Adsorption of the Defective Phage PBS Z1 to Bacillus subtilis 168 Wt

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SUMMARY

Three aspects of the adsorption of the defective phage PBS Z1 to Bacillus subtilis 168 Wt have been investigated. These are the kinetics, the number of receptors on the cell wall and the character of these receptors. The reaction constants for the binding of phages onto receptors, for the dissociation of the phage–receptor complex and for the transition from reversible to irreversible binding of the phages were calculated from adsorption curves obtained by an enzyme-linked immunosorbent assay (ELISA). They were $1.8 \times 10^{-13}$, $6.7 \times 10^{-2}$ and $9.0 \times 10^{-3}$ respectively. The maximum number of phages adsorbed per cell was 2700, a number limited by the surface area of the cells. Apart from the receptors on the cell wall, receptors on the cell membrane were found. This was concluded from additional adsorption experiments with stable L-forms and contracted phages. Based on these results, together with data from the literature on bacteriocins, phage ghosts and yeast killer factors, a hypothesis concerning the first stage of killing by defective phages has been formulated.

INTRODUCTION

Bacillus subtilis strains harbour a range of defective phages known as PBS X, PBS Y, PBS Z and PBS W (Eiserling, 1964; Seaman et al., 1964; Ionesco et al., 1964; Stickler et al., 1965; Steensma et al., 1978). Similar phages have been reported for Bacillus licheniformis (Huang & Marmur, 1970) and Bacillus pumilus (Bradley, 1965; Steensma et al., 1978). These phages share a common morphology and are serologically related. They are distinguished by their tail length, the number of cross-striations on the tail and their killing range (Steensma et al., 1978). The latter has been shown to be dependent on the teichoic acids in the bacterial cell wall (Glaser et al., 1966). Furthermore, it has been established that within 10 min of the addition of PBS X to sensitive cells, the synthesis of protein, RNA and DNA is halted (Okamoto et al., 1968). Similar phenomena have been found with phage ghosts (Duckworth, 1970), bacteriocins (for reviews, see Hardy, 1975; Tagg et al., 1976) and yeast killer factors (Pietras & Bruenn, 1976). It has been suggested that these agents kill bacteria in a comparable way (Nomura, 1967; Changeux & Thiéry, 1967). They have in common an initial cell wall adsorption stage during which rescue is often possible. This stage is followed by either active or passive transfer of the killing agent to the cytoplasmic membrane and the biological target within the cell. Apart from the studies by Glaser et al. (1966) and Okamoto et al. (1968), only genetic approaches to the killing by PBS X have been reported (Buxton, 1976; Yasin & Ledbetter, 1978). To elucidate the mechanism by which these defective phages kill sensitive bacteria, the adsorption of PBS Z1 on to cells of B. subtilis 168 Wt has been investigated.
METHODS

Organisms. *Bacillus subtilis* 168 Wt (LMD 69-3) and *B. subtilis* W23 str (LMD 69-2) were obtained from the culture collection of the Delft Laboratory of Microbiology. Stable L-forms were made according to Young et al. (1970), but with the T medium replaced by TGT as the L-forms grew better in this medium. L-forms were stored in liquid nitrogen or maintained by serial passage at intervals of 2 or 3 days. After 20 transfers in medium lacking penicillin, the L-forms had not reverted to the bacillary form. Protoplasts were prepared by treatment of young, exponentially growing cultures in TGT with 2000 units/ml penicillin G and 100 μg/ml lysozyme. After 60 to 120 min at 37 °C, the resulting protoplasts were resuspended in fresh TGT.

The induction and purification of PBS X1 and PBS Z1 have been described elsewhere (Steensma & Sondermeijer, 1977). The purified suspensions in tris-1.5 M-KCl were stored at 4 °C. Phages were contracted by dialysis against 0.01 M-EDTA, pH 8 (Konopa & Taylor, 1975; Haas & Yoshikawa, 1969) or 0.01 M-tris, 0.001 M-EDTA, pH 8.

Media. TY contained per litre: 10 g tryptone, 5 g yeast extract and 10 g NaCl and was adjusted to pH 7.2. TGT consisted of 10 g tryptone, 5 g yeast extract, 70 g NaCl, 1 g KH₂PO₄ and 3 g K₂HPO₄ per litre and 0.005 M-MgSO₄ (separately sterilized). TY agar was TY with 2% agar. DP agar has been described by Landman et al. (1968). Tris-1.5 M-KCl contained 0.01 M-tris, 0.005 M-MgSO₄ and 1.5 M-KCl, pH 7.2.

