 50 mM-tris-maleate buffer, pH 6·0, containing 10 mM-CaCl₂. At intervals, 0·2 ml of the mixture was centrifuged, without dilution, at 10000 rev/min for 15 min at 2 °C to sediment the phage-adsorbing cell walls. A portion of the supernatant fluids was diluted appropriately and plated with the host cells as indicator in a soft agar layer for determining the amounts of unadsorbed free phages. The phage counts were made in triple sets of Petri dishes, calculated to contain about 200 plaques/dish where there was no adsorption. Percentage of unadsorbed phage was computed as (unadsorbed phages/input phages) × 100. The amount of total phage (unadsorbed and cell wall-adsorbed phages) was determined by plating the adsorption mixture directly, without centrifuging.

To test the possible phage-adsorbing ability of the isolated cell walls of Lactobacillus casei ATCC 27092, various concentrations of cell walls were incubated with phages and at intervals both the amounts of unadsorbed and total phages were assayed. At their steady state, the proportion of unadsorbed phages decreased with the increasing concentrations of cell walls (Fig. 1). However, it was independent of the incubation time. After mixing of phages with cell walls, a steady state was accomplished almost immediately according to the respective cell wall concentrations, after which the amounts of unadsorbed phages did not change with time. One of the distinctive features of this phage-cell wall system was that the adsorbed phages were not inactivated by the cell walls; namely, phage adsorption was not irreversible. The amounts of total phages, therefore, did not decrease, but held the level of input ones regardless of the cell wall concentrations. In most other cases, as shown for instance by Takeda & Uetake (1973), incubation of phages with the isolated cell wall preparations from sensitive bacteria caused them to be exponentially inactivated with incubation time. That is, the amount of total phages decreased in proportion to the cell wall
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Fig. 1. Proportion of both the unadsorbed and total phages to the input ones at the steady state after mixing PL-1 phages with various concentrations of cell walls isolated from the host Lactobacillus casei ATCC 27092. PL-1 phages (1.5 × 10^6 p.f.u./ml) were mixed with cell walls at 37 °C in MR medium, pH 6.0. Samples were taken at intervals (0, 10, 20, 30 and 40 min) and assayed for both unadsorbed and total (unadsorbed plus adsorbed) phages. Each point is the average of five separate assays, each done in triplicate. Vertical bars show the standard deviation of the mean (±). Standard deviation $s = \sqrt{\frac{\sum(x_i-\bar{x})^2}{n}}$.

- •, Unadsorbed phages; ○-○, total phages; ........., a theoretical curve of unadsorbed phages in the case where the value of $K_{eq}$ in equation (2) is constant [in this case, $= 0.1$ (μg/ml)^{-1}].

concentrations, and the inactivation curve of total phages coincided with that of unadsorbed phages. As a control experiment, the cell walls of phage-resistant strains were substituted for those of sensitive strain. The cell walls (50 μg/ml) of Lactobacillus casei ATCC 7469 showed no tendency to adsorb phages. Those of a phage-resistant mutant derived from the sensitive strain adsorbed phages much less than those of sensitive strain. This result shows that phage adsorption was specific to the strain from which cell walls were prepared. We have now detected, by paper-chromatography, rhamnose, glucose and some hexosamines in the acid hydrolysates of cell walls of these strains. The crude cell wall preparations, not treated with trypsin and RNase, did not inactivate the phages. The addition of the cell-free extracts sonically obtained again did not cause cell walls to inactivate the adsorbed phages. Purified cell walls heated at 100 °C for 30 min produced the same results as in Fig. 1, indicating that the phage-adsorbing site of cell walls is heat-resistant and may not be associated with such heat-sensitive materials as protein. Once again, the proportion of unadsorbed phages in the equilibrium state at 0 °C was almost identical to that at 37 °C, showing that significant activation energy is not required in phage adsorption to cell walls and that ionic groups of the two surfaces may interact primarily. When tris-maleate buffer was used as adsorption medium instead of MR medium, almost the same result was obtained.

