Sporulation-Converting Bacteriophages for Bacillus pumilus

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Received for publication 17 April 1978

Thirty-three sporulation-converting bacteriophages for Bacillus pumilus NRS576 were assigned to two apparently unrelated groups on the basis of morphology and antiserum neutralization. Bacterial sporulation mutants responded similarly (conversion or nonconversion) to representatives of both phage groups. Evidence is presented indicating that PMB1 and related phages specify a restriction and/or modification system.

We have considered the possibility that certain sporulation mutations might be complemented by naturally occurring nonchromosomal replicons, such as bacteriophage genomes (1). Genetic and biochemical manipulations of this class of phage might then yield information as to the nature of the bacterial function complemented by the virus. We previously demonstrated the existence of two distinct sporulation-converting phages isolated from soil (1, 2). One of the phages, PMB1, converts certain spore-negative mutants of Bacillus pumilus strains to spore positive, whereas the other phage, PMB12, complements the sporulation defect in RNA polymerase mutants of B. subtilis. In the present study we have examined additional spor-converting phage isolates for B. pumilus and present information on the properties of this unusual type of bacteriophage.

B. pumilus strain NRS576 harbors a plasmid, pPL576, whose presence correlates with the sporogoropgenic phenotype of the host (6). The pseudotemperate phage PMB1 was initially selected on the basis of its ability to enhance the sporulation frequency of strain NRS576 (1). Thus, PMB1 appears to relieve the "repression" of sporulation presumably mediated by pPL576. It was subsequently demonstrated that several sporulation mutants of plasmid-negative derivatives of NRS576 were also converted to spore positive by infection with PMB1. Thus, selection of a phage that enhanced the frequency of sporulation of NRS576 simultaneously yielded a virus which was capable of complementing (or suppressing) specific chromosomal sporulation mutations. In the present study, 32 additional phages were isolated from soil by selecting for viruses capable of enhancing the sporulation frequency of wild-type (i.e., pPL576+) NRS576. Eighteen of these phage isolates were neutralized by PMB1 antiserum at rates similar to the inactivation rate of PMB1. The remaining 14 phage isolates were not neutralized by PMB1 antiserum. One of the latter class was examined in detail.

PMJ1 produces turbid-centered plaques on all PMB1-sensitive strains of B. pumilus (1). In addition, PMJ1 formed plaques on B. pumilus strains ATCC 7061 and ATCC 7065, which are not sensitive to plaque formation by PMB1. PMJ1 did not form plaques on B. subtilis strain 168 or W23 or on B. licheniformis 9945A. When B. pumilus NRS576 cells were used as the indicator for PMJ1 plaque assays in the manner previously described (1), the turbid center of each plaque yielded a spore-positive clone during incubation at 30°C for 3 days. PMJ1 lysates contain a high proportion of spontaneously occurring clear-plaque variants. The frequent appearance of clear-plaque variants is a characteristic common to all B. pumilus spor-converting phages that we have tested. However, the frequency of occurrence of clear-plaque variants of PMJ1 (~1/500 PFU) is higher than we have detected with the other spor-converting phages.

Cells isolated from the turbid centers of PMJ1 plaques can carry PMJ1 infectivity for several successive single-colony isolations. However, each restreaking of a PMJ1-carrying colony generates several daughter colonies (5 to 10%) that are PMJ1-free. Uninfected colonies are morphologically distinct from infected colonies. In addition, inoculation of a PMJ1-infected colony in Penassay broth (Difco Laboratories) containing PMJ1 antiserum (K = 20) eliminated detectable PFU when the culture was assayed after 20 h of incubation. Thus, PMJ1 is maintained by cells in an unstable carrier state. The clear-plaque variants of PMJ1 appear to be incapable of establishing this carrier state.

The morphology of PMJ1 (Fig. 1) is distinct
from that of PMB1 (1). PMB1 antiserum \((K = 200)\) prepared and processed as previously described (3), did not detectably neutralize PMJ1 \((K = <2)\). Similarly, PMJ1 antiserum \((K = 300)\) did not inactivate PMB1 \((K = <2)\). Thus, PMB1 and PMJ1 are quite dissimilar. Moreover, the 14 spore-converting phages isolated in the present study which were not neutralized by PMB1 antiserum were neutralized by PMJ1 antiserum at rates similar to the rate of inactivation of PMJ1. Thus, the 33 spore-converting phages that we have isolated by \emph{B. pumilus} NRS576 fall into two serological groups which are represented by PMB1 and PMJ1.