Enumeration. Bacteria were counted microscopically, or by plating. Plate counts were made by spreading 0.1 ml of a dilution in TY on TY agar plates. Colonies were counted after 18 to 24 h incubation at 37 °C. The phage:bacteria ratios for the kinetic experiments and for the estimations of the number of receptors were determined from chamber counts of the bacteria with cells in chains being counted as separate individuals.

For the experiments with wall-free cells, L-forms were preferred to protoplasts as the latter may carry traces of cell wall material. Cultures of L-forms contained individuals of various dimensions (from 4 μm to barely visible under the light microscope), which made chamber counts extremely difficult. Consequently, phage:L-form ratios were based on plating of the L-forms on DP agar. Ratios exceeding 100 were used routinely, but the plate counts were generally 10% of the chamber counts in which many of the small cells were not counted. Electron microscopy showed that these small vesicles could adsorb phages. The actual phage:cell ratios, therefore, were much lower than 100.

L-forms were plated by mixing 0.1 ml of a dilution in TGT with 10 ml DP agar held at 45 °C. Colonies were counted after 48 to 72 h at 37 °C.

The number of unadsorbed phage was found by centrifugation of 1 ml samples for 1 min at 14000 g, followed by an enzyme-linked immunosorbent assay (ELISA) (Steensma & Duermeyer, 1979), of the supernate.

Kinetic experiments. An overnight culture of *B. subtilis* 168 Wt in TY was diluted 1/100 into fresh medium and incubated for 2 h at 37 °C. Growth generally started after 2.5 h. Bacteria were counted in a chamber and PBS Z1 was added to give the desired phage:bacteria ratio. The number of free phages was assayed at various times. The initial phage concentration was obtained from a control in which bacteria had been omitted. Phages were stable under the experimental conditions and it was not necessary to correct for bacterial growth during the experiment.

Determination of the number of receptors. Bacteria were grown as above. PBS Z1 was added to give various phage:bacteria ratios and the mixtures were incubated for 60 min at 37 °C. Unadsorbed phages were then determined by the ELISA. The phage:bacteria ratios were calculated from the number of phages in control samples without bacteria and the chamber counts of the bacteria immediately before the addition of phages. During an experiment the bacterial counts increased by less than 15%.
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Fig. 1. Adsorption curves. Phages were added to bacteria to give various phage:bacterium ratios: ○——○, 13·0; △——△, 3·8; □——□, 14·4. At the times indicated samples were taken and the number of free phages assayed and plotted as a ratio of the final titre P to initial titre P0.

Fig. 2. Maximum number of adsorbed phages. Phages were added to bacteria to give various phage:bacterium ratios. After 60 min incubation the number of free phages was assayed. The results of two independent determinations are combined. Only the higher phage:bacterium ratios are shown but the lower values lay on the same line.

Table 1. Reaction constants

<table>
<thead>
<tr>
<th>Phage:bacterium</th>
<th>R (ml⁻¹)*</th>
<th>k₁ (ml min⁻¹)</th>
<th>k₋₁ (min⁻¹)</th>
<th>k₂ (min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13·0</td>
<td>2·0 × 10¹²</td>
<td>1·6 × 10⁻¹³</td>
<td>8·3 × 10⁻²</td>
<td>1·48 × 10⁻³</td>
</tr>
<tr>
<td>3·8</td>
<td>3·5 × 10¹²</td>
<td>1·7 × 10⁻¹³</td>
<td>6·2 × 10⁻²</td>
<td>7·7 × 10⁻³</td>
</tr>
<tr>
<td>14·4</td>
<td>4·3 × 10¹²</td>
<td>2·0 × 10⁻¹³</td>
<td>5·5 × 10⁻²</td>
<td>4·5 × 10⁻³</td>
</tr>
<tr>
<td>Mean</td>
<td>1·8 × 10⁻¹³</td>
<td>6·7 × 10⁻²</td>
<td>9·0 × 10⁻³</td>
<td>5·3 × 10⁻³</td>
</tr>
<tr>
<td>S.D.</td>
<td>0·2 × 10⁻¹³</td>
<td>1·5 × 10⁻²</td>
<td>5·3 × 10⁻³</td>
<td>5·5 × 10⁻³</td>
</tr>
</tbody>
</table>

* The receptor concentration R is the product of the bacterial concentration and 2700, the mean number of receptors/cell.