The infectivity of the phage-cell wall complexes was neutralized by specific antiphage sera to the same extent and with the same first order kinetics as free phages having the same titres. This result suggests that the nature of phage adsorption may be reversible and that
Table 1. Approaching equilibrium from opposite directions

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Direction</th>
<th>Cell walls (μg/ml)</th>
<th>Total phages (p.f.u./ml)</th>
<th>Free phages (%)</th>
<th>Kq (μg/ml)⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>A</em> P+CW →</td>
<td>10</td>
<td>1.8 x 10⁶</td>
<td>44</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td><em>B</em> P-CW</td>
<td>10</td>
<td>1.0 x 10⁶</td>
<td>45</td>
<td>0.12</td>
</tr>
<tr>
<td>2</td>
<td>A P+CW →</td>
<td>50</td>
<td>2.1 x 10⁶</td>
<td>31</td>
<td>0.044</td>
</tr>
<tr>
<td></td>
<td>B P-CW</td>
<td>50</td>
<td>1.3 x 10⁶</td>
<td>33</td>
<td>0.042</td>
</tr>
</tbody>
</table>

* (A) PL-1 phages were incubated with cell walls at the indicated concentrations at 37 °C in MR medium, pH 6.0, for 20 min. After centrifuging the mixture, the supernatants were assayed for unadsorbed free phages.

† (B) The centrifuged cell walls from (A) were resuspended in the same vol. of medium and maintained for another 20 min, after which the suspensions were centrifuged and again the supernatants were assayed for unadsorbed free phages.

The phages are eluted from the phage-cell wall complexes upon resuspending them in the serum solution. In addition, with glutathion, which had been shown by Murata et al. (1972) to inactivate various kinds of phages, the inactivation curve of phage-cell wall complexes was identical with that of free phages. Having inferred that phage adsorption might be a reversible process, an experiment was then carried out to determine whether the resuspension procedure was causing the adsorbed phages to be eluted from the phage-cell wall complexes. A 10 min incubation mixture of phages (2.5 x 10⁶ p.f.u./ml) and cell walls (50 μg/ml) in MR medium, pH 6.0, was centrifuged, to separate the unadsorbed phages, and the pellets containing phage-cell wall complexes were resuspended in the equal vol. of the fresh medium, and centrifuged again in the same manner. This procedure was repeated for four consecutive cycles on a given phage stock, and the phage titres of both the pellet suspensions (remaining) and the supernatants (eluted) were assayed after each cycle. This experiment was designed to determine the amount of cell wall-adsorbed phage which is subsequently removable by repeated washing of the phage-cell wall complex. The result showed that upon repeated washing with fresh media, there was a gradual elution of the adsorbed phages as infective virions, which could readсорb to the intact cells and cause lysis. The ratio of eluted phages to those remaining was roughly constant. Similar results were obtained with tris-maleate buffer as the washing and suspending medium. This behaviour is compared with those of other phage-cell wall systems: T4 phages, which had been adsorbed to the purified membranes of Escherichia coli B, were eluted by diluting the mixture into a nutrient broth with 0.5% added NaCl (Christensen & Tolmach, 1955); N1 phages adsorbed to the isolated cell walls of Micrococcus lysodeikticus strain 1 were eluted by a chloroform-containing cold tris-buffer (Lovett & Shockman, 1970); J1 phages adsorbed to the isolated cell walls of Lactobacillus casei strain S1 were eluted by 0.2 M-L-rhamnose (Yokokura, 1971). In the case of whole cells, Garen & Puck (1951) and Garen (1954) reported earlier that under the following conditions T4 phage attached only reversibly to its host cells; lowering of the reaction temperature to 0 °C, ultraviolet irradiation of the cells, previous treatment of the cells with zinc ions and substitution of the phage-resistant mutant.

