Infection by Bacteriophage P1 and Development of Host-controlled Restriction and Modification and of Lysogenic Immunity

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Shigella dysenteriae cells were infected with phage P1 or P1cl. The outcome of superinfection of these cells with phage T1.Sh or T1.Sh(P1) or P1cl was studied as a function of time after the initial infection. Cells undergoing either a lytic response or a lysogenic response to the primary infection develop the ability to specifically restrict T1.Sh between 30 and 45 min. Between 15 and 30 min, the cells seem to develop the ability to produce T1.Sh(P1) after infection by T1.Sh. However, reasons are given for believing that this apparent time difference is consistent with a simultaneous development of the two capacities (restriction and modification) within the cell. This development occurs between 30 and 45 min. Cells infected with P1cl and superinfected 45 or more min later with T1.Sh(P1) can yield both P1cl and T1. Cells infected with P1 become resistant to infection by P1cl within 5 to 10 min. It is argued that this early immunity is not necessarily different in mechanism from true lysogenic immunity.

Bacteriophage P1, as a prophage, confers on the host cell the ability to restrict and to modify certain other infecting phage. For example, Shigella dysenteriae strain Sh lysogenic for P1 (Sh(P1)) will restrict T1 or λ phage unless the superinfecting phage has been modified by previous growth on Sh(P1) (2, 10). What is the relationship between the restricting and the modifying abilities controlled by P1? Are the genes for these two abilities located within a single operon and is their functioning initiated by the same physiological signal? What is the relationship between the ability to restrict T1 and lysogenic immunity as expressed toward superinfecting P1?

Experiments performed by Arber and Dussoix (2) indicate a difference in the time of onset of restricting and modifying ability (as expressed toward superinfecting λ) within a cell which has been infected with P1 phage. In these experiments, modifying ability seems to develop well in advance of restricting ability. It has also been shown that Shigella Sh cells infected with P1vir are able to modify superinfecting T1 (4). Arber and

1 Based on a Ph.D. Thesis submitted by E.R.W. to The University of Rochester. A preliminary report of this work was presented at the 67th Annual Meeting of the American Society for Microbiology (New York, 30 April to 4 May 1967).

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Dussoix (2) suggested the following hypothesis for P1 functions: restriction and modification are separate processes, independently controlled. The ability to modify other phages develops soon after P1 infection of sensitive hosts, but the capacity to restrict other phages develops much later and perhaps parallels the establishment of the P1 prophage. These ideas functioned as our working hypothesis and led to the experiments described in this paper.

Reported here are the results of the kinetic studies on the development of restriction and modification by P1-infected Sh, as expressed toward superinfecting T1, and of immunity as expressed toward superinfecting P1cl. The results presented include (i) an apparent difference in the kinetics of development of restricting and modifying ability, (ii) the ability of cells infected with a nonlysogenizing mutant of P1 to restrict T1, and (iii) the very early development of apparent immunity to superinfection by P1cl. The possibility is emphasized that the difference in the time of onset of restricting ability and modifying ability seen in these results and in those of Arber and Dussoix (2) is not necessarily due to different times of onset of action of the respective genes. An alternative explanation for these results is presented.
MATERIALS AND METHODS

Phage assays were by standard double-layer technique. Plates for the assay of P1 and its derivatives were incubated at 41°C to obtain more distinct plaques. All other phage assays were incubated at 37°C. Ti.Sh (P1) was selectively assayed on Sh (P1); Ti.Sh or total T1 (T1.Sh(P1) plus T1.Sh) was assayed on Sh.

Bacterial strains, bacteriophage strains, and media. The origin or composition of each of these has been previously reported (4-7). Nutrient broth (NB), L broth (LB), and LB without added NaCl and with added CaCl2 (LB-b) were used.

