Mixed Infections of *Bacillus subtilis* Involving Bacteriophage SPO2c₁

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The synthesis of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) was studied in *Bacillus subtilis* cells mixedly infected with phages SPO2c₁ and either SP82 or β 22. It was found that cells preinfected 5 min with either β 22 or SP82 could not support significant amounts of RNA or DNA synthesis from the genome of superinfecting SPO2c₁. Conversely, cells preinfected with SPO2c₁ remained susceptible to superinfection even at the midpoint of the latent period. When SP82 was added to cells preinfected 5 min with SPO2c₁, the former greatly inhibited the replication and transcription of the SPO2c₁ DNA, but if superinfection with SP82 was delayed until the 15th min of the SPO2c₁ latent period, RNA and DNA specific for both phages were synthesized. Both viral genomes were transcribed throughout the remainder of the lytic cycle when β 22 was added to cells preinfected either 5 or 15 min with SPO2c₁. In both the SPO2c₁-SP82 and the SPO2c₁- β 22 mixed infections, the types of phage-specific RNAs synthesized at a given time in the latent period were similar to those synthesized in single infections with each phage. The association of SPO2c₁ DNA with the host cell membrane in certain of the doubly infected cells was different from that observed in singly infected cells. In the SPO2c₁-SP82 infection, SPO2c₁ DNA was not released from the membrane; in the SPO2c₁- β 22 mixed infections, significantly less SPO2c₁ DNA was membrane-bound when β 22 was added before SPO2c₁. However, binding and release of SPO2c₁ DNA was normal in cells infected first with SPO2c₁ and then with β 22. The synthesis of phage-specific nucleic acids and the binding of phage DNA to the membrane are discussed with respect to dominance relationships among these phages.

Bacteria simultaneously or consecutively infected with more than one kind of phage often produce only one parental type as progeny. The mechanisms responsible for such dominance relationships, termed mutual exclusion (3, 4), have not been established except in *Escherichia coli* superinfected with phage T2 or T4. Bacteria infected with either of the latter phages rapidly undergo changes in the surface properties which prevent productive superinfection with the same or unrelated phages. In this instance, the deoxyribonucleic acid (DNA) of the second phage is degraded at or near the cell surface, but the precise nature of the phage-induced surface changes have not been elucidated (1, 8, 20). Superinfection exclusion does not occur in all mixed

infection systems, however, and most examples of mutual exclusion require other explanations.

Bacillus subtilis cells mixedly infected with various combinations of phages β 22, SP82, and SPO2c₁ have shown a dominance hierarchy among the three phages (17). In strain 168 of *B. subtilis*, SPO2c₁ (a clear plaque mutant of the thymine-containing temperate phage SPO2) is subordinate to both SP82 (a hydroxymethyluracil-containing virulent phage) and β 22 (a thymine-containing virulent phage) and does not produce significant numbers of progeny when infected simultaneously with either of these phages. Likewise, SP82 is dominant over β 22 in simultaneous mixed infections of these two phages in *B. subtilis* 168 but has less effect on β 22 development when mixed infections are made in *B. subtilis* strain W23. The dominance of several of the phages can be altered by adding phages sequentially, rather than simultaneously, to the host cell.

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This paper presents data on the synthesis of nucleic acids and the attachment of phage DNA to the membrane in *B. subtilis* cells mixedly infected with SPO2c₁ and SP82 or β 22. The results indicate that mutual exclusion is related to differing levels of viral genome expression.

MATERIALS AND METHODS

Bacterial strains, phage, and medium. *B. subtilis* strain 168 (*try*⁻), obtained from E. W. Nester, was used for the production of phage lysates. Phages SPO2c₁, SP82, and β 22 were kindly supplied by W. R. Romig, C. H. Stewart, and R. H. Doi, respectively. A modified M medium (25) was used for cell growth, isotopic labeling of infected cells, and preparation of phage lysates as described previously (15).

