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Dominance Relationships in Mixedly Infected *Bacillus subtilis*

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The progeny released from *Bacillus subtilis* cells mixedly infected with bacteriophages β22, SP82, and SP02c₁ have been studied at varying multiplicities of infection and orders of addition and with different host strains of the bacterium. In *B. subtilis* 168, SP02c₁ was subordinate to both SP82 and β22 and did not yield significant numbers of progeny even when added 5 min before the superior phage. Dominance in mixed infections of β22 and SP82 was host-dependent. In *B. subtilis* 168, SP82 was dominant and greatly reduced the yield of β22 if added simultaneously or before the subordinate partner. However, in the same mixed infection in *B. subtilis* SB11, β22 was the dominant phage and totally suppressed the production of SP82 even when added 5 min after the latter.

Bacteria of the genus *Bacillus* are hosts for a wide variety of bacteriophages of diverse morphology, chemistry, and host range. Our initial interest in these phages centered on an analysis of host and phage nucleic acid synthesis in infected cells. Several investigators have noted that certain *B. subtilis* phages, like the bacteriophages of other bacteria, bring about a suppression of host macromolecular syntheses during the latent period (9, 17, 23), and a number of theories related to the structural chemistry and physiology of *B. subtilis* phages have been proposed to explain how this might occur (14, 15).

In the course of studying the biochemistry of infection of phages SP02c₁, β22, and SP82, we concluded that it might be possible to assess the role of deoxyribonucleic acid (DNA) base substitutions and certain phage-induced physiological changes by observing the action of one phage on another in mixed infection. Studies of simultaneous and consecutive infections with two different kinds of phages have been made in a number of bacteria with major emphasis on the bacteriophages of *Escherichia coli*. Investigations in these mixed infection systems reveal that in most instances only one phage partner in any given mixedly infected cell is successful in producing progeny; this phenomenon is mutual exclusion. The subordinate partner, however, often greatly reduces the yield of the dominant phage in what is referred to as the depressor effect (3, 4, 22). A review of mixed infections between unrelated, related, and mutants of the same phage is contained in reference 1.

From the physiological standpoint, the most extensively studied mixed infections are those involving T2 and T4 with each other or with other phages. Consecutive mixed infections with these T-even phages, however, are complicated by a unique mutual exclusion phenomenon termed superinfection exclusion. In this instance, preinfection with T2 or T4 induces changes in the surface properties of the *E. coli* cell such that DNA of a superinfecting phage does not penetrate beyond the cell envelope (2, 7).

As will be discussed below, we have found mutual exclusion and depressor effects to be common in a number of *B. subtilis* mixed-phage infections, but we and others (11, 13) have not found evidence of superinfection exclusion. One factor we have found to be important in mixed infections in this bacterium are definite host range differences of some of the phages and slight host preferences of others. In the following report, we have examined the host ranges of several *B. subtilis* phages and some of the factors influencing host specificity. On the basis of results from single infections, an analysis was made of progeny released in mixedly infected cells with varying times of addition of the second phage, multiplicities of infection (MOI), and hosts.

MATERIALS AND METHODS

Bacterial strains and media. *B. subtilis* strain SB11*try*⁻, a derivative of *B. subtilis* W23, was obtained from E. W. Nester; *B. subtilis* 168 M and 168 M (SP02) were provided by W. R. Romig. Bacteriophage β22 was supplied by Roy Doi, SP82 was sup-

plied by Charles Stewart, and SP02c1 was supplied by W. R. Romig.

The medium used in the present experiments for growing cells and preparing phage lysates was a modified M medium (23) containing per liter of water: 10 g of tryptone (Difco), 5 g of yeast extract, and 10 g of NaCl. Sterile salt solutions were added to give a final concentration of 5×10^{-3} M $CaCl_2$ and $MgSO_4$ and 2×10^{-5} M $MnCl_2$. The same medium made with 1.5% agar served as the base medium for plating phages, cells, or both. The modified M medium with 0.7% agar was used for soft-agar overlays.

Growth and infection of cells. Experiments in the liquid medium were carried out at 37 C with cultures grown in flasks on a rotary shaker. Optical density (OD) at 600 nm was measured in a Spectronic 20 colorimeter on cells growing in 16-mm diameter screw-cap tubes. Cultures to be infected with phages were grown to an OD of 0.3 to 0.35 (about 2×10^8 cells/ml) and infected with phages at the desired MOI.

Fresh lysates were prepared as needed and used as the source of phages for given experiments. Allowing for the problems in accurately quantitating *B. subtilis* phage preparations (*see below*), stocks of phage particles remained at approximately the same titer for about a week.