Infection procedure. The host bacteria in all experiments were log-phase cultures of Sh grown in LB at 37°C. Primary infection with P1 or Plc12 was carried out in aerated LB-b at 37°C; usually 10 min was allowed for attachment of the phage. Except as noted later, dilution was then made into an aerated growth tube containing LB at 37°C.

Superinfection procedure. At various times, samples from the growth tube were diluted into superinfection tubes at 37°C. These contained phage Ti.Sh or Ti.Sh(P1) suspended in NB or phage T2 suspended in NB plus 0.5% NaCl. The multiplicity of infection in the superinfection tubes was approximately 100 phage per cell. Five minutes was allowed for attachment of the superinfecting phage, after which samples were diluted as necessary and collected on membrane filters (0.45 μm pore size, type HA; Millipore Corp., Bedford, Mass.) for assay of surviving cells or infectious centers. The filters were then washed three times with 5-ml portions of NB plus 0.2% Tween 80.

In the case of superinfection with Plc12, a slight modification was made. No growth tube was employed. At various times, samples were taken directly from the original attachment tube, which was maintained with aeration at 37°C. These were diluted into superinfection tubes containing Plc12 at a multiplicity of infection of 4 to 8. Subsequent procedures were the same for the other experiments.

Survivor assay. Samples of infected cells were collected, usually on black filters, which were then washed as above. The filters were transferred to the surface of LB agar plates which were incubated overnight at 37°C for the development of colonies.

Infectious center assay. Samples were collected and washed as for the cell assay. The filters were transferred to the surface of the LB agar plates and then overlaid with soft agar containing an appropriate plating culture for the phage to be assayed.

Operational definitions. The following terms are used throughout this paper with the limited meanings indicated. (i) Host-controlled restriction: the activity of a cell which prevents replication of infecting Ti.Sh phage while allowing replication of infecting Ti.Sh(P1) phage; (ii) host-controlled modification: the activity of a cell which causes it to produce one or more Ti.Sh (P1) particles after infection by Ti.Sh; (iii) cellular refractoriness: the inability of a cell, shortly after infection by P1, to support replication of either Ti.Sh or Ti.Sh(P1) particles; (iv) lysogenic immunity: the ability of a PI-infected cell to survive superinfection by Plc12.

RESULTS

Development of restricting ability. To observe the onset of restricting ability, Sh bacteria were infected with P1 phage and portions were subsequently superinfected at various times with Ti.Sh. For each sample, colony-forming cells were assayed before and after superinfection with Ti.Sh. Assays made before superinfection represent the cells that survived the P1 infection to become lysogens. Under our conditions, this was generally 80 to 90% of the total cell population. The counts after superinfection should measure the ability of the potential lysogens, at a given time after P1 infection, to restrict Ti.Sh.

Controls were included in each experiment to determine the extent to which uninfected Sh and Sh(P1) bacteria would be killed by the procedure used for superinfection. These controls showed little or no killing of Sh(P1), whereas at least 95% killing of Sh was usually achieved.

For most of the population, restricting ability develops between 30 and 45 min after P1 infection (Fig. 1). With P1-infected Sh, it has been shown that the infected cell's decision to become a lysogenic cell rather than a lytic cell occurs at approximately 20 min after P1 infection (3). It is expected that the establishment of the P1 genome as a prophage would occur at some time subsequent to this decision. Therefore, it is possible that the onset of restricting ability at 30 to 45 min is a reflection of the establishment of the P1 prophage, in agreement with our working hy-

![Fig. 1. Development of host-controlled restriction. The curve shows the proportion of potential PI lysogens that survive superinfection by Ti.Sh at various times after P1 infection. Early resistance to Ti.Sh infection is not due to host-controlled restriction.](http://jvi.asm.org/Downloaded from http://jvi.asm.org/)

Downloaded from jvi.asm.org on October 19, 2017 by guest.
potothesis that restriction is a property of an established lysogen.