Nucleic acid hybridization. The method of Gillespie and Spiegelman (10) was used for ribonucleic acid (RNA)-DNA hybridization and that of Denhardt (5) for DNA-DNA hybridization. Hybridization experiments were conducted by using 0.5 ml total volume of $2 \times \text{SSC}$ ($\text{SSC} = 0.15 \text{ M NaCl plus } 0.015 \text{ M sodium citrate}$) and an incubation time of 16 hr at 63 C. Conditions for competition-hybridization experiments have been described previously (14). The data are reported as the average percentage of input radioactivity bound to each DNA calculated from four or more determinations made at increasing ratios (0.1 to 20) of H³-labeled RNA or DNA/filter-bound DNA. The extent of cross-hybridization in control experiments between nucleic acids specific for one phage and those specific for either another phage or the host did not exceed 0.5% of input radioactivity hybridized.

Radioactively labeled nucleic acids used in hybridization experiments were prepared from cultures infected at multiplicities of 5 phages per cell. Radioactive RNA was obtained by pulse-labeling cells for 5 min with 1 μCi of H³-uridine/ml of culture; radioactive DNA was prepared from cells pulse-labeled for 5 or 10 min with 2 μCi of either H³-thymidine or H³-adenosine per ml of culture. H³-Adenosine was used to label DNA in cells infected with SP82 since the DNA of this phage contains 5-hydroxymethyluracil (HMU) in place of thymine (12).

Preparation of radioactively labeled phage. Phage labeled with ³²P_o were prepared as previously described (15). Tritium-labeled phage were obtained by infecting cells grown in M medium with a multiplicity of infection of 4 to 5 and adding 2 μCi of H³-thymidine per ml 5 to 10 min after infection. After cell lysis, the labeled phage were purified as described previously (15). The phage preparations contained about 1 count per min per 5×10^4 plaque-forming units.

Miscellaneous methods. The procedures for preparation of unlabeled phage and for determination of membrane-DNA complexes have been described previously (15). The M-band procedure for measuring association of DNA with host cell membrane was a modification of the Mg²⁺-sarkosyl method of Earhart et al. (7).

RESULTS

DNA synthesis in singly infected *B. subtilis* cells. Previous studies on DNA synthesis in *B. subtilis* infected with phage β 22 and SPO2c₁ (14, 15) showed that phage DNA synthesis and host DNA synthesis are probably mutually exclusive events and that at some point in the latent period host DNA replication ceases. As a prelude to studies of DNA synthesis in mixed infections, we have attempted to define more precisely the periods of host and phage DNA replication in cells singly infected with these three phages. To do this, *B. subtilis* 168 cells were infected with β 22, SPO2c₁, or SP82 and then pulse-labeled with H³-thymidine or H³-adenosine for overlapping 10-min intervals throughout the latent period. The radioactive DNA was isolated from the infected cells, denatured, and hybridized with denatured phage and host DNA immobilized on nitrocellulose filters. The data in Fig. 1 compare the amounts of radioactivity that bound to the *B. subtilis* DNA and the DNA of the infecting phage in samples taken at the different time intervals.

In all three infections, bacteriophage DNA replication was initiated about one-fourth of the way through the latent period, and host DNA synthesis stopped at about the same time. After the first third of the latent period, essentially no detectable host DNA was synthesized in the infected cells, although it has been shown in other studies that preexisting host DNA is either only slightly degraded or not degraded at all in some *B. subtilis* phage infections (14, 18, 19, 26).

RNA synthesis in singly infected *B. subtilis* cells. *B. subtilis* 168 cells were infected with the desired phage and pulse-labeled with H³-uridine at 5-min intervals throughout the latent periods. The radioactive RNA was isolated from the infected cells and hybridized to filter-bound *B. subtilis* DNA or phage DNA, and a comparison was made of the relative amounts of input radioactivity binding to the two DNAs.

The results of this study (Fig. 2A, B, and C) indicate that the synthesis of phage messenger RNA (mRNA) began early in the latent period and was detectable in RNA preparations obtained from cells pulse-labeled with H³-uridine from 0 to 5 min after addition of the phage. In cells infected with SPO2c₁, phage RNA synthesis remained a minor component of total cellular RNA production throughout the first third of the infection, although it is possible that quantitatively it was made in an amount comparable to host mRNA. Shortly

FIG. 1. Hybridization of pulse-labeled DNA isolated from phage-infected cells to phage and host DNA. Cells infected with SPO2c₁ (A) and β 22 (C) were labeled with H³-thymidine (55.7 Ci/mmole), and cells infected with SP82 (B) were labeled with H³-adenosine (17.7 Ci/mmole) for 10-min intervals at various times during the latent period. The points are plotted in the middle of each pulse interval. The specific activities of the isolated DNA ranged from 100 to 1,000 counts per min per μ g of DNA. Symbols: O, host DNA; □, phage DNA.