Titer of phages and determination of MOI. Phage stocks were titered on M agar with 0.7% soft-agar overlay containing the indicator strain. We have noted previously (9) that some *B. subtilis* phages, particularly β22, give small plaques, and the titer of the same stock may show marked fluctuations from day to day. Moreover, the titer of many bacteriophage preparations as determined by plaque-forming units is substantially lower than predicted from burst size or by applying the Poisson distribution to surviving cells at various MOI.

For these reasons we do not believe it is possible to precisely determine the MOI in our experiments. The MOI given in mixed infection experiments are estimated over a twofold range (4–8 or 10–20) and were based on direct measurement of PFU, known burst size, or the Poisson distribution, or all three, as described previously (9).

Determination of relative burst size and cell killing. Relative burst sizes of the various phages on different hosts were determined by infecting cultures of *Bacillus* species at a MOI of 0.2 with the desired phage. The phages were allowed 1 min to absorb, the cultures were returned to the shaker for 5 min, and the cells were then diluted 1:10,000. After lysis, the number of phage particles was determined by plating on appropriate indicator strains.

For the determination of cell killing, host bacteria were infected at the desired MOI with the appropriate phage, and at 1 and 5 min after the addition of the phage particles the surviving cells were diluted and plated for colony-forming units in the presence of specific phage antiserum.

Determination of phage adsorption. To determine the ability of phages to adsorb to various *Bacillus* strains, bacteria were grown to an OD of 0.3 at 600 nm and infected at a MOI of 0.2. One minute was allowed for adsorption without shaking. Adsorption was continued with shaking for 5 additional min, the culture was diluted 1:10,000 and treated with chloroform, and unadsorbed phages in the supernatant fraction were titered on appropriate indicators.

Progeny released in mixed infections. Methods for determing the relative number of progeny produced in mixed infections closely paralleled the one-step growth experiments used in single infections, except that the experiments were conducted at high MOI. *B. subtilis* strains were grown to an OD of 0.3 and infected with the desired MOI and sequence of phages. Five minutes after the second phage was added, the cultures were diluted 1:10,000 and allowed to lyse. The proportions of the two progeny were then determined by neutralizing one of the phages with specific antiserum or plating on selective indicators. Examples of selective indicators are as follows. The number of SP82 progeny produced in a mixed infection of SP02c1 and SP82 were determined by plating the lysates on *B. subtilis* 168 (SP02), a lysogenic strain on which SP02c1 cannot grow. In mixed infections of SP02c1 and β22 or SP82 and β22, the number of β22 progeny were determined by plating on *B. subtilis* SB11 on which SP82 and SP02c1 either formed plaques at greatly reduced efficiency or did not form plaques at all.

Determination of phage released from single cells. Experiments were also conducted to determine the number of cells producing phage particles in single infections or releasing a given phage in mixed infections. In the former instance, infected cells were diluted 1,000-fold, treated with specific antiserum to neutralize unadsorbed phages, and further diluted as desired, and the number of infective centers producing a given phage was determined by plating the diluted cells with an indicator strain of bacteria. Appropriate controls were made to demonstrate that the antiserum was sufficiently concentrated to neutralize unadsorbed phages and that the antiserum carried over into the plate had no effect on the titer of infective centers.

Analysis of infective centers in mixed infections was done in a similar manner. Five minutes after the addition of the second phage, the cells were diluted and treated with antisera to neutralize unadsorbed phage. The cells were then further diluted and plated before lysis on an appropriate selective strain of *Bacillus* (*see above*) or plated with a nonselective indicator in the presence of the appropriate heterologous antiserum.

Preparation of phage antiserum. Each phage preparation was purified by CsCl density gradient centrifugation, mixed with complete Freund's adjuvant, and injected subcutaneously into two rabbits. Intramuscular injections were made at weekly intervals of 4 weeks. The rabbits were then exsanguinated, and the sera were pooled. The neutralizing capacities of the sera (*K* values), determined by standard methods (1), were 1,300 for β22, 510 for SP02c1 and 250 for SP82. Each antiserum was completely specific, i.e., it neutralized only the homologous phage.

RESULTS

Host ranges of three B. subtilis phages. The three bacteriophages chosen for this study are representative of the wide variety of phages known to infect various strains of *B. subtilis*. SP82 is a virulent phage which contains the DNA base substitution hydroxymethyluracil in place of thymine (8, 11, 12, 18); β22 is also a virulent phage but has a longer latent period than the other phages used and has no known base substitutions (23); $SP02c_1$ is a clear-plaque mutant of temperate *B. subtilis* phage SP02 (16).