If the adsorption of phages to cell walls is reversible with an equilibrium state between adsorbed and unadsorbed phages, the reaction can be expressed as follows:

\[ P + CW \rightleftharpoons P \cdot CW, \]

\[ K_{eq} = \frac{k_1}{k_2} = \frac{(P \cdot CW)}{(P) \times (CW)} \times \frac{(T)-(P)}{(P) \times (CW)} \]

where P represents unadsorbed free phages, CW cell walls, P-CW reversibly associated phage-cell wall complexes and T total phages (P + P-CW). The symbols k1 and k2 refer to
the specific rate constants for the indicated reactions and $K_{eq}$, the equilibrium constant. These equations apply under the conditions where the total number of phage particles adsorbed is small compared to the total number of potential cell wall receptor-sites. The following experiments were then run to confirm that PL-I phage adsorption to cell walls is a reversible reaction as expressed in equation (1). At first, in order to prove the achievement of a state of equilibrium with respect to reversible adsorption of phages to cell walls, experiments were carried out where the reactants concerned were allowed to approach equilibrium from opposite directions. Table 1 shows that the same value for the $K_{eq}$ was obtained regardless of whether the reaction was started from phage-cell wall complexes which contained originally only adsorbed phages or when free phages were mixed with equal concentrations of cell walls. With different concentrations of cell walls (Expt. 1 and 2), the value for the $K_{eq}$ was not always constant as could be inferred from Fig. 1. The proportion of unadsorbed phages in the equilibrium position deviated from the theoretical values (the dotted line in Fig. 1 is a theoretical curve of unadsorbed phages when the $K_{eq}$ value is constant, $K_{eq} = 0.1 \text{ (mg/ml)}^{-1}$). The $K_{eq}$ value tended to decrease when the proportion of unadsorbed phages was below 50% and increase when it was above 50%.

In equation (2), under the conditions where the ratio of phages to potential cell wall receptor sites was kept low, the equilibrium constant should be independent of the total concentration of phages. This was observed to be the case for a thousand-fold variation in total concentration of phages. When various concentrations of phages ($10^8$ to $10^6$ p.f.u./ml) were adsorbed to cell walls (50 $\mu$g/ml) at 37 °C in a tris-maleate buffer, pH 6.0, for 20 min, $K_{eq}$ values of 0.033 to 0.035 ($\mu$g/ml)$^{-1}$ were obtained. If a mixture of phages and cell walls which has reached an equilibrium state is diluted, the amount by which the denominator, $(P \times CW)$, is decreased, will be greater than that of numerator, $(P-CW)$. Therefore, the amounts of $(P-CW)$ must decrease and those of $(P)$ increase to readjust the system to a new equilibrium position in accordance with the change in the concentrations of phages and cell walls. As a test of this prediction, a mixture of phages ($2.1 \times 10^6$ p.f.u./ml) and cell walls (45 $\mu$g/ml) at an equilibrium state was diluted in fresh medium by factors of 10 and 100, and the amounts of free phages $(P)$ in the supernatant fluid were measured. The values for $(P)$ actually increased from 30 to 57 and 84%, respectively. We therefore conclude that the adsorption of PL-I phages to the isolated cell walls of host Lactobacillus casei behaves as a reversible system with an equilibrium state expressed by equations (1) and (2). Because of the reversible nature, the removal of free phages from the adsorption mixture leads to the dissociation of phage-cell wall complexes so as to readjust the equilibrium state.

The term phage receptor has been used for a specific cell surface structure which adsorbs phages irreversibly and inactivates them (Salton, 1964; Tokunaga, 1972). From this standpoint, the cell walls of Lactobacillus casei can be regarded as ‘incomplete’ or ‘primitive’ phage receptors for phage PL-1. The inability of the cell walls to inactivate phage PL-1 implies that other cellular components in addition to the cell walls prepared here may be required for the irreversible adsorption of phage PL-1.

The authors wish to thank Dr K. Asou of Yakult Institute for his generous supply of Lactobacillus casei ATCC 7469. We also thank the following undergraduate students of Fukuoka University for their technical assistances in part of the experiments: E. Yukawa, Y. Wada, T. Kitahara, E. Hashimoto and A. Tsukamoto.

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(Received 11 May 1976)