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SP-10 Bacteriophage-Specific Nucleic Acid and Enzyme Synthesis in *Bacillus subtilis* W23

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Bacillus subtilis W23 was infected with a clear-plaque variant of SP-10 phage, namely, SP-10c. Exogenous thymidine was not incorporated into phage DNA (even in the presence of deoxyadenosine), nor was there any transfer of thymidine nucleotides from bacterial to viral DNA. The lytic program was unaffected by concentrations of 5-fluorodeoxyuridine sufficient to reduce bacterial DNA synthesis by >95%. Although these data are consistent with the interpretation that thymidine nucleotides are excluded from phage DNA, formic acid digests of SP-10c DNA contained what appeared to be the four conventional bases; however, adenine and thymine were not recovered in equimolar yields. DNA-RNA hybridization and hybridization competition experiments were done. Synthesis of host RNA started to wane moments postinfection and stopped completely by 36 min. SP-10c coded for discrete classes of early and late RNA. The possibility of discrete subclasses of early RNA exists. Replication of the bacterial genome appeared to terminate 12 min postinfection. Degradation of the host DNA to acid-soluble material started at 36 min and, by the end of the latent period, >90% of the host chromosome was hydrolyzed. Four apparent phage-coded enzymes have been identified. A di- and triphosphatase degraded dUTP, dUDP, dTTP, and dTDP (and, to a lesser extent, dCDP and dCTP) to the corresponding monophosphates; the enzyme had no apparent activity on dATP and dGTP. SP-10c also coded for a DNA-dependent DNA polymerase, lysozyme, and a nuclease that degrades native bacterial DNA. Judging from the dependence of enzyme synthesis on the time of addition of rifampin (an inhibitor of the initiation of RNA synthesis), messengers for the di- and triphosphatase, as well as the nuclease, are transcribed from promoters that start to function 6 min postinfection. Promoters for polymerase and lysozyme did not become functional until 8 and 16 min postinfection, respectively.

SP-10 is a moderate-size, pseudolysogenic phage that productively infects the "Marburg" and W23 strains of *Bacillus subtilis* (4, 26, 36). This virus has been widely used in transduction experiments (4, 33, 34, 37), yet virtually nothing is known about its intracellular development (4).

For some time now, it has been thought that SP-10 DNA contains an unusual or modified base, because estimates of guanine plus cytosine content from buoyant density and thermal denaturation parameters differed (4, 26). However, formic acid digests of SP-10 are reported to contain only the four conventional bases in molar ratios similar to those obtained for host DNA (26). Nevertheless, *Bacillus* and SP-10 DNAs are separable in Cs_2SO_4 as well as CsCl gradients (4, 26).

This paper is the first of a projected series dealing with the biochemical and genetic characterization of SP-10. In the present communication, we report on general aspects of DNA, RNA, and enzyme synthesis in phage-infected bacteria. Several lines of evidence argue strongly that thymidine nucleotides are not a major component of phage DNA. Notwithstanding, we, like the earlier workers (26), fail to detect unusual bases in formic acid digests of SP-10 DNA. In contrast to the earlier report, we find that formic acid hydrolysates of phage DNA do not contain equimolar amounts of adenine and thymine.

MATERIALS AND METHODS

Phage and bacteria. *B. subtilis* W23 (ATCC 23059) was used as host. SP-10c is a clear-plaque variant present in ATCC 23059B. SP-10c was readily inactivated by antiserum prepared against SP-10c and provided by K. Bott (Table 1). The eclipse and latent periods of SP-10c at 37°C were 25 and 55 min,

TABLE 1. *Inactivation of SP-10c phage by antiserum*[a]

Condition	Time (min)	PFU/ml
Control	−1	7.0×10^9 (100%)
Control	5	6.8×10^9 (97%)
Antiserum	−1	6.9×10^9 (100%)
Antiserum	5	5.1×10^7 (0.74%)

[a] Phage were diluted 10-fold into tubes containing 1 ml of previously warmed (30°C) NBS. A 0.1-ml sample was removed from both tubes. One minute later, one tube received 0.1 ml of NBS and the other received 0.1 ml of NBS plus antiserum ($K \simeq 10$). The tubes were incubated at 30°C for 5 min and sampled. Theoretically, a final antiserum level of $K = 1$ should yield 0.9% survival in 5 min (32).

respectively; the burst size was 93 ± 12 (data not shown).

Medium. NBS (4, 36) was the medium in all experiments.

Preparation of extracts for enzyme assay. Cultures (usually 100 ml) of bacteria were grown at 37°C to a density of 2×10^8/ml, at which time phage were added to a final concentration of 1×10^9 PFU/ml. At the desired time, an entire culture was poured over an equal volume of finely crushed, frozen medium. The cells were harvested by centrifugation at 5,000 × *g* for 5 min and resuspended to a final concentration of 1 g/ml in ice-cold 100 mM phosphate buffer (pH 7.0)-4 mM phenylmethylsulfonylfluoride (an inhibitor of seryl proteases). The cells were broken by grinding with 3 g of glass beads in a cell homogenizer (Bronwill). Glass beads and large debris were removed by centrifugation at 15,000 × *g* for 30 min.

Enzyme assays. DNase activity was measured in a reaction mixture that contained (in 1 ml) 50 μmol of Tris-hydrochloride (pH 7.9), 5 μmol of $MgCl_2$, 200 μmol of KCl, 1 μmol of dithiothreitol, 25 μg of *B. subtilis* W23 [^{3}H]DNA (14,500 cpm/μg), and 100 μg of extract proteins. The reaction was started by addition of the extract. Samples (0.1 ml) were removed at 2-min intervals and diluted into 1 ml of ice-cold water. Each diluted sample was made 20 μg/ml for calf thymus DNA, and the amount of label in cold acid-precipitable, alkali-stable material was determined (18). A unit of enzyme degraded DNA at an initial rate of 1% per min at 37°C.

Deoxynucleoside di- and triphosphatase activity was measured in reaction mixtures that contained (in 0.1 ml) 10 μmol of Tris-acetate (pH 9.1), 5 μmol of Mg acetate, 1 μmol of dithiothreitol, 0.5 μmol of labeled substrate (see Table 6 for specifics), and 5 μg of extract proteins. Product and substrate were separated by thin-layer chromatography (2). A milliunit of enzyme activity converted 1 nmol of di- or triphosphate to the corresponding monophosphate in 1 h at 37°C.

DNA-dependent DNA polymerase activity was measured as described by Aposhian and Kornberg (1). The labeled substrate was [5-^{3}H]dCTP (80,000 cpm/μmol) and the template was heat-denatured calf thymus DNA. The reaction mixtures also contained 5 mM NaN_2 to suppress triphosphatase activity. A milliunit of enzyme activity incorporated 1 nmol of dCTP into DNA in 1 h at 37°C.

Lysozyme activity was measured spectrophotometrically. W23 was grown at 37°C to a final density of 3×10^8/ml. The cells were harvested by centrifugation, washed twice with 10 volumes of ice-cold 50 mM Tris-hydrochloride (pH 7.9)-20 mM EDTA (pH 8), and resuspended to their original density in the same buffer. The reaction mixtures contained (per milliliter) 2×10^8 washed cells and 1 mg of extract proteins. The reaction mixtures were incubated at room temperature in a spectrophotometer (Gilford Model 240). The absorbance at 660 nm was read at 1-min intervals for at least 15 min. The reaction followed simple exponential kinetics, so only the rate constant (K) is reported. All values were corrected for the small amount of autolysis observed in enzymeless controls.

Isolation of RNA. Unlabeled RNA was isolated from 2-liter cultures by the method of Bolle et al. (3), as modified by Legault-Demare and Chambliss (17). [^{3}H]RNA was isolated from 50-ml cultures labeled with 5 μCi of [5-^{3}H]uridine per ml.

Other procedures. DNA-RNA hybridization and hybridization competition were carried out by the method of Gillespie and Spiegelman (11). Phage DNA was isolated by the method of Thomas and Abelson (35). Formic acid digests were prepared by the method of Okubo et al. (26). Protein determinations were made by the method of Lowry et al. (19).

RESULTS

DNA synthesis. Uninfected *B. subtilis* W23 readily incorporated a variety of exogenous precursors into DNA (Fig. 1a). Upon infection by SP-10c, incorporation of exogenous thymidine abruptly stopped at 12 min, whereas incorporation of all other precursors into DNA continued until 40 or 50 min (Fig. 1b). Such data implied that exogenous thymidine was not incorporated into phage DNA under the conditions tested. To confirm this, incorporation was monitored in W23 infected with SP-10c in the presence of 6-(*p*-hydroxyphenylazo)-uracil (HPUra), a potent inhibitor of bacterial DNA polymerase III (7, 25); this drug selectively inhibits bacterial DNA synthesis in a number of *Bacillus* phage systems (7, 14, 28-30). Control experiments showed that concentrations of HPUra higher than those employed in the present experiments were without effect on SP-10c phage development (data not shown). Presence of HPUra eliminated incorporation of any precursor during the initial 12 min postinfection (Fig. 2). No incorporation of thymidine was evident in the presence of HPUra, although incorporation of the other precursors tested started at 12 min and continued until 40 to 50 min (Fig. 2).

Under normal circumstances, exogenous thymidine is not incorporated into coliphage T5 DNA (W. Mego and A. Fox, personal communi-

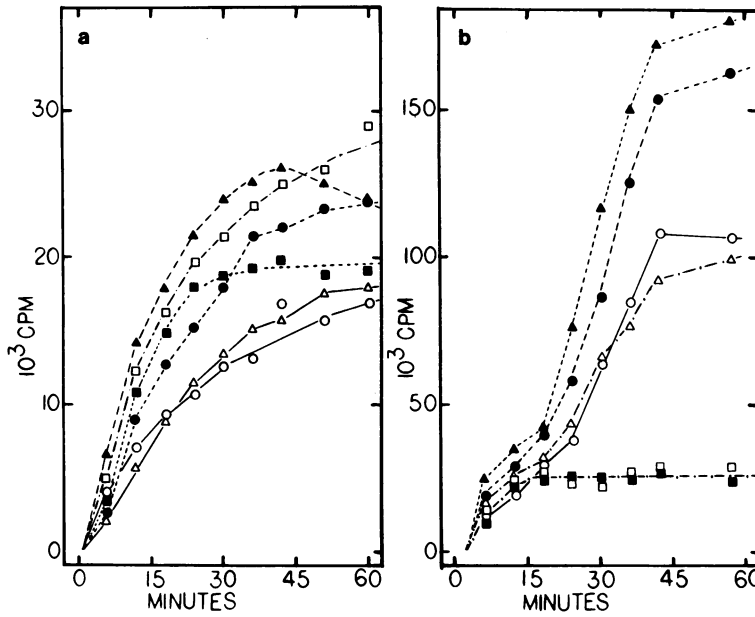

FIG. 1. *DNA synthesis in uninfected and SP-10c phage-infected Bacillus subtilis W23. (a) DNA synthesis in uninfected cells. Cells were grown to a density of 2 × 10^8/ml. The culture was split into several equal portions. Each portion received a different radioactively labeled precursor at a final concentration of 4 μCi/ml. At the times shown in the figure, 0.5-ml portions were removed, and the amount of label incorporated into DNA was estimated (18). Zero time is the time of addition of labeled precursor. (b) DNA synthesis in phage-infected cells. The experimental protocol was identical to the one described above, except that cells were infected with SP-10c and labeled precursors were not added until 2 min postinfection. Zero time is the time of infection. Symbols: (●) [8-^{3}H]adenine, (○) [G-^{3}H]cytidine (i.e., cytidine generally labeled with ^{3}H), (■) [methyl-^{3}H]thymidine, (□) [6-^{3}H]thymidine, (▲) [6-^{3}H]uridine, and (△) [5-^{3}H]uridine.*

cation). Evidently, enzymes encoded by this virus seem to minimize the contribution to deoxythymidylate biosynthesis made by the salvage pathway (38). Deoxyadenosine is known to enhance the amount of deoxythymidylate synthesized via the salvage pathway (5, 16). Indeed, presence of deoxyadenosine permits incorporation of exogenous thymidine into T5 DNA (38). However, even in the presence of deoxyadenosine, exogenous thymidine was not incorporated into SP-10c phage DNA, although a sevenfold stimulation of thymidine uptake into bacterial DNA was observed (Fig. 3). Such data imply that the lack of incorporation of thymidine into SP-10c was not attributable to a viral-induced "turnoff" of the salvage pathway.

Most thymidine nucleotides used in DNA synthesis are made de novo (16). Replication dependent upon a de novo source of thymidine nucleotides is readily inhibited by 5-fluorodeoxyuridine (FdUrd), a potent inhibitor of deoxythymidylate synthetase (16). In our hands, 100 μg of FdUrd per ml sufficed to reduce bacterial DNA synthesis by >95%, whereas that and higher concentrations of the analogue were ineffective against SP-10c phage DNA synthesis (Fig. 4). Thus, de novo synthesis of thymidine nucleotides plays no vital role in a normal SP-10c program.

Degradation of host DNA. In the case of some phages (e.g., coliphage T4), hydrolysis of the host chromosome provides a significant source of nucleotides for viral DNA synthesis (13, 40). Other phage, like T5, extensively degrade the host genome, but the mononucleotides thus generated are not reused during a normal infection (41); interestingly, a small amount of T5 DNA synthesis occurs in the presence of FdUrd and, in this situation, nucleotides originally present in host DNA are reincorporated into phage DNA (41).

To see whether hydrolysis of the host chromosome provided a significant source of nucleotides for SP-10c phage DNA synthesis, the following experiments were done. *B. subtilis* W23 were labeled with either [8-^{3}H]adenine or [6-^{3}H]thymidine for three generations before infection by SP-10c, and the amount of DNase I-resistant label in lysates was determined. This experiment is predicated on the assumptions that progeny viruses will be labeled only if hydrolysis of the host chromosome provides nucleotides for phage DNA synthesis and that DNA, packaged into phage heads, is nuclease

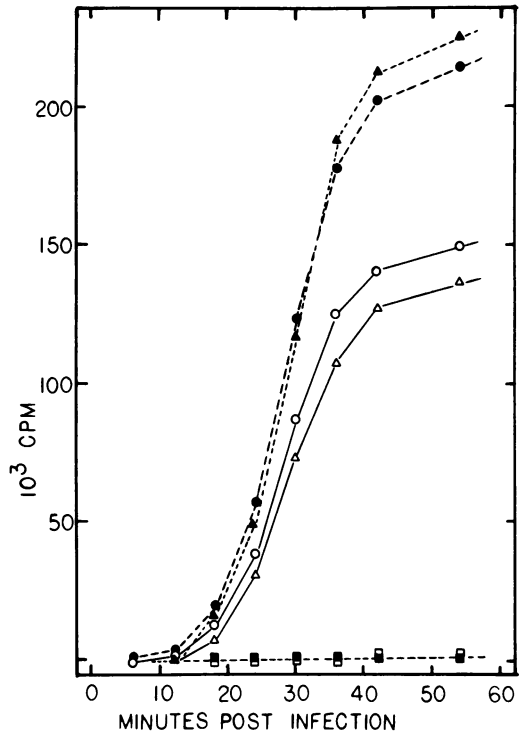

FIG. 2. *Effect of 6-(p-hydroxyphenylazo)-uracil (HPUra) on DNA synthesis in SP-10c phage-infected cells. The experimental protocol was identical to the one described under Fig. 1b except that the cultures were made 200 μM for HPUra 5 min before infection. Symbols: (●) [8-^{3}H]adenine, (○) [G-^{3}H]cytidine, (■) [methyl-^{3}H]thymidine, (□) [6-^{3}H]thymidine, (▲) [6-^{3}H]uridine, and (△) [5-^{3}H]uridine.*

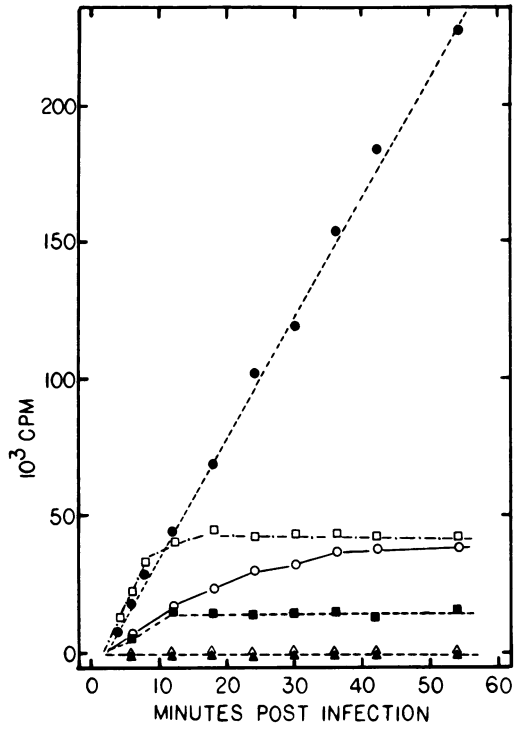

FIG. 3. *Effect of deoxyadenosine on the incorporation of [6-^{3}H]thymidine into bacterial and SP-10c DNA. Cells were grown to a density of 2 × 10^8/ml. The culture was split into six equal portions. Four portions were infected with SP-10c, and two were sham-infected. [6-^{3}H]thymidine was used at a concentration of 10 μCi/ml and was added 2 min postinfection. When present, deoxyadenosine (250 μg/ml) was added 10 min before infection. Symbols: (○) sham-infected cells, no deoxyadenosine, (●) sham-infected cells, plus deoxyadenosine, (■) infected cells, no HPUra, no deoxyadenosine, (□) infected cells, no HPUra, plus deoxyadenosine, (△) infected cells, plus HPUra, no deoxyadenosine, (▲) infected cells, plus HPUra, plus deoxyadenosine.*

resistant (13, 40). By the time of cell lysis, 85 to 90% of the bacterial chromosome had been rendered soluble in cold acid (Table 2). Nevertheless, only 1.8% of the adenine and 0.25% of the thymidine originally in host DNA were present in lysates in a nuclease-resistant form (Table 2). For comparison, Table 2 also shows the result of an experiment in which addition of labeled precursors was delayed until 2 min postinfection; here, over 60% of the label incorporated into DNA was nuclease resistant. Basically the same results were obtained when infections were carried out with FdUrd (Table 3). Considered together, these results indicate that, under the conditions tested, hydrolysis of the host chromosome was not a principal source of nucleotides for viral DNA synthesis.

Figure 5 shows the kinetics of degradation of host DNA upon infection with SP-10c. Even in the presence of chloramphenicol, a slow rate of hydrolysis was evident, suggesting that preexisting host enzymes serve a role in the process under consideration. During a normal infection or an infection carried out in FdUrd, a slow rate of degradation was evident until 36 min, at which time the rate of hydrolysis abruptly increased. By 36 min, SP-10c DNA had accumulated to 75% of its maximum level (Fig. 2). Hence, the rather belated onset of significant host DNA degradation may explain why this is not a meaningful source of nucleotides for viral-specific replication.

Component bases of SP-10c phage DNA. The data related above strongly argued that thymidine nucleotides are not a major component of SP-10c DNA (Fig. 1 to 5; Tables 2 and 3). Okubo et al. (26), however, reported only the conventional four bases in formic acid hydrolysates of SP-10 DNA. Consequently, we believed that a reexamination of this point was merited.

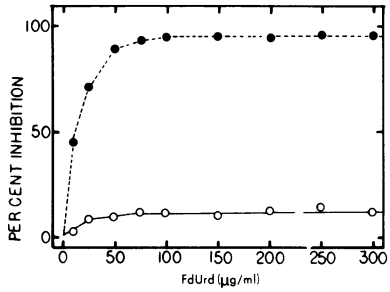

FIG. 4. *Effect of 5-fluorodeoxyuridine (FdUrd) on bacterial and SP-10c phage DNA synthesis. In the case of uninfected cells, the protocol was identical to the one described under Fig. 1a except that FdUrd was added 5 min before the labeled precursor, in this case, [G-³H]adenine. In the case of infected cells, the protocol was the same as the one described under Fig. 2, except that FdUrd was added 5 min before infection; the labeled precursor was [G-³H]adenine and it was added 2 min postinfection. In all instances, a fivefold weight excess of uridine was added with the FdUrd. Samples were removed at 10-min intervals for 1 h; however, we report here only the 60-min readings. Symbols:* (●) *Bacillus subtilis W23,* (○) *SP-10c phage.*

TABLE 2. *Transfer of nucleotides from host to SP-10c DNA*[a]

Sample	cpm/ml[b]			
	A	B	C	D
Before infection[c]	268,870	666,693		
Lysate (before DNase I)	40,451	70,788	255,938	111,161
Lysate (after DNase I)	4,727	1,716	166,408	71,884

[a] Four cultures of *B. subtilis* W23 were grown in NBS. Cultures A and B were labeled with, respectively, 10 μCi of [8-³H]adenine or [6-³H]thymidine per ml for three generations. All cultures were harvested by centrifugation, washed once with 20 volumes of ice-cold medium, and resuspended to their original density in warm (37°C) NBS. The cultures were made 200 μM for HPUra and were infected 5 min later. Cultures C and D received, respectively, 10 μCi of [8-³H]adenine or [5-³H]uridine per ml at 2 min postinfection. The cultures were incubated until complete lysis (ca. 65 min). A 1-ml portion of each lysate was incubated with 50 μg of DNase I per ml for 90 min.

[b] Amount of label in DNA was determined by the method of Lembach and Buchanan (18).

[c] Sample (1 ml) taken 1