Table 1 summarizes the host ranges of these phages as determined by plaque-forming efficiency on three hosts: *B. subtilis* SB11, *B. subtilis* 168, and *B. subtilis* 168 (SP02). The results demonstrate that β22 phage titered with equal efficiency on all three hosts, whereas $SP02c_1$ formed plaques only on *B. subtilis* 168. Preparations of SP82 gave the same number of PFU on *B. subtilis* 168 and 168 (SP02) but formed plaques 5,000 times less efficiently on *B. subtilis* SB11. Moreover, plaques of SP82 on the latter host were only about 25% of the diameter of those observed on *B. subtilis* 168.

We have investigated the possibility that the plaques of SP82 which were observed on *B. subtilis* SB11 resulted from a minor population of phages which was genetically or phenotypically adapted to grow on the restrictive host. Plaques formed on SB11 were picked and eluted, and lysates were prepared by adding the eluted phages to liquid cultures of SB11. The lysates were again cloned on SB11, and the process was repeated. After the second recloning, the adapted phages titered about 500 times less efficiently on *B. subtilis* SB11 than on *B. subtilis* 168. These results may indicate a slight increase in the titering efficiency on the restrictive host, but the improvement was variable from one experiment to another and was much less than commonly seen in host range mutants or host-induced modifications.

TABLE 1. *Relative plaque-forming efficiency of Bacillus subtilis phages on three Bacillus strains*[a]

Host bacterium	Bacteriophage tested		
	β22	$SP02c_1$	SP82
Bacillus subtilis 168	1	1	1
B. subtilis 168 (SP02)	1	$<10^{-7}$	1
B. subtilis SB11	1	$<10^{-7}$	5×10^{-4}

[a] Fresh lysates of the phages were serially diluted and plated on the desired host. A relative efficiency of 1 was equal to approximately 2×10^{10} plaque-forming units per ml. The highest concentration titered was a 10^{-3} dilution of the phage. If no plaques or evidence of cell killing was noted at this dilution, a plaque-forming efficiency of less than 10^{-7} was recorded.

Adsorption to and killing of host cells. Our interest in using phages SP82, $SP02c_1$, and β22 in mixed infections led us to make a more detailed study of the host-phage interaction of these viruses. Table 2 indicates that β22 and SP82 adsorbed to *B. subtilis* 168 and SB11 with high efficiency, whereas $SP02c_1$ adsorbed poorly to *B. subtilis* SB11. The latter observation explains why $SP02c_1$ did not form plaques on SB11 nor kill this strain of *B. subtilis* in liquid cultures (Table 4).

Table 3 presents an analysis of the number of progeny released in one-step growth experiments when *B. subtilis* strains were infected at low multiplicity with phages $SP02c_1$, β22, and SP82. The most important observation is that under the conditions of the experiment less than 1% as many SP82 progeny were produced in the restrictive host *B. subtilis* SB11 as in the permissive 168 strain. In other experiments, we have determined that this reduced yield reflects a decline in the number of cells producing any progeny rather than a reduction of burst size. Thus, the low plaque-forming efficiency of SP82 on *B. subtilis* SB11 (Table 1) would appear to reflect a

TABLE 2. *Adsorption of Bacillus subtilis phages to two hosts*

Phage	Per cent total input phage adsorbing in 5 min	
	B. subtilis 168	*B. subtilis* SB11
SP82	88	99
$SP02c_1$	99	16
β22	80	99

TABLE 3. *Relative numbers*[a] *of phage released from Bacillus cultures infected at a multiplicity of infection of 0.2*

Phage	Host	
	B. subtilis 168	*B. subtilis* SB11
SP82	100	<1
$SP02c_1$	100	0
β22	100	80

[a] A normalized value of 100 = 148×10^8 plaque-forming-units (PFU) per ml for SP82, 44×10^8 PFU/ml for $SP02c_1$; 42×10^8 PFU/ml for β22.

relatively small number of productive infections at low MOI. When the MOI was increased to 10, however, the numbers of progeny produced on strains 168 and SB11 as measured in one-step growth experiments were approximately equal, and at least 90% of the infected SB11 cells released progeny. Therefore, productive infection of *B. subtilis* SB11 by SP82 would appear to be favored when the host bacterium is simultaneously infected with more than one phage particle.

The rapidity with which cells were infected with various phages at high MOI was also investigated (Table 4). In most combinations of phage and host, at least 90% of the colony-forming units were lost in 1 min. The exception to this was β22 which adsorbed to and killed *B. subtilis* 168 somewhat more slowly than *B. subtilis* SB11.