**Refractory state.** In some experiments of this type, including the one illustrated in Fig. 1, a refractory state, unrelated to host-controlled restriction, was seen in cells during the first several minutes after P1 infection. Cells superinfected with T1.Sh at 10 or at 15 min after P1 infection showed a greater ability to survive the superinfection than did cells superinfected at 30 min after the primary infection.

That this early refractory state of P1-infected Sh toward T1 infection was not true restriction is shown by the fact that T1.Sh(P1) was also unable to infect the cells at this time (Table 1). During the early part of the experiment, the number of survivors after exposure to T1.Sh(P1) is comparable to the number surviving T1.Sh exposure at comparable times. The number of survivors of T1.Sh(P1) superinfection begins to decline after approximately 15 to 20 min. This decline marks the end of the refractory state, and the decline continues during the time that restriction specific for T1.Sh develops within the cell.

The refractory state does not extend to T2, however, as is also shown in a separate experiment in Table 1.

Investigation of the nature of the refractory state was frustrated by its inconsistent occurrence. Whether or not the refractory state occurred, however, the kinetics of the appearance of true restriction in P1-infected Sh was the same.

**Development of modifying ability.** To measure the development of modifying ability in P1-infected Sh, we examined the type of T1 produced after superinfection by T1.Sh. A suspension of Sh was infected with P1 and incubated at 37°C. Samples were superinfected with T1.Sh after various intervals. The superinfected cells were plated in a soft agar overlay which contained either Sh or Sh(P1); they were examined for plaque formation after incubation (Fig. 2).

At 15 min, about 20% of the superinfected cells yield T1. This low value is presumably due to the refractory state described above. Of these yielders, only about 1 in 10 produce any modified phage. By 30 min, however, more of the cells produce T1 progeny, and essentially all of these produce at least one T1.Sh(P1) particle. At later times, as restriction develops, the number of T1-producing cells declines with all T1 yielders continuing to produce T1.Sh(P1).

In considering these results, it should be borne in mind that T1-producing cells may be either those that would have become lysogens or those that would have yielded P1 had they not been superinfected with T1.Sh. Thus, there appears to be a difference between the time of onset of restricting ability and that of modifying ability: restricting ability develops between 30 and 45 min after P1 infection, while modifying ability seems to develop between 15 min and 30 min after P1 infection.

**Restriction capacity of lytic cells.** One aspect of our working hypothesis is the supposition that cells undergoing lytic infection with P1 do not restrict. To study this point further, we examined the T1-plaque-forming ability of Sh infected with P1cl5, which does not lysogenize Sh (6). The

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**Table 1.** Refractory state of P1-infected Sh toward T1/Sh(P1) infection

<table>
<thead>
<tr>
<th>Time of superinfection (min)</th>
<th>No. of P1-infected cells surviving superinfection/no. of viable cells prior to superinfection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Superinfection by T1/Sh(P1)</td>
</tr>
<tr>
<td>5</td>
<td>0.64</td>
</tr>
<tr>
<td>12</td>
<td>0.48</td>
</tr>
<tr>
<td>15</td>
<td>0.67</td>
</tr>
<tr>
<td>18</td>
<td>0.48</td>
</tr>
<tr>
<td>22</td>
<td>0.25</td>
</tr>
<tr>
<td>45</td>
<td>0.10</td>
</tr>
<tr>
<td>60</td>
<td>0.02</td>
</tr>
</tbody>
</table>

* T2 control: fraction of uninfected Sh cells which survive T2 infection = 0.03; fraction of Sh(P1) cells which survive T2 infection = 0.03.

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**Fig. 2.** Development of host-controlled modification. Curves show the type of T1 produced by cells initially infected by P1 and superinfected by T1/Sh at times indicated. Curve A: cells producing at least one T1/Sh (P1) particle. Curve B: cells producing any T1.
experiment was performed by infecting Sh with P1cl and superinfecting with T1.Sh or T1.Sh(P1) at various intervals. The superinfected cells were then assayed for the total number of T1-producing cells present. Figure 3 shows the results of a typical experiment. Comparing curve A, representing superinfection with T1.Sh(P1), to curve B, representing superinfection with T1.Sh, we can see that P1 lytic deciders as well as lysogenic deciders develop a restriction toward superinfecting T1. This can be seen from the fact that T1.Sh(P1), but not T1.Sh, can grow in the late stages of P1cl infection of an Sh cell. Mutual exclusion would not discriminate between these T1 mnemotypes. This restriction begins approximately 30 min after P1cl infection, a time which coincides with the onset of restriction in lysogenic deciders after infection by P1 (Fig. 1). Thus, both lytic deciders and lysogenic deciders develop the ability to restrict. Although we have demonstrated an apparent difference in the time of onset of restricting and modifying functions, the ability of lytic cells to restrict indicates that, while restriction and modification may be separately controlled, the establishment of P1 as a prophage is not the factor controlling restricting ability.

Phage yield from cells infected by P1cl and by T1.Sh. In Fig. 3 we can also see the development of T1 modification ability in Sh infected with P1cl (curve C). In this experiment, there was no early refractory state toward T1 infection. Early after P1cl infection, few of the T1.Sh-superseded infected cells produced any T1.Sh(P1), but by 30 min essentially all of them did. In this way, too, P1cl-infected Sh resemble P1-infected Sh.

It is noteworthy that some P1-infected cells can yield T1 phage after superinfection with T1.-Sh(P1), even when superinfection occurs 60 min after P1cl infection (Fig. 3). We found that some superinfected cells are able to yield at least one P1 phage particle and at least one T1 phage particle. The experiment was performed by infecting cells at a multiplicity of infection of 9 with P1cl, and then diluting fivefold into a growth tube containing LB without added NaCl. At 45 and 55 min after initial infection, T1.Sh was added at a multiplicity of infection of 11. After 5 min for T1 attachment, samples were diluted and plated in a soft agar overlay, with a mixture of E. coli B and Sh/T1 as the plating culture. Since T1 cannot form plaques on Sh/T1 and P1cl cannot form plaques on E. coli B, both T1 and P1cl produce turbid plaques on the mixed plating culture, with T1 plaques being much larger than plaques formed by P1cl. Cells which lyse and yield both T1 and P1cl form large turbid plaques with small central clear areas. Mixed plaques were scored as coming from a single cell only if the P1cl plaques appeared to be exactly in the center of a T1 plaque. To minimize random overlapping of plaques, samples were diluted prior to plating so that only 5 to 10 T1 plaques per plate would be expected (Table 2).

**Development of lysogenic immunity.** What is the correlation between the development of restricting and modifying ability in a P1-infected cell undergoing a superinfection with P1cl and T1.Sh? The development of restriction is shown by the fall in curve B. Development of modification is shown by the rise in curve C.

### Table 2. Phage production from cells infected with P1cl and superinfected with T1.Sh(P1)

<table>
<thead>
<tr>
<th>Time of superinfection</th>
<th>45 min</th>
<th>55 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of plates examined</td>
<td>16</td>
<td>22</td>
</tr>
<tr>
<td>No. of doubly infected cells represented</td>
<td>19</td>
<td>24</td>
</tr>
<tr>
<td>No. of pure T1 plaques observed</td>
<td>80</td>
<td>154</td>
</tr>
<tr>
<td>No. of pure P1 plaques observed</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No. of mixed plaques observed</td>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td>No. of mixed plaques/doubly infected cell</td>
<td>0.2</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* Estimated from the Poisson distribution.

* Includes unattached T1 and cells yielding only T1.
cell and the development of lysogenic immunity, which can be viewed as the ability of lysogenic cells to survive superinfection by weak virulent mutants of the carried phage.

Superinfection experiments were carried out in which the primary infection of Sh was with PI and the secondary infection with P1c12. Results (Fig. 4) are expressed in terms of the fraction of viable cells at any time which are resistant to P1c12 superinfection. This fraction considers only that portion of the cell population which would become lysogenic if it were not superinfected.