Killing. Young, exponentially growing, bacterial cultures in TY or actively growing cultures of L-forms in TGT were incubated with phages for 60 min at 37 °C. The percentage of killing was calculated from post-incubation plate counts of the mixture and of a blank without phages to which the same volume of tris–1·5 M-KCl had been added.

Electron microscopy. Preparations for the electron microscope were made on 200 mesh formvar-coated copper grids. Phages and mixtures of cells and phages were washed on the grids with distilled water and negatively stained with a solution of 2% uranyl acetate in 50% ethanol. The adsorption of phages to L-forms was also studied in platinum-shadowed preparations and in ultra-thin sections. For the former, samples were prefixed with 3% glutaraldehyde before application to the grids. All three methods gave essentially similar results.

RESULTS

Kinetics of phage adsorption

With the experimental conditions used, adsorption was rapid and approx. 90% of the phages were bound within 60 min (Fig. 1). In two cases this occurred within 10 min. The shape of the curves primarily varied with the receptor concentration (R) as may be concluded from the comparison of Fig. 1 and Table 1. The same conclusion may be drawn from the
The formulae below. In these experiments phage:bacteria ratios between 3 and 14 were used. Despite these relatively high values, the reaction constants could be calculated from a pseudo first order reaction by considering the reaction between the phages and the receptors on the cell wall. This is represented by:

$$P + R \xrightarrow{k_1} PR \xrightarrow{k_2} PR^*$$

where $PR$ and $PR^*$ stand for receptors with reversibly and irreversibly bound phage respectively. Assuming that each receptor only binds one phage, that the number of receptors is much higher than that of phages and that the phage-receptor complex does not influence neighbouring receptors, it may be deduced that:

$$P = P_0 e^{-k_1 R t} + \left( k_1 k_2 R / K \right) e^{-k_1 k_2 R / K}$$

in which $K = k_{-1} + k_2 + k_1 R$, $P_0$ is the initial phage concentration, $P$ the phage concentration at time $t$, and $R$ the concentration of receptors which equals the product of the mean number of available receptors/bacterium and the bacterial concentration. The mean number of receptors was determined in a separate experiment using cells grown under similar conditions as those used for the kinetics experiments. A value of 2700 was used to calculate $k_1$, $k_{-1}$ and $k_2$ (Table 1).

Number of receptors/bacterium

The mean number of receptors/bacterium was determined for two reasons: (i) this figure was required for the calculation of the reaction constants in the preceding paragraph and (ii) if the number of available receptors on a cell was low, the phages could not kill the bacteria by lysis from without. Such a hypothesis concerning the killing mechanism of the defective phages could then be refuted. Fig. 2 displays the results of two assays, which gave a value of 2700, with a standard deviation of 300. A greater variation was found between cultures of different ages. This probably reflects the differences in surface area, as will be discussed later.

Characterization of the receptors

The adsorption of PBS Z1 and other defective phages is specific, as shown by the killing pattern of these phage (Steensma et al., 1978) and electron microscopy (EM). The present work is an extension of that research, involving the quantitative measurement of adsorption using an ELISA. It showed that PBS Z1 does not adsorb to its own resistant host, B. subtilis W23 str', whereas more than 95% adsorbed to B. subtilis 168 Wt, killing at least 80% of the cells (Table 2).

When L-forms of B. subtilis 168 Wt were used, the ELISA indicated only 25% adsorption and many free phage were seen under the electron microscope. This was not due to the high salt concentration in the medium (TGT), because vegetative cells of 168 Wt gave similar adsorption and killing whether grown and incubated in TGT or TY. Moreover, phages that had adsorbed to L-forms were not contracted, whereas phages on vegetative cells were always seen to be contracted (Fig. 3). This suggests that phages on L-forms are reversibly bound, which also explains why, at such a low level of adsorption, 89% of the bacteria were killed (Table 2). The latter figure means that the contraction process is not, in itself, responsible nor essential for the bacteriocidal effect of the defective phages.

Because the first event after adsorption is the contraction of the phage tails, the presence of a receptor on the cell membrane was further investigated by using contracted phages. These
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Table 2. Characterization of the receptors

<table>
<thead>
<tr>
<th>Bacteriophage*</th>
<th>Organism</th>
<th>Adsorption‡</th>
<th>Killing (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS Z (extended)</td>
<td>Vegetative cells of B. subtilis W23 strf</td>
<td>--</td>
<td>&lt;10</td>
</tr>
<tr>
<td>PBS Z (extended)</td>
<td>Vegetative cells of B. subtilis 168 Wt</td>
<td>+</td>
<td>96</td>
</tr>
<tr>
<td>PBS Z (extended)</td>
<td>L-forms B. subtilis 168 Wt</td>
<td>+</td>
<td>25</td>
</tr>
<tr>
<td>PBS Z (extended)</td>
<td>Protoplasts of B. subtilis W23 strf</td>
<td>--</td>
<td>ND†</td>
</tr>
<tr>
<td>PBS Z (contracted)</td>
<td>Vegetative cells of B. subtilis W23 strf</td>
<td>--</td>
<td>ND</td>
</tr>
<tr>
<td>PBS Z (contracted)</td>
<td>Vegetative cells of B. subtilis 168 Wt</td>
<td>--</td>
<td>0</td>
</tr>
<tr>
<td>PBS Z (contracted)</td>
<td>L-forms B. subtilis 168 Wt</td>
<td>+</td>
<td>44</td>
</tr>
<tr>
<td>PBS Z (contracted)</td>
<td>Protoplasts of B. subtilis W23 strf</td>
<td>--</td>
<td>ND</td>
</tr>
<tr>
<td>PBS X (extended)</td>
<td>Vegetative cells of B. subtilis W23 strf</td>
<td>+</td>
<td>80</td>
</tr>
<tr>
<td>PBS X (extended)</td>
<td>Vegetative cells of B. subtilis 168 Wt</td>
<td>--</td>
<td>0</td>
</tr>
<tr>
<td>PBS X (extended)</td>
<td>L-forms B. subtilis 168 Wt</td>
<td>--</td>
<td>0</td>
</tr>
<tr>
<td>PBS X (contracted)</td>
<td>L-forms B. subtilis 168 Wt</td>
<td>--</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Phages, which are normally present in extended form, were contracted as described in Methods. Upon contraction the sheaths of the tails become shorter and thicker and the typical cross-striations disappear.

† The adsorption determined by ELISA, and the killing varied with the number of phage/bacterium. The values given are representative for phage:bacterium ratios between 10 and 500. Because of the presence of many small vesicles, the phage : cell ratios for the L-forms are uncertain.

‡ ND, Not determined.

do not adsorb to or kill vegetative cells of B. subtilis W23 strf and 168 Wt but they do so with L-forms of B. subtilis 168 Wt (Table 2). Electron microscopy revealed that the majority of the contracted phages adsorb to L-forms (Fig. 3). The lower value found with the ELISA could be explained by the fact that many small vesicles are present in the cultures of L-forms. These adsorb phages but perhaps do not sediment on centrifugation, thus giving an erroneously high value for the unadsorbed phages in the assay. Alternatively, this may be caused by reversible adsorption, but this is less probable because of the low number of free phages observed under the electron microscope. These results justify the conclusion that a receptor is present on both the cell wall and the cell membrane.

The specificity of the receptors on the cell membrane was tested by adding extended or contracted PBS Z1 to protoplasts of B. subtilis W23 strf (Table 2). Vegetative cells of this strain did not adsorb this phage and neither did the protoplasts. But as the experiments with PBS Z1 and L-forms showed that killing could occur at a low level of adsorption (Table 2), experiments were done using extended and contracted PBS X1 with vegetative cells and L-forms of its host 168 Wt. Table 2 shows that adsorption and killing were not observed in these experiments. From these results it was concluded that the receptors on the cell membrane are specific.

This section may be summarized by stating that PBS Z1 and other closely related defective phages (Steensma et al., 1978) adsorb to receptors on the cell wall and on the cell membrane, and that both are specific.

Although it was not the direct purpose of this investigation, three conclusions regarding the killing mechanism may be drawn from our observations. (i) The number of receptor sites does not exclude a lysis from without mechanism and this hypothesis requires further research. (ii) The contraction of the phage tail is neither essential nor responsible for the killing of bacteria as demonstrated by the lethal effect of extended phages of L-forms of B. subtilis 168 Wt. (iii) The bactericidal action of the phages is not caused by damage to the cell wall and the concomitant lysis of the cells in hypotonic medium as described for a bacteriocin from Clostridium perfringens (Mahoney et al., 1971). Such a mechanism was refuted because both cells and L-forms in hypertonic medium (TGT) were killed (Table 2).
Fig. 3. Adsorption of PBS Z to vegetative cells and L-forms of *B. subtilis* 168 Wt. (a) Vegetative cell incubated with extended phages. All phages are contracted (negatively stained). (b) L-form incubated with extended phages. No contraction took place and many phages are unadsorbed (negatively stained). (c) L-forms incubated with contracted phage, the majority of the phage are bound (platinum-shadowed after fixation with glutaraldehyde).

**DISCUSSION**

The number of receptor sites for PBS Z1 was determined for cells in the late lag and early exponential growth phases. Cells in these cultures are approx. $1 \times 3.5 \mu m$. Taking the diam. of the head of PBS Z1 as 45 nm and the tail length as 270 nm (Steensma *et al.*, 1978), and
assuming that the phage heads are closely packed in a layer 270 nm away from the cell wall, it may be calculated that the cells can accommodate a maximum of 2525 phages. This suggests that the maximum number of adsorbed phages is limited by the available surface area on the cell rather than by the number of receptors. This was confirmed by electron microscopy which showed that at very high phage:bacteria ratios the cells were completely covered with phages. It is therefore probable that the maximum number of phages adsorbed to cells from other stages of growth or in other media varies proportionately with the size and thus the surface area of the cells. As at very high phage:cell ratios L-forms were seen completely covered with phages, the maximum number of adsorbed phages on these bodies will probably also depend on their surface area and thus will vary with their diameter. A similar conclusion has been drawn for other phages (Schwartz, 1976).

Isolated tails give a similar killing and adsorption pattern to that for complete phages. They also correspond morphologically with some bacteriocins. For example, the structure of the R-type pyocins from \textit{Pseudomonas aeruginosa} resembles a contractile phage tail with a sheath, base plate and tail fibres. Phages which react with pyocin antiserum have been described (Ito & Kageyama, 1970; Kageyama \textit{et al.}, 1979). The cell wall receptors for PBS Z and those for the pyocins are scattered over the cell surface, whereas in both bacterial species the receptors for certain phages are located on the older parts of the cell wall (Archibald, 1976; Umeda \textit{et al.}, 1980). Despite these similarities, the killing mechanism seems to be different. In contrast to PBS Z, contraction is a prerequisite for killing by the pyocins (Shinomiya \textit{et al.}, 1975), although it was not reported whether contracted pyocins were tested with sphaeroplasts. Killing without irreversible adsorption, demonstrated in this study by the action of extended phages on L-forms, has also been reported for a yeast killer factor (Bussey \textit{et al.}, 1973).

The characterization of the receptor showed that on both the cell wall and cell membrane, specific receptors are present. These results, which correspond with existing data on the adsorption of other bacteriophages, bacteriocins and yeast killer factors, suggest the following sequence of events. Adsorption primarily takes place on the cell wall receptor. The composition of the teichoic acid in the bacterial cell wall (Glaser \textit{et al.}, 1966) and possibly the six tail fibres of the phages are responsible for the specificity of this adsorption. The only arguments for the latter are the analogy with T4 (Arscott & Goldberg, 1976; Crawford & Goldberg, 1980) and the presence of a specific line in immunodiffusion tests, i.e. PBS X gave two bands with antiserum against PBS X and PBS Z one, whereas PBS X gave one and PBS Z two with antiserum against PBS Z (H. Y. Steensma, unpublished results). Tail fibres are detached relatively easily from the phages and, if they are specific, the extra band could be explained. Immediately after adsorption, contraction takes place. This is deduced from the fact that phages adsorbed to vegetative cells are always seen to be contracted. Contraction might be due to the affinity of a cell wall component, possibly the teichoic acid, for Mg$^{2+}$ (Heptinstall \textit{et al.}, 1970; Beveridge & Murray, 1980) which is essential for stability of the phages (Haas & Yoshikawa, 1969). Removal of Mg$^{2+}$ results in contraction of the phage tails. Phage contraction releases a component of the tail which adsorbs specifically on to the receptor of the cell membrane. By analogy with T4, this could be a part of the core or base plate (Dawes, 1976). After this adsorption, the phage may kill either directly or by transfer of molecules or information that will be lethal for the cells.

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