Mixed infections. An understanding of host range restrictions and other differences in *B. subtilis* phage infections may be revealed by studies of events occurring in the first few minutes subsequent to phage infection. One approach to observing changes in cellular physiology following phage infection is to study bacteria infected concurrently with more than one kind of bacteriophage. The ability of one phage to dominate another when both are added simultaneously (mutual exclusion) and the method of achieving superiority may be related to how the phage controls cellular metabolism in single infections. Delayed addition of the second phage may reveal the nature of changes directed by the first phage; for example, studies of delayed mixed infections in *E. coli* cells infected with T-even phages led to the discovery that infection with the first phage quickly resulted in changes in the surface properties of the cell which excluded the DNA of the second phage from participating in the infective process (6, 21).

TABLE 4. *Efficiency with which various phages kill Bacillus subtilis strains*

Phage[a]	Time after infection (min)	Per cent survivors	
		B. subtilis 168	*B. subtilis* SB11
SP82	1	10	2
	5	2	1
SP02c₁	1	10	100
	5	2	100
β22	1	12	5
	5	5	2

[a] Multiplicity of infection in each experiment was 4 to 8.

In the following experiments (Tables 5–8), the progeny released in mixed infections with pairs of *B. subtilis* phages were quantified under conditions of varying MOI values, hosts, and times of addition of the second phage. The standard format for the experiments included mixed infections in which both phages were added simultaneously and others in which the phages were added consecutively at intervals of 5 and 15 min. Results in the tables are presented in terms of number of progeny normalized to the singly infected controls.

Table 5 presents the analysis of mixed infections of SP82 and $SP02c_1$. The results obtained at two MOI values demonstrated that SP82 dominated the infection and prevented the production of any $SP02c_1$ progeny even when added 5 min after the latter. It is notable, however, that the presence of $SP02c_1$ did reduce the relative numbers of progeny SP82 produced in the mixed infection. A similar depression in numbers of progeny released by the dominant phage (the depressor effect) has also been noted in mixed infections of T_1 and T_7 in *E. coli* (3). Interestingly, the present experiments show that $SP02c_1$ seemed to have greater effect when added 5 or 15 min after SP82 than when added simultaneously. This has been repeatedly noticed in this mixed infection and may indicate that the physical act of secondary phage penetration had a disruptive effect on the already developing SP82. Superinfection with $SP02c_1$ was not, however, accompanied by detectable lysis of cells.

Addition of SP82 to cells 15 min after infection with $SP02c_1$ greatly reduced the number of $SP02c_1$ released, but this was not accompanied by production of a significant number of SP82

TABLE 5. *Relative number[a] of progeny released in SP82-SP02c₁ mixed infections in Bacillus subtilis 168*

Time of addition of SP02c₁ (min)	Time of addition of SP82 (min)	MOI of 4 to 8		MOI of 10 to 20	
		SP02c₁ Progeny	SP82 Progeny	SP02c₁ Progeny	SP82 Progeny
0		100		100	
	0		100		100
0	0	<1	78	<1	75
0	+5	<1	59	<1	32
0	+15	37	8	11	5
+5	0	2	47	<1	53
+15	0	<1	70	<1	63

[a] A value of 100 = 222 × 10^8 plaque-forming units (PFU) per ml for $SP02c_1$, and 268 × 10^8 PFU/ml for SP82. MOI = multiplicity of infection.

phage, i.e., the cells seemed unable to produce a high yield of either phage. There was other evidence that SP82 was unable to gain control of the $SP02c_1$; infection at this time in the lytic cycle. Although there was no significant premature lysis of cells upon the secondary addition of SP82, the mixedly infected cells lysed at precisely the same time as control cells singly infected with $SP02c_1$; the effect of SP82 appeared to be limited to reducing the yield of $SP02c_1$ progeny. The observation that only a very small number of SP82 progeny were produced could also be explained by the time of lysis of the cells. Even if the latter phage began a normal sequence of ribonucleic acid (RNA) transcription and DNA replication, the mixedly infected cells would lyse in the 20th min of the SP82 portion of the infection. In single infections, the first intracellular SP82 phage would be appearing at about this time.

A similar but not identical picture is seen in mixed infections of β22 and $SP02c_1$ in *B. subtilis* 168 (Table 6). Addition of β22 simultaneously or preceding the addition of $SP02c_1$ led to a reduced but nevertheless high burst of β22 with almost no production of $SP02c_1$, i.e., β22 dominated the infection. Preinfection with $SP02c_1$, however, substantially changed the picture. When β22 was added 5 min after $SP02c_1$, only 10% of the normal yield of either phage was produced. Later addition of β22 even at high MOI had little effect on $SP02c_1$ synthesis, and again almost no β22 was produced.

The lengths of the lytic cycles of β22 and $SP02c_1$ (50–55 min and 30–35 min, respectively) were sufficiently different so that an interpretation of the course of mixed infection could be obtained by following lytic curves. Such data for a series of $SP02c_1$ and β22 mixed infections are given in Fig. 1, and the results are of interest with respect to the progeny studies (Table 6). The course of the lytic cycle appeared to be controlled by whichever phage first infected the cell with a mixed result being obtained in simultaneous infection. It is especially notable that, although the addition of β22 5 min after $SP02c_1$ prevented the latter from releasing a significant number of progeny, it did not change the course of the lytic cycle.

The third series of mixed infections, involving β22 and SP82, were conducted in two hosts: *B. subtilis* SB11 and *B. subtilis* 168. It will be recalled that SP82 has a preference for the latter strain, and the results (Tables 7 and 8) appear to reflect this difference. In mixed infections in *B. subtilis* SB11, β22 clearly dominated the infection, being the only phage to produce progeny when added simultaneously or before SP82. In the second host, however, SP82 appeared to play a stronger role and greatly reduced the burst of β22 even when added after the latter phage. Since the latent periods for these phages were similar (35–40 min for SP82 and 50–55 min for β22), it was not possible to determine whether the time of

TABLE 6. *Relative number[a] of progeny released in β22-$SP02c_1$ mixed infections of Bacillus subtilis 168*

Time of addition of β22 (min)	Time of addition of $SP02c_1$ (min)	MOI of 4 to 8		MOI of 10 to 20	
		β22 Progeny	$SP02c_1$ Progeny	β22 Progeny	$SP02c_1$ Progeny
0		100		100	
	0		100		100
0	0	38	7	45	9
0	+5	50	3	74	2
0	+15	74	4	62	3
+5	0	10	9	10	8
+15	0	4	51	5	95

[a] A value of 100 = 96 × 10^8 plaque-forming units (PFU) per ml for β22 and 300 × 10^8 PFU/ml for $SP02c_1$. MOI = multiplicity of infection.

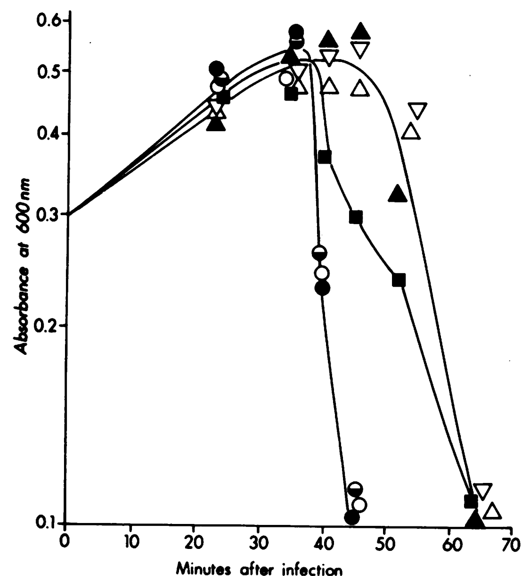

FIG. 1. *Lytic curves of B. subtilis 168 singly and mixedly infected with phages β22 and $SP02c_1$. (▲) Culture singly infected with β22; (△) culture preinfected 5 min with β22 and then superinfected with $SP02c_1$; (▽) culture preinfected 15 min with β22 and then superinfected with $SP02c_1$; (●) culture singly infected with $SP02c_1$; (○) culture preinfected 5 min with $SP02c_1$ and then superinfected with β22; (◒) culture preinfected 15 min with $SP02c_1$ and then superinfected with β22; (■) culture simultaneously infected with β22 and $SP02c_1$.*

TABLE 7. *Relative number[a] of progeny released in SP82-β22 mixed infections in Bacillus subtilis 168*

Time of addition of β22 (min)	Time of addition of SP82 (min)	MOI of 4 to 8		MOI of 10 to 20	
		β22 Progeny	SP82 Progeny	β22 Progeny	SP82 Progeny
0		100		100	
	0		100		100
0	0	4	30	9	41
0	+5	20	8	21	22
0	+15	24	8	22	11
+5	0	1	59	8	60
+15	0	1	84	3	69

[a] A value of 100 = 125 × 10^8 plaque-forming events (PFU) per ml for β22 and 93 × 10^8 PFU/ml for SP82. MOI = multiplicity of infection.