Our results show that within 5 to 10 min after PI infection, most cells which will become lysogenic have already become resistant to superinfection by P1c12. This resistance seems to develop, then, before the cell has made an irreversible commitment to lysogeny.

DISCUSSION

As measured in these experiments, T1-restricting and T1-modification capacities seem to develop at different times after PI infection (Fig. 1 and 2). We believe, however, that this difference represents a difference in the times during the T1 life cycle at which the two capacities can or must operate on the T1, rather than a difference in the times during the PI life cycle at which the mechanisms responsible for the two capacities are developed.

Let us assume that the capacities for modification and restriction are under the same genetic control and thus appear together after PI infection. There is considerable evidence (1, 3, 13) that a necessary step in the restriction mechanism must occur very early during the life cycle of the phage that is to be restricted, perhaps during the penetration of the phage deoxyribonucleic acid (DNA). If this step is somehow circumvented the phage cannot be restricted. Therefore, in our experiments, in order to register as a restricting cell, the cell would have had to develop the capacity for restriction before the T1 superinfection.

Modification, on the other hand, as shown by Arber and Dussoix (2), can be applied to a phage DNA molecule after it has been synthesized. Thus, in a particular cell, modification can be effectively expressed at any time prior to the maturation of the last T1 particle to be assembled before lysis of that cell. The capacity for modification could, therefore, appear near the end of the T1 latent period and still allow the cell to register as a modifier.

The minimum latent period for T1 phage is 13 min, and the average latent period is perhaps a few minutes longer. This agrees with the approximate difference of 15 min in the times of the apparent development of modification and the development of restriction, as our model would require.

Our hypothesis does not require that restriction and modification be effected by the same agent, merely that the responsible agents be produced at the same time. The possibility of a single agent has, however, recently been suggested (11).

Restricting capacity develops not only in lysogenic deciders but also in lytic cells. This restricting ability in lytic cells can be seen in the fact that P1c12-infected Sh cells will prevent the growth of T1.Sh but not of T1.Sh(P1) (Fig. 3). This property begins to appear about 30 min after the PI infection; this is also the time of the onset of restricting ability among lysogenic deciders (Fig. 1). Therefore, restricting capacity is like modifying capacity in that it develops without requiring that P1 become established as a prophage.

Late in the latent period, some P1c12 infectious centers survive even T1.Sh(P1) superinfection. These cells produce both P1c12 and T1 (Table 2). The cells may have contained mature P1c12 particles before the T1.Sh(P1) infection. We believe this to be the first report of a single cell producing two unrelated DNA phages, although there is a
report of a single cell producing both DNA and ribonucleic acid phages (9).

It is not easy to arrive at a simple interpretation of the experiments in which P1-infected Sh are infected with P1cl2. We do not know the stages in the life cycle at which lysogenic immunity can act. Even if immunity ordinarily blocks an early stage in the life cycle of the superinfecting phage, it may be true that it can act to block late functions, and that this is sufficient to prevent the superinfecting phage from growing. If this were the case, and recalling that the latent period for P1 is considerably longer than that for T1, it could be possible that the mechanism which suppressed P1cl2 superinfection in our experiments actually develops at the same time as the mechanisms responsible for the restriction and modification of T1. (It is even possible to imagine a temperate phage that could infect a sensitive cell after an infection by its weak virulent mutant, and still be able, in some cells, to suppress the growth of the mutant and eventually establish lysogeny. This would give the appearance of a preinfection disposition of a portion of the cell population toward a “decision not to lyse.”) Under this hypothesis, early immunity or “paraimmunity” (14) is not necessarily different in mechanism from true lysogenic immunity. Only further knowledge about the process of lysogenization by P1, and of the mechanistic basis of lysogenic immunity, paraimmunity, and the restriction of heterologous phage will allow a more certain interpretation.

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LITERATURE CITED