FIG. 2. Hybridization of pulse-labeled RNA isolated from phage-infected cells to phage and host DNA. Cells infected with SPO2c₁ (A), SP82 (B), and β 22 (C) were labeled with H³-uridine (39.6 Ci/mmole) for 5-min intervals during the latent period. The points are plotted in the middle of each pulse interval. The specific activities of the isolated RNA ranged from 100 to 1,000 counts per min per μ g of RNA. Symbols: O, binding to host DNA; □, binding to phage DNA.

after the initiation of SPO2c₁ DNA synthesis, there was a dramatic increase in the proportion of newly synthesized RNA which was phage related. β 22-specific and SP82-specific RNAs, on the other hand, constituted a major component of RNA synthesized early in the infection, but again there was a marked increase in the proportion of phage RNA after DNA synthesis began.

Studies of RNA synthesis in phage-infected

B. subtilis cells are complicated by the fact that many *B. subtilis* phages do not completely inhibit ribosomal RNA synthesis or do so only slowly (9, 14, 19), and the stable host RNA species make up the bulk of the newly synthesized RNA accumulated in 5-min pulses. In hybridization experiments, the excess stable RNA quickly saturates the few sites on DNA homologous to these RNAs, and thereafter only a low percentage of input radioactivity of the host RNA binds to homologous DNA. However, it has been shown that host mRNA synthesis is eventually inhibited in at least one of the phage infections, that of *B. subtilis* infected with β 22 (14).

Nucleic acid synthesis in mixedly infected cells. In the present study, all mixed infections were done sequentially, that is, the second partner was added 1, 5, 10 or 15 min after the first phage. Sequential rather than simultaneous infections were carried out to lessen the possibility that mutual exclusion in some situations might result from interference by the dominant phage in adsorption or penetration of the subordinate phage. Our previous studies indicated that all three phages adsorb rapidly to cells (17), and the investigations of single infections discussed above demonstrate that all phages initiate RNA synthesis within the first 5 min of the latent period. Thus, by adding one phage at a given time interval before the second partner, one can probably separate temporal control from physical or steric effects.

DNA synthesis in SPO2c₁-SP82 mixed infections. The proportions of host and phage DNAs synthesized in mixedly infected *B. subtilis* cells when SP82 was added at progressively longer time intervals after the addition of SPO2c₁ are compared in Table 1. In the first set of experiments, the cells were pulse-labeled with H³-adenosine at a constant time interval (15–25 min) after SPO2c₁ infection. In single infections of SPO2c₁, large quantities of phage DNA were synthesized at this point in the latent period, and host DNA replication had ceased (Fig. 1A).

The results in Table 1 demonstrate that cells superinfected with SP82 1 or 5 min after infection with SPO2c₁ produced much less SPO2c₁ DNA at 15 to 25 min after infection and that replication of SP82 DNA was the major component of DNA synthesis. However, addition of SP82 to SPO2c₁-infected cells at later time intervals had little immediate effect on the synthesis of SPO2c₁ DNA. For example, when SP82 was added to cells preinfected 15 min earlier with SPO2c₁, the

TABLE 1. Comparison of quantities of host and phage DNA synthesized at various times after infection of *Bacillus subtilis* with SPO2c₁ and SP82

Time of addition of phage (min)		Time of pulse ^a	Percent input radioactivity hybridized to DNA from:		
SPO2c ₁	SP82		SPO2c ₁	SP82	Host
0		15-25	47		0.8
	0	10-20		43	5.2
0	1	15-25	9.7	65	2.5
	5	15-25	11	66	3.0
	10	15-25	29	35	12
	15	15-25	48	0.5	7.2
0	15	25-30	19	13	0.7

^a Minutes after the addition of the first phage.

level of SPO2c₁ DNA synthesized 15 to 25 min after infection was equal to that made in single infected cells at that same time in the latent period, and no SP82 DNA was replicated. However, later pulses (25-30 min) in this same mixed infection demonstrate that SP82 DNA synthesis was eventually initiated, indicating that SPO2c₁-preinfected cells that are actively replicating SPO2c₁ DNA are still capable of synthesizing SP82 DNA.