TABLE 8. *Relative number[a] of progeny released in SP82-β22 mixed infections in Bacillus subtilis SB11*

Time of addition of β22 (min)	Time of addition of SP82 (min)	MOI of 4 to 8		MOI of 10 to 20	
		β22 Progeny	SP82 Progeny	β22 Progeny	SP82 Progeny
0		100		100	
	0		100		100
0	0	78	<1	74	<1
0	+5	50	<1	51	<1
0	+15	95	<1	77	<1
+5	0	21	<1	2	2
+15	0	6	21	5	36

[a] A value of 100 = 46 × 10^8 plaque-forming units (PFU) per ml for β22 and 214 × 10^8 PFU/ml for SP82. MOI = multiplicity of infection.

lysis in this mixed infection (40 to 50 min) was controlled by one of the phages.

Progeny released from single cells. As noted earlier, in most mixed infections even the yield of the dominant phage was less than that obtained in single infections. This raised the question as to whether the lower yield of the dominant phage or subordinate phage, or both, represented a uniform decrease in the number of progeny per cell or a reduction in the number of cells releasing any progeny. This question was investigated in several mixed infections with varying results.

The first mixed infection so analyzed employed SP02c$_1$ and SP82 in which the latter phage was added 15 min after SP02c$_1$. It will be recalled from Table 5 that the yield of SP02c$_1$ phage released under these conditions was 11 to 37% that in single SP02c$_1$ infections. A comparison of the number of infectious centers releasing any SP02c1 progeny in single and 15-min mixed infections revealed that 70 to 90% of the superinfected cells released at least one SP02c$_1$ phage. Thus, the greatly reduced yield of the subordinate partner appears to be due to a combination of a smaller number of SP02c$_1$ being released by each infected cells and slightly fewer infected cells producing any progeny.

In mixed infections of SP82 and β22 in *B. subtilis* 168, SP82 was the only partner to produce a significant number of progeny if it was added 5 min before or simultaneously with β22. However, the number of SP82 progeny released as measured in one-step growth experiments was reduced in both instances. We have compared the number of cells releasing SP82 in both conditions of mixed infection with a control culture singly infected with this phage. When the two phages were added simultaneously, the number of infective centers releasing any SP82 was 36% that in singly infected controls. Since the yield of SP82 in the mixed infection was 30 to 40% normal, it would appear that those cells which produced SP82 progeny released a normal burst, whereas the majority of cells produced neither β22 nor SP82. When SP82 preceded β22 by 5 min, approximately the same number of cells appeared to release SP82 as in the singly infected control. Thus the 40% reduction in total yield of phages in this mixed infection (Table 7) would appear to be due to a reduction in number of progeny per cell rather than cells releasing phages.

The most closely examined of the mixed infections from the standpoint of progeny released from single cells was that of β22 and SP02c$_1$ in *B. subtilis* 168. In this infection (Table 6), β22 was the dominant phage in that it suppressed the development of SP02c$_1$ when added simultaneously with or 5 min after the subordinate partner. However, the yield of β22 was also greatly reduced in these mixed infections.

Studies of the number of infective centers releasing β22 progeny or SP02c$_1$ progeny, or both, in the simultaneous mixed infection indicated that compared to singly infected controls 77% as many cells released at least one β22 progeny, and 27% released at least one SP02c$_1$. Thus the reduced total yield of both phages seen in one-step growth experiments (Table 6) would appear to reflect a combination of fewer phages per productive infection and fewer total cells producing phages.

A similar study of the number of cells releasing β22 or SP02c$_1$, or both, was made using cells preinfected 5 min with SP02c$_1$ and then superinfected with β22 In these experiments, a special effort was made to determine whether any mixedly infected cells released both types of progeny. A culture of *B. subtilis* 168 was infected with SP02c$_1$ and β22

in the sequence described, centrifuged to remove unadsorbed phage, resuspended in prewarmed growth medium, diluted, and plated for infective centers on *B. subtilis* SB11 and *B. subtilis* 168 with β22 antiserum. The number of plaques formed on the two selective indicator systems were approximately equal to each other and to the number of colony-forming units found in the sample of uninfected cells treated in parallel. These results suggest that most of the infective centers released both phages and that the overall reduction in the yield of progeny β22 and SP02c_1 was due to fewer phages being released per cell rather than fewer productive infections.

To further substantiate these results, a sample of mixedly infected cells was plated on *B. subtilis* 168 under conditions in which both β22 and SP02c_1 could grow. The plaques formed on these plates were picked and respotted on selective indicators. Results from this experiment indicated that 100% of the infective centers containd SP02c_1 and 10 to 60% contained β22. We were unable to detect more than 60% of the infective centers producing both phages with this method, but this may simply mean that the more rapidly lysing SP02c_1 frequently overgew the plaques. The present experiment further suggests that a substantial number of the cells, mixedly infected with β22 and SP02c_1, produced both progeny.

Adsorption of superinfecting phage to preinfected cells. The suppressive effect of the dominant phage on the development of the subordinate partner, even when the former is added after the latter, leaves little doubt but that the superinfecting phage rapidly adsorbs to the preinfected cells. Nevertheless, an independent check was made of the possibility that the first phage may saturate all adsorption sites or sterically hinder the second partner. In these experiments, cells were preinfected with one phage at a MOI of 10 to 20, and 15 min later the second phage was added at a MOI of 0.2 to 1. After allowing 5 additional min for adsorption, the number of superinfecting phages remaining in the supernatant fraction was determined. In none of the variously mixed infection combinations did we find evidence that the second phage was significantly less well adsorbed to preinfected cells than to uninfected controls. Presumably *B. subtilis* has many general phage-receptor sites or not all phages adsorb to the same sites. A detailed discussion of phage receptor sites in *B. subtilis* may be found in reference 25.

DISCUSSION

Studies with *E. coli* phages have indicated that with rare exception only very closely related phages can develop concurrently in the same cell; in most instances only one partner produces progeny. The selection of which phage actually controls the infection depends on several parameters. In mixed infections of T1 and T2, the latter dominates the infection even when added 6 min after the former (3, 5, 22); this may reflect the tendency of T2 to disrupt certain host functions that are required for T1 but not T2 development. In simultaneous infections of T1 and T7, about 33% of the cells release T1, and 66% liberate T7. The reason for this peculiar ratio is unknown; however, if either phage is added a few minutes ahead of the other, it is the only one to produce progeny (4).

Studies of double infections of mutants of T2 or T4 indicate that primary infection with one phage results in a rapid change in the surface properties of the bacterium, so that a second phage added even 1 or 2 min later is prevented from participating in the infection or adding its genome to the genetic pool. This phenomenon of superinfection exclusion is also accompanied by developing resistance to lysis from without; if *E. coli* cells are first infected at low MOI with T2, within 6 min they are resistant to lysis from without even at MOI of 500 (21).

The results of mixed infection studies in *B. subtilis* are superficially similar to those previously done with *E. coli* and its phages. In most experiments, only one partner yielded progeny in significant numbers, with the choice of successful partner depending on the time of addition and, in some instances, on the host bacterium. On the basis of these experiments, the phages may be ranked in the following order of dominance: in *B. subtilis* strain 168, SP82 $>$ β22 $>$ SP02c_1, whereas, in strain SB11, β22 predominates in a mixed infection of β22 and SP82. Exclusion in mixedly infected *B. subtilis* cells differs, however, from superinfection exclusion noted in T2 and T4 infections of *E. coli*, for in the current study the second phage was adsorbed, entered the cell, and, as will be shown in later papers, RNA may have been transcribed from the genome of the excluded phage.

Another question that may be raised concerns the failure of a dominant phage to release progeny when added 5 or 15 min after infection with a subordinate phage. For example, SP82 fails to produce progeny if added in the 15th min of the SP02c_1 latent period. It appears likely that several factors may be involved. In the mixed infections of SP82 and SP02c_1, the addition of SP82 15 min after SP02c_1 failed to prevent the cells from lysing 20 min later in harmony with cells singly infected with SP02c_1. Thus, the cells lysed in the 20th min of the hypothetical SP82 latent period

prior to the appearance of intracellular SP82. It is also possible that the products of the SP02c$_1$ genome may have altered the structure of RNA polymerase in such a way that SP82 cannot be transcribed in an orderly fashion. Similar interpretations could be offered in SP02c$_1$-β22 infections. Cells preinfected 5 min with SP02c$_1$ lyse at about 35 min (the 30th min of the β22 infection). This would be almost precisely the time of appearance of the first intracellular progeny in single infections of β22 (10, 23). These explanations, however, do not resolve why SP02c$_1$ maintains control of the time of lysis while producing few progeny or why β22-SP82 mixed infections are so strongly dependent on the order of addition. These observations may suggest that the clock is set for lysis time very early in the infection. Genetic evidence in T4 infections of *E. coli* suggests that there may be a limited number of membrane sites to which the DNA of the incoming phage can attach, and failure to gain one of these sites precludes active participation in the infection (19, 20). It is possible that, in our mixed infections, the membrane sites are usurped by the first phage. The second phage, if it is dominant, may interrupt the development of the first phage, but its failure to gain a membrane site could result in an inability to carry out the orderly transcription of RNA replication, DNA replication, or both, prerequisite for development of the normal number of progeny.

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LITERATURE CITED

1. Adams, M. H. 1959. Bacteriophages. Interscience Publishers, Inc., New York.
2. Anderson, C. W., J. R. Williamson, and J. Eigner. 1971. Localization of parental deoxyribonucleic acid from superinfecting T4 bacteriophage in *Escherichia coli*. J. Virol. **8**: 887–893.
3. Delbrück, M. 1945. Interference between bacterial viruses. III. The mutual exclusion effect and the depressor effect. J. Bacteriol. **50**:151–170.
4. Delbrück, M., and W. T. Bailey, Jr. 1946. Induced mutations in bacterial viruses. Cold Spring Harbor Symp. Quant. Biol. **11**:33–37.
5. Delbrück, M., and S. E. Luria. 1942. Interference between bacterial viruses. I. Interference between two bacterial viruses acting upon the same host, and the mechanism of virus growth. Arch. Biochem. **1**:111–117.
6. Dulbecco, R. 1952. Mutual exclusion between related phages. J. Bacteriol. **63**:209–217.
7. French, R. C., S. M. Lesley, A. F. Graham, and C. E. van Rooyen. 1951. Studies on the relationship between virus and host cell. III. The breakdown of P^{32}-labelled T2r$^-$ bacteriophage adsorbed to *E. coli* previously infected by other coliphages of the T group. Can. J. Med. Sci. **29**:144–148.
8. Green, D. M. 1964. Infectivity of DNA isolated from *Bacillus subtilis* bacteriophage SP82. J. Mol. Biol. **10**:438–451.
9. Hemphill, H. E., and H. R. Whiteley. 1970. Nucleic acid synthesis in *Bacillus subtilis* infected with bacteriophage β22. J. Virol. **6**:381–392.
10. Hemphill, H. E., H. R. Whiteley, L. R. Brown, and R. H. Doi. 1969. The effect of rifampin on the production of β22 phage by *Bacillus subtilis*. Biochem. Biophys. Res. Commun. **37**:559–566.
11. Kahan, E. 1966. A genetic study of temperature-sensitive mutants of the *subtilis* phage SP82. Virology **30**:650–660.
12. Kallen, R. G., M. Simon, and J. Marmur. 1962. The occurrence of a new pyrimidine base replacing thymine in a bacteriophage DNA: 5-hydroxymethyl uracil. J. Mol. Biol. **5**:248–250.
13. McAllister, W. T. 1970. Bacteriophage infection: which end of the SP82G genome goes in first? J. Virol. **5**:194–198.
14. Nishihara, M., A. Chrambach, and H. V. Aposhian. 1967. The deoxycytidylate deaminase found in *Bacillus subtilis* infected with phage SP8*. Biochemistry **6**:1877–1886.
15. Nishihara, M., N. Friedman, and H. V. Aposhian. 1969. Biological activity of 5-hydroxymethyluracil and its deoxynucleoside in noninfected and phage-infected *Bacillus subtilis*. J. Virol. **3**:164–170.
16. Okuba, S., and W. R. Romig. 1965. Comparison of ultraviolet sensitivity of *Bacillus subtilis* bacteriophage SPO2 and its infectious DNA. J. Mol. Biol. **14**:130–142.
17. Roscoe, D. H. 1969. Synthesis of DNA in phage-infected *Bacillus subtilis*. Virology **38**:527–537.
18. Roscoe, D. H., and R. G. Tucker. 1964. The biosynthesis of a pyrimidine replacing thymine in bacteriophage DNA. Biochem. Biophys. Res. Commun. **37**:559–566.
19. Snustad, D. P. 1966. Limited genome expression in bacteriophage T4-infected *Escherichia coli*. I. Demonstration of the effect. Genetics **54**:924–935.
20. Snustad, D. P. 1966. Limited genome expression in bacteriophage T4-infected *Escherichia coli*. II. Development and examination of a model. Genetics **54**:937–954.
21. Visconti, N. 1953. Resistance to lysis from without in bacteria infected with T2 bacteriophage. J. Bacteriol. **66**:247–253.
22. Weigle, J. J., and M. Delbrück. 1951. Mutual exclusion between an infecting phage and a carried phage. J. Bacteriol. **62**:301–318.
23. Yehle, C. O., and Roy H. Doi. 1967. Differential expression of bacteriophage genomes in vegetative and sporulating cells of *Bacillus subtilis*. J. Virol. **1**:935–947.
24. Yelton, D. B., and C. B. Thorne. 1971. Comparison of *Bacillus cereus* bacteriophages CP-51 and CP-53. J. Virol. **8**:242–253.
25. Young, F. E. 1967. Requirement of glucosylated teichoic acid for adsorption of phage in *Bacillus subtilis*. Proc. Nat. Acad. Sci. U.S.A. **58**:2377–2384.