NOTES

Characterization of a Virulent Bacteriophage for Bacillus subtilis (var. amyloliquefaciens)

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A phage, designated PBA12, has been isolated from the soil and found to be virulent on Bacillus subtilis (var. amyloliquefaciens). PBA12 has a large cylindrical head that is 100 nm long and 35 nm in diameter and a tail that is 200 nm in length. The phage contains double-stranded DNA and demonstrates chloroform sensitivity. The processes of both adsorption and replication appear to be slow and inefficient.

Our laboratory has isolated a number of bacteriophages capable of virulent infection of Bacillus subtilis (var. amyloliquefaciens) strain BaM-1, a host cell which produces large amounts of extracellular proteases and amylases. In this report, we describe some properties of bacteriophage PBA12, a phage that demonstrates unusual morphological and growth characteristics.

The phage was isolated from approximately 5 g of soil sample by a procedure described by Romig and Brodetsky (5) except that a streptomycin-resistant mutant of BaM-1 was used as the host cell and brain heart infusion broth (BHI; Difco) was used as the growth medium. PBA12 plaques were purified by three successive single-plaque isolations.

Concentrated phage stock preparations were prepared by growing PBA12 on strain BaM-1 in a soft agar overlay (0.7% agar [Difco] in BHI) at 37 C and scraping off the cell lysate after 12 h of incubation. The lysate was centrifuged for 15 min at 2,000 × g, and the supernatant fluid was collected and centrifuged again at 5,000 × g for 15 min. This supernatant fluid was then treated with deoxyribonuclease I (20 μg/ml) for 30 min at 37 C and then centrifuged at 30,000 × g for 90 min at 4 C. After this centrifugation, the supernatant fluid was discarded and the pellet was gently resuspended in 0.15 M potassium phosphate buffer (pH 7.4) at 4 C. The bacteriophage preparations were sterilized by passage through a membrane filter (HA; 0.45 μm pore size; Millipore Corp.) and stored at 4 C.

Host range of PBA12 was determined by placing a drop containing approximately 10^8 phage particles on BHI containing 0.7% agar in which the bacterial species to be tested was growing. Areas of lysis were observed with strain BaM-1 and B. subtilis (var. amyloliquefaciens) strain H, but B. subtilis 168, B. subtilis W23, B. licheniformis FDO-12, and B. pumulis NRRL B-3275 were not sensitive to phage PBA12. (See reference 2 for the original derivation of these bacterial strains.)

The nucleic acid of the phage was extracted by shaking a concentrated phage preparation with an equal volume of phenol that had been saturated with 0.1 M borate buffer (pH 7.5). The aqueous phase was removed and dialyzed against 1,000 volumes of 0.15 M NaCl and 0.015 M sodium citrate (SSC) for 48 h at 4 C. The resultant material produced an A_{260}/A_{240} ratio of 0.54 and reacted positively with diethylarnine (1). Analysis of the thermal denaturation of the PBA12 nucleic acid by using a Beckman T_m analyzer showed a T_m in SSC of 85.2 C and a 37% hyperchromicity. These results indicate that bacteriophage PBA12 contains a double-stranded deoxyribonucleic acid (DNA) genome with a guanosine + cytosine content of approximately 39% (3). This compares with the host cell DNA duplex which has a guanosine + cytosine content of 43% and a T_m of 87.1 C (7).

A sample of concentrated bacteriophage was examined with a Hitachi MUIIA electron microscope by the pseudoreplication technique (4) and was stained with uranyl acetate. Figure 1a shows an intact phage with a dense cylindrical head approximately 110 nm in length and 35 nm.
in width. The long filamentous tail is about 200 nm in length, and one can observe striations along this structure from head to the tail terminus. At the terminus a small structure, presumably a tail plate, is discernible. In Fig. 1b, an intact phage with an electron-dense head is observed next to the tail of a phage that appears to have lost its DNA genome. It is of interest to note that the head structure increases in size in the absence of the phage nucleic acid.

The growth of phage PBA12 on BaM-1 is illustrated in Fig. 2A. The phage was added at a concentration of about $4 \times 10^5$ plaque-forming units (PFU) per ml to a log-phase culture of the host cell growing in BHI. The multiplicity of infection was 0.01, and the cultures were incubated at 37 C in a water-bath shaker at 60 rpm. At the designated time interval, a portion of the culture was removed, diluted in growth medium, and allowed to form plaques in a BHI-soft agar overlay that had been seeded with uninfected host cells. A very long latent period was observed, about 2 h, followed by a slow rise in the titer of the virus. Growth of the virus was also a very slow process, and after 6 h of incubation only 180 phage particles were produced per input phage.

To gain some insight into the process of penetration, we attempted to estimate the ratio of adsorbed to unadsorbed phage by destroying the infective centers by exposure to chloroform. Initial experiments showed that virtually 100% of the phage added to a log-phase BaM-1 culture immediately became chloroform sensitive; however, control experiments showed
PBA12 to be inactivated by chloroform (e.g., 99.90% reduction in PFU), and this method of studying penetration had to be abandoned.

Figure 2B represents the results of studies on the penetration of PBA12 into the host cell by using a washing procedure. At the beginning of the experiment, about $3.6 \times 10^4$ PFU/ml were added to a log-phase culture of strain BaM-1 at a multiplicity of infection of 0.01. At defined intervals, four samples were removed from the culture. Two of these were diluted and plated as described in Fig. 2A, and the remaining samples were washed with sterile BHI three times by centrifugation. After the last wash, the cells were resuspended, diluted, and assayed as previously described. During the first 60 min of the PBA12 latent period, the association between phage and host cell appeared to be a very inefficient process. Less than 1% of the PFU added to the culture became irreversibly adsorbed to the cells after 1 min at 37 °C. This value increased to about 10% after 30 min of incubation. Thus, 90% of the phage capable of initiating infectious centers in the BHI-0.7% agar are lost by washing. This washing (i.e., $4,000 \times g$ for 10 min at room temperature) procedure does not affect cell viability.

We have attempted to alter this pattern of phage growth by utilizing a low-protease-producing mutant and by using a defined growth medium (Spizizen minimal salts solution [6], 0.5% glucose and 5 $\mu$g of thiamine hydrochloride per ml). Neither of these changes in experimental procedure altered our results.

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