RNA transcription in SPO2c₁-SP82 mixed infections. As noted in Table 1, the addition of SP82 to cells preinfected 5 min with SPO2c₁ decreased the level of replication of the latter phage, whereas SP82 DNA synthesis reached the same level as in single infections. The data in Table 2 demonstrate that under the same conditions of sequential infections a more pronounced inhibition of SPO2c₁ RNA transcription was observed. Although small quantities of SPO2c₁ RNA were made in the first few minutes after superinfection, thereafter only SP82 RNA was produced. Indeed, the pattern of RNA synthesis closely resembled that seen in single infections of SP82. In the mixed infections, however, it seems clear that SP82 was inhibiting the expression of the SPO2c₁ genome, indicating that infection by this phage can preempt the RNA synthesizing capability of the cell or prevent the synthesis of other RNA species. It is possible that SP82 has a similar effect on the synthesis of host mRNA but, as stated earlier, the synthesis of stable RNA is not shut off.

When the addition of SP82 was delayed until 15 min after SPO2c₁ infection, the superinfecting phage did not prevent further transcription of SPO2c₁ RNA, but rather both RNAs were made. This observation is inter-

TABLE 2. Comparison of quantities of host and phage RNA synthesized at various times after infection of *Bacillus subtilis* with SPO2c₁ and SP82

Time of addition of phage (min)		Time of pulse ^a	Percent input radioactivity hybridized to DNA from:		
SPO2c ₁	SP82		SPO2c ₁	SP82	Host
0		5-10	2.8		4.9
0		10-15	2.1		4.5
0		25-30	40		2.6
	0	1-6		9.7	3.2
	0	5-10		9.1	5.5
	0	20-25		26	4.2
	0	25-30		26	3.9
0	5	6-11	2.8	8.0	4.9
0	5	10-15	0.64	16	2.2
0	5	25-30	0.65	27	1.5
0	15	20-25	13	30	2.2
0	15	24-29	23	39	1.5

^a Minutes after the addition of the first phage.

esting for more than one reason. It is known that by the 15th min of the latent period there are many SPO2c₁-mediated changes in the infected cells: host DNA synthesis has ceased, phage DNA replication has been initiated, and SPO2c₁ RNA is the major product of transcription. Yet the present studies of RNA and DNA synthesis in mixedly infected cells indicate that even at the mid-point of the latent period none of the SPO2c₁-directed changes prevent the superinfecting phage from penetrating the cell and directing its own biosynthetic functions. In an attempt to pursue this point more closely, we examined the RNA products synthesized by SP82 in a mixed infection with SPO2c₁ to determine whether the RNA populations differed from those made in single infections. To do this, unlabeled competitor RNA was isolated from cells which had been preinfected 15 min with SPO2c₁ prior to the addition of SP82 and then harvested at 20 min (5 min after the addition of SP82). Unlabeled competitor RNAs were also isolated from 5-min and 30-min singly infected cells. These RNAs were then used in competition-hybridization experiments with radioactive RNA isolated from singly infected cells pulse-labeled with H³-uridine at 0 to 5 and 25 to 30 min after infection. Transcription of SP82 DNA in the mixed infection followed the same sequence as in singly infected cells (Fig. 3). It can be observed that the 5-min unlabeled RNA from singly infected cells and the 20-min unlabeled RNA from the mixed infection competed with RNA from 0- to 5-min pulse-labeled

FIG. 3. Competition between unlabeled RNA isolated from single and mixed infections of SP82 and radioactive RNA from (A) cells singly infected with SP82 and pulse-labeled with H^3 -uridine from 0 to 5 min and (B) cells singly infected with SP82 and pulse-labeled with H^3 -uridine from 25 to 30 min after infection. In both figures: (●) competitor RNA from cells infected 5 min with SP82; (○) competitor RNA from cells infected 30 min with SP82; (▲) competitor RNA from cells infected at time 0 with SPO2c₁, superinfected with SP82 15 min later, and harvested at 20 min (5 min after the addition of SP82). Each reaction mixture contained 8.5 μ g of filter-bound SP82 DNA, 50 μ g of 0- to 5-min or 25 μ g of 25- to 30-min H^3 -RNA and the indicated amount of competitor RNA in 0.5 ml of $2 \times$ SSC. Reactions were incubated at 63 C for 16 hr. In the absence of competitor, 2% of the input counts per min bound in the experiments using 0- to 5-min H^3 -RNA and 19% of the input counts per min bound in those using 25- to 30-min H^3 -RNA.

singly infected cells but had few RNA species in common with 25- to 30-min pulse-labeled RNA. Thus, the RNA from the mixed infection had early, but few late, RNA species. These results again emphasize that the cellular changes mediated by SPO2c₁ to further its own development do not affect directly the early development of a second phage. As will be noted later, this does not appear to be true when β 22 or SP82 is added first in a mixed infection.

DNA synthesis in cells mixedly infected with SPO2c₁ and β 22. Earlier progeny studies (17) showed that the outcome of mixed infections of SPO2c₁ and β 22 are, to some degree, dependent on the order of addition of the phages. If SPO2c₁ is added to cells 5 min before β 22, both phages are produced in very low yields. When the order of addition is re-

versed, a high yield of β 22 progeny is obtained, but few if any SPO2c₁ are released.

The pattern of DNA synthesis in this mixed infection was also dependent on the order of addition of the two phages (Table 3). When SPO2c₁ was added first, β 22 DNA synthesis was delayed compared to the time of DNA synthesis in singly infected cells, and considerable amounts of both phage DNAs were produced. Studies of progeny released from single plaque-forming units in this particular mixed infection (17) indicate that infected cells do release small numbers of both β 22 and SPO2c₁. Thus, it is likely that both phage DNAs were actually replicated in the same cells. When the order of addition was reversed and SPO2c₁ was added 5 min after β 22, much less SPO2c₁ DNA was made.

RNA synthesis in cells mixedly infected with SPO2c₁ and β 22. The patterns of transcription in cells mixedly infected with SPO2c₁ and β 22 were also dependent on the order of addition of the phages (Table 4). When β 22 was added to cells preinfected 5 min with SPO2c₁, both DNAs were transcribed, although β 22-specific RNA synthesis appeared to exceed SPO2c₁-directed transcription early in the mixed infection. Late in the infection, neither RNA was clearly the dominant product of overall RNA synthesis, and total phage-directed RNA production did not reach the level noted in either single infection. When the order of infection was reversed and β 22 had a 5-min advantage, SPO2c₁ DNA transcription was almost totally inhibited, whereas β 22-directed RNA synthesis approached that

TABLE 3. Comparison of quantities of host and phage DNA synthesized at various times after infection of *Bacillus subtilis* with SPO2c₁ and β 22

Time of addition of phage (min)		Time of pulse ^a	Percent input radioactivity hybridized to DNA from:		
SPO2c ₁	β 22		SPO2c ₁	β 22	Host
0		5-15	3.7		12
0		15-25	47		0.8
0		20-30	48		0.5
	0	0-10		0.8	22
	0	10-20		27	17
	0	15-25		54	4.6
0	5	5-15	13	0.4	15
0	5	15-25	18	5.6	1.0
0	5	20-30	22	32	0.7
5	0	15-25	10	12	3.8
5	0	35-45	2.7	22	0.1

^a Minutes after the addition of the first phage.

TABLE 4. Comparison of quantities of host and phage RNA synthesized at various times after infection of *Bacillus subtilis* with SPO2c₁ and β 22

Time of addition of phage (min)		Time of pulse ^a	Percent input radioactivity hybridized to DNA from:		
SPO2c ₁	β 22		SPO2c ₁	β 22	Host
0		5-10	2.8		4.9
0		10-15	2.1		4.5
0		20-25	19		3.8
0		25-30	40		2.6
	0	5-10		15	2.0
	0	10-15		18	2.3
	0	20-25		22	2.8
	0	25-30		34	2.0
0	5	10-15	1.5	8.7	3.0
0	5	25-30	8.5	14	2.6
0	15	25-30	37	7	2.6
5	0	10-15	0.7	21	3.0
5	0	25-30	1.6	30	2.4

^a Minutes after the addition of the first phage.

of single infections.

The results of these studies of DNA replication and transcription in cells mixedly infected with β 22 and SPO2c₁ indicate that when β 22 was added first it dominated SPO2c₁ by almost completely preventing the synthesis of SPO2c₁-specific nucleic acids. Moreover, apart from the fact that the yield of β 22 progeny was reduced (17), there was little evidence that the addition of SPO2c₁ had any effect on the course of the β 22 life cycle. When SPO2c₁ was added first, however, both RNAs were synthesized, but neither phage was produced in high yield. These observations prompted an examination of the populations of SPO2c₁ RNA present in cells which had been infected sequentially first with SPO2c₁ and then with β 22 5 min later. The results in Fig. 4 demonstrate that transcription 10 to 15 min after infection yielded early RNA comparable to that observed in a similar time interval in single infections (15), and that RNA made 25 to 30 min after infection was late RNA. Thus, although the quantity of SPO2c₁ RNA was reduced in the mixed infection, the populations produced at given times were those characteristic of single infections.

Fate of DNA in mixedly infected cells. Several explanations for dominance relate to changes in the physical state or cellular processing of the DNA of the phages. In an attempt to follow the fate of DNA in mixedly infected cells, we employed the M-band tech-

FIG. 4. Competition between phage-specific RNA isolated from SPO2c₁- β 22 mixedly infected cells pulse-labeled with H³-uridine at 5 to 10 min (A) and 25 to 30 min (B) after infection and unlabeled RNA from 5-min (O) and 30-min (□) SPO2c₁-infected cells. Pulse-labeled cells were infected with β 22 5 min after infection with SPO2c₁. Each reaction mixture contained 10 μ g of filter-bound SPO2c₁ DNA, 61 μ g of 10- to 15-min H³-RNA, or 18 μ g of 25- to 30-min H³-RNA and the indicated amount of unlabeled competitor RNA isolated from cells singly infected with SPO2c₁ in a total volume of 0.5 ml of 2 $\times$ SSC. Reactions were incubated at 63 C for 16 hr. In the absence of competitor, 1.2 and 15% of the input radioactivity was bound using 10- to 15-min H³-RNA and 25- to 30-min H³-RNA, respectively.

nique of Tremblay et al. (22) to examine the association of phage DNA with the cell membrane. In these studies, radioactive phage were first added to cells in single infections, and the amount of input DNA binding to membrane at various times after infection was determined. A similar series of experiments was then carried out by using the radioactive phage in mixed infections, and a comparison was made to the single infections.

The results of these analyses showed that in single infections SP82 (Fig. 5A), SPO2c₁ (Fig. 5B), and β 22 (Fig. 5C) DNAs became associated with the cell membrane soon after infection but then were released after initiation of phage DNA replication. The amount of dissociation was greater in SPO2c₁-infected cells than in SP82-infected cells, but the smallest degree of detachment was observed in cells infected with β 22.

Figure 5D presents M-band data for a mixed infection of SP82 and SPO2c₁ in which SP82 was added 5 min after SPO2c₁. It will be recalled that, under these conditions of mixed infection, SP82 inhibits SPO2c₁ RNA and DNA synthesis. The data show that SP82

FIG. 5. Fractions of radioisotope-labeled phage DNA associated with the M band at various times in the latent period of single and mixed infections. The M-band procedure is described in Materials and Methods. Total radioactivity is equal to the sum of the trichloroacetic acid-precipitable radioactivity in the supernatant and the M-band fractions. SP82 was radioactively labeled with $^3\text{H}^{32}\text{PO}_4$, SPO2c₁ with either $^3\text{H}^{32}\text{PO}_4$ or H^3 -thymidine, and β22 with H^3 -thymidine. A, (Δ) SP82 single infection; B, (□) SPO2c₁ single infection; C, (○) β22 single infection; D, SPO2c₁ (□) added 5 min before SP82 (Δ); E, SPO2c₁ (□) added 5 min before β22 (○); F, β22 (○) added 5 min before SPO2c₁ (□).

DNA became associated with the membrane shortly after infection and then was slowly released from the membrane in a pattern similar to that observed in single infection (Fig. 5A). The SPO2c₁ DNA, however, became attached to the membrane and then remained attached throughout the mixed infection. It has been reported in T4 infections that detachment of parental phage DNA requires a late phage function (6). If a late gene function is also involved in detachment of the parental SPO2c₁ DNA, the parental DNA may remain attached because little or no SPO2c₁ products were made in the mixed infection. However, the existence of a constant amount of high-molecular-weight, membrane-bound SPO2c₁ DNA throughout the infection indicates that the genome of the subordinate phage was not extensively degraded.

Studies of membrane-associated DNA in SPO2c₁-β22 mixed infections were made under conditions in which β22 was added 5 min before SPO2c₁ and experiments in which the order of phage addition was reversed (Fig. 5E and F). In both situations, β22 DNA quickly became associated with the membrane and remained bound throughout the infection in a pattern similar to that noted in single infections (Fig. 5C). Thus, the replication of β22 DNA appears different since a smaller amount of detachment was detected in both single and mixed infections. The complexing

of SPO2c₁ DNA with the membrane showed differences related to the order of addition of the two phages. If SPO2c₁ was added 5 min before β22 (Fig. 5E), then the SPO2c₁ DNA associated and disassociated from the membrane in a pattern similar to that in single infection. However, it should be noted that if SPO2c₁ was added 5 min after β22 (Fig. 5F), significantly less SPO2c₁ became attached to the membrane although some release was observed.

The results of these studies of membrane-bound DNA in mixed infections indicate that the DNA of the subordinate phage (i.e., SPO2c₁) attached to the membrane although, as stated above, differences in the degree of attachment were observed. Once the SPO2c₁ DNA became associated with the membrane, the dominant phage apparently could not displace the subordinate phage DNA from the membrane. The SPO2c₁ DNA either remained associated throughout the mixed infection or detached at a time equivalent to the time it is released from the membrane in single infections.

DISCUSSION

It has been widely observed in both bacteriophage and animal virus infections that, at some point in the latent period, the infecting agent inhibits one or more of the host's macromolecular syntheses. Despite extensive stud-

ies, no generalized mechanisms have yet been proposed to explain how the virus specifically inhibits host functions while the synthesis of viral nucleic acid and protein can continue. A number of investigators (2, 11, 21) have noted parallels between the inability of host DNA to function during phage infections and the failure of heterologous phage genomes to be expressed in certain mixed infections. It seems possible, therefore, that an elucidation of the molecular basis of dominance would be intrinsically interesting and might also shed some light on the mechanisms by which viruses control host functions.

Mixed infections of T4 and the RNA phage f2 in *E. coli* have been studied to observe specifically the action of T4 on protein synthesis (11, 13). Previous studies of translation in *E. coli* singly infected with T4 have been difficult to interpret because this phage also suppresses host DNA replication and transcription. The mixed infection studies indicate that T4 may direct the synthesis of an endoribonuclease which degrades f2 RNA and, by extrapolation, perhaps also degrades host mRNA.

Double and mixed infections of ϕ X174 and S13 have been studied (21) in an attempt to determine the nature of superinfection exclusion and the role of membrane binding sites in the propagation of these phages. By use of appropriate mutants of S13, evidence has been obtained that the gene A product (a protein required for synthesis of progeny replication forms) is associated with superinfection exclusion.

The most thoroughly studied mixed infections have been those involving the T-even bacteriophages of *E. coli*. When this bacterium is infected with T2 or T4, the surface properties of the cell are quickly altered so that even an identical superinfecting phage cannot infect productively. Although the superinfecting phage can adsorb and inject its DNA, the DNA does not enter the host cytoplasm, and within a few minutes the genome of the superinfecting phage is degraded (superinfection breakdown; 1, 3, 4, 8). Vallée et al. (23) have noted that it is possible to obtain mutants of T4 which do not exclude superinfecting phage. This and other evidence indicates the involvement of a specific phage product (24) which may alter the membrane of the cell within the first moments of the latent period (20).

Most of the mixed infections in *B. subtilis* discussed in the present paper exhibited mutual exclusion (i.e., a mixed infection in which both phage entered the cell but only one partner succeeds in producing progeny) rather

than superinfection exclusion. In some mixed infections, mutual exclusion could be explained by the fact that only one phage was observed to synthesize RNA and DNA. For example, when SP82 was added before or up to 5 min after SPO2c₁, the biosynthetic activities of the latter were almost completely inhibited. We do not yet know which changes mediated by HMU-containing SP82 were important in preventing SPO2c₁ development, but a reduction in the level of host DNA polymerase (26) has been reported in cells infected with SPO1, a phage closely related to SP82. The same mechanism involved in excluding thymine from DNA in infections by another HMU-containing phage (18) may also function to prevent the synthesis of thymine-containing SPO2c₁ DNA in SPO2c₁-SP82 mixed infections. Alternatively, it is possible that HMU was incorporated into SPO2c₁-specific DNA and that its presence may have prevented or inhibited the synthesis of SPO2c₁-specific RNA (Table 2) and protein. Such alterations accompanying base substitutions in T4 DNA have been reported: the rate of synthesis of at least one late T4 phage protein was less efficient with T4 progeny DNA containing 20% of the hydroxymethylcytosine replaced by cytosine than with normal hydroxymethylcytosine-containing DNA (16).

It is notable, however, that the SPO2c₁ DNA was probably not destroyed since it remained membrane-bound presumably in a high-molecular-weight form throughout the infection. Moreover, once the replication of SPO2c₁ DNA was initiated, then superinfection at 15 min with SP82 and the transcription of the SP82 genome did not arrest the synthesis of SPO2c₁-specific nucleic acids. Thus, if enzyme modifications were involved in inhibiting SPO2c₁-specific DNA synthesis and transcription, they apparently could not completely inhibit late development. Alternatively, it is also possible that if SPO2c₁-mediated enzyme modifications occurred prior to superinfection at 15 min with SP82, then dominance by SP82 would not be fully expressed.

An analysis of infections of β 22 and SPO2c₁ indicates that the outcome of this mixed infection was influenced by the order of addition of the phages. If β 22 was added 5 min before SPO2c₁, RNA synthesis specific for SPO2c₁ was completely prevented, although some SPO2c₁ DNA synthesis did occur. The time of lysis of the cells was controlled by β 22, and high yield of β 22 progeny was released (17). However, if the order of infection was reversed, the two phage DNAs were both repli-

cated, both DNAs were transcribed, and small quantities of both progeny were released. Moreover, the cells lysed at the same time as control cultures singly infected with SPO2c₁, demonstrating that the presence of β 22 in the same cells did not alter SPO2c₁ control of the lytic cycle. It seems unlikely that RNA or DNA polymerase modifications would be sufficient to explain β 22 dominance in these infections even when this phage was added before SPO2c₁. This is so because the same modifications presumably would occur to permit β 22 development in cells preinfected with SPO2c₁, yet β 22 failed to completely inhibit transcription or replication of the latter phage. It seems more likely that, when cells were preinfected with β 22, the physiology of the host cell was altered so that SPO2c₁ could not gain access to the mechanisms normally synthesizing macromolecular components in the cell. In this respect, it should be noted that in infections in which β 22 preceded SPO2c₁, SPO2c₁ DNA did not bind to the cell membrane to the degree noted in infections in which SPO2c₁ was added first.

SPO2c₁ was the common partner in all the mixed infections reported in this paper. Cells infected with this phage undergo a complex series of changes during the latent period; host DNA replication is completely inhibited, and by the mid-point of the infection, most RNA synthesis is phage related. Moreover, a series of temporal changes (15) occurs in the populations of phage RNA in the course of the infection. Unlike mixed infections involving SP82 or β 22, however, these changes did not prevent the establishment of a second phage infection. Both SP82 and β 22 could superinfect SPO2c₁-infected cells at mid-latent period and begin to synthesize RNA and replicate DNA. Thus, the mechanisms involved in SPO2c₁-mediated shut-off host functions did not cause superinfection exclusion of unrelated phage.

The mixed infections discussed in the present paper offer experimental systems in which it is possible to add known DNAs to cells and to determine which genomes will be expressed by altering the order of addition and the specific combination of phages. The precision by which phage functions can be measured, and the fate of phage DNA examined may permit a determination of the changes mediated by the first phage and how they affect the second phage. We are now isolating RNA polymerase from phage-infected cells to determine what alterations, if any, take place after infection and whether changes in the properties of the

enzymes can be correlated with the dominance relationships described above. Among other points to be established is whether superinfecting phage of the same type also fail to infect productively in situations where a given phage excludes development of a heterologous phage. If mutual exclusion involves modification of host biosynthetic functions in such a way that they cannot act upon heterologous DNA, then one might reasonably expect that homologous secondary infections could still contribute to the genetic pool of the first phage. Appropriate mutants to test this are now being isolated.

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