In a one-step growth curve, using \emph{B. pumilus} NRS576 as host, PMJ1 had a latent period of \(~75\) min and a burst size of 85. DNA extracted from PMJ1 particles had a buoyant density of \(1.696 \text{ g/cm}^3\) in CsCl and sedimented at \(65 \pm 3\)S in 5 to 20\% neutral sucrose gradients. If PMJ1 DNA is assumed to be a linear duplex molecule, then the molecular weight of the PMJ1 genome is on the order of \(2 \times 10^7\) (8). Sedimentation of PMJ1 DNA through alkaline sucrose gradients fragmented the DNA into numerous small peaks, suggesting that the DNA has an extensive number of single-stranded interruptions or alkaline-labile links.

PMB1 was previously shown to both enhance the sporulation frequency of strain NRS576 and to convert the sporulation defect in several sporulation mutants of W20 (a plasmid-negative variant of NRS576 [7]). The sporulation mutants of W20 that were converted to spore positive by PMB1 were also converted to spore positive by PMJ1. Significantly, spore-negative mutants of W20 not converted to spore positive by PMB1 were also not converted by PMJ1. Thus, although PMB1 and PMJ1 appear serologically and morphologically distinct, both show the same pattern of spore conversion.

PMB1 and PMJ1 are maintained by strain NRS576 in a relatively stable carrier state. Because both phages promote sporulation of specific sporulation mutants of NRS576, we suspected that the phages might also confer on carrier cells some additional biological functions. Studies of PMJ1-carrying derivatives of NRS576 have not yet revealed any unique host trait that is phage specified other than activities associated with sporulation (K. M. Keggins, unpublished data). However studies of NRS576 derivatives carrying PMB1 (and related phages) have provided evidence of a restriction-modification-like system that is specified in part or in total by PMB1.

Cells of NRS576 or the sporulation mutant derivative GW2 carrying PMB1 are resistant to plaque formation by PMB1 or clear-plaque-forming mutants of PMB1. Thus, PMB1-infected cells appear “immune” to superinfection by PMB1. When several phages which are se-
rologically unrelated to PMB1, such as the clear-plaque-forming phages BIC and PMJ1C, were assayed for plaque-forming activity on GW2 and GW2 (PMB1), the phages exhibited a 10⁻⁵ reduction in plating efficiency on the PMB1 pseudolysogen (Table 1). One or two clear-plaque plaques detected on each indicator host were transferred to 0.5 ml of Penassay broth to which a drop of chloroform was added. To the resulting aqueous phase (~0.4 ml), a drop of PMB1 antiserum (K = 3) was added, and the solution was incubated for 10 min, and then diluted and assayed for plaque formation on GW2 and GW2 (PMB1). As shown in Table 1, phage BIC grown on the PMB1 pseudolysogen (B1C-M) exhibited a similar plating efficiency on both GW2 and GW2 (PMB1). Similar results were obtained with PMJ1 (data not shown). Phage modified by growth on GW2 (PMB1) lost the modification during growth on nonlysogenic GW2. Thus, plaques of B1C-modified (B1C-M) on GW2 indicator cells yielded phage (designated B1C⁺) that again exhibited a reduced plating efficiency on GW2 (PMB1) (Table 1). These experiments have also been performed without treating the cloned phages with PMB1 antiserum. The results are comparable to those reported above, except that lysates generated by cloning B1C plaques from lawns of GW2 (PMB1) indicator cells contain PMB1-infectious particles. These PMB1 particles are easily detected because they produce turbid plaques that are morphologically distinct from B1C plaques.

All phages serologically related to PMB1 which we have tested (six) appear to specify either the restriction-modification phenotype, or only the modification phenotype. Unmodified B1C phage (grown on GW2) and B1C-M (grown on GW2[PMB1]) were assayed for plaque-forming activity using as indicator GW2, and GW2 pseudolysogenic for PMB1 or phage 5-2, or phage 2-2 (Table 1). The data show that the restriction phenotype is exhibited by GW2 carrying PMB1 or 2-2. Although unmodified B1C is not restricted by GW2 (5-2), phage 5-2 seems to confer a modification phenotype. Individual B1C phage plaques cloned from indicator lawns of GW2 (5-2) showed a similar plating efficiency on GW2 (efficiency of plating = 1) and GW2 (PMB1) (efficiency of plating = 0.3). Similarly, B1C plaques cloned from lawns of GW2 (2-2) exhibited a similar plating efficiency on GW2 and GW2 (PMB1).

These results suggest that PMB1 and related phages specify an activity which phenotypically resembles restriction-modification systems detected in other bacteria. A restriction-modification system has been previously demonstrated in B. subtilis (4, 5, 10). However, in the B. subtilis system the genetic determinants for the restriction-modification activity appear to be located on the bacterial chromosome.

B. Reilly (personal communication) initially found that phage PMB1 is neutralized by antiserum to the pseudotemperate phage SP10. We have found that PMB1 antiserum (K = 301) neutralizes phage SP10 (K = 115). Similarly, SP10 antiserum (K = 920), neutralizes PMB1 infectivity (K = 117). SP10 is infectious for strains of B. subtilis (e.g., W23 and ATCC 7003) and B. licheniformis (9). However, we have not been capable of demonstrating that SP10 enhances the sporulation of W23, and more than 50 independently isolated sporulation mutants of W23 were not converted to spore positive by SP10 infection.

Phages capable of enhancing the sporulation frequency of the oligosporogenic B. pumilus

**Table 1. Efficiency of plating of B1C on GW2 and GW2 derivatives pseudolysogenic for PMB1 and related phages**

<table>
<thead>
<tr>
<th>Host</th>
<th>BIC</th>
<th>EOP</th>
<th>BIC-M</th>
<th>EOP</th>
<th>BIC⁺</th>
<th>EOP</th>
</tr>
</thead>
<tbody>
<tr>
<td>GW2</td>
<td>8 × 10⁶</td>
<td>1</td>
<td>6 × 10⁷</td>
<td>1</td>
<td>4 × 10⁷</td>
<td>1</td>
</tr>
<tr>
<td>GW2 (PMB1)</td>
<td>2 × 10⁶</td>
<td>2.5 × 10⁻⁵</td>
<td>3 × 10⁶</td>
<td>0.5</td>
<td>1 × 10⁶</td>
<td>2.2 × 10⁻⁵</td>
</tr>
<tr>
<td>GW2 (5-2)</td>
<td>1 × 10¹¹</td>
<td>1.25</td>
<td>4 × 10⁶</td>
<td>0.6</td>
<td>1 × 10⁶</td>
<td>0.25</td>
</tr>
<tr>
<td>GW2 (2-2)</td>
<td>7 × 10⁶</td>
<td>8.7 × 10⁻⁵</td>
<td>1 × 10⁸</td>
<td>0.16</td>
<td>3 × 10⁶</td>
<td>7.5 × 10⁻⁵</td>
</tr>
</tbody>
</table>

*Table 1: Efficiency of plating of B1C on GW2 and GW2 derivatives pseudolysogenic for PMB1 and related phages. B1C is partially characterized virulent phage isolate which appears infectious only for strains of B. pumilus. B1C was used in these studies because it is sensitive to the PMB1-specified restriction-like activity and because B1C is serologically unrelated to PMB1 and PMJ1. B1C was assayed for plaque formation on GW2 and GW2 pseudolysogenic for PMB1, 5-2, or 2-2 as previously described (1). B1C plaques cloned from GW2 (PMB1) indicator lawns were shaken into 0.5 ml of Penassay broth containing a drop of chloroform. The aqueous phase was incubated for 10 min with 1 or 2 drops of PMB1 antiserum (K = 3). The infectious phage in these preparations are released as B1C-modified (or B1C-M). Phage isolated from plaques of B1C-M on GW2 indicator lawns are referred to as B1C⁺. That is, B1C⁺ phages are B1C-M passed once on GW2. Bacteriophage B1C, a lytic phage which is not neutralized by PMB1 or PMJ1 antiserum, was used for these studies because B1C plaques are distinct from those of PMB1. EOP, Efficiency of plating.
strain NRS576 represented more than 10% of the pseudotemperate viruses isolated from soil for this bacterial host. Each of 33 such sporulation-converting phage isolates was assigned to one of two apparently unrelated groups which are distinguishable on the basis of morphology and serology. Representatives of both groups converted the same sporulation mutants of NRS576. It, therefore, seems likely that the fundamental mechanism for spore conversion may be similar for both phage groups, despite their apparent unrelatedness.

This investigation was supported by National Science Foundation grant PCM 75-1771. K.M.K. is recipient of a University of Maryland Predoctoral Fellowship. P.S.L. is recipient of Public Health Service Research Career Development Award 1 KO4 AI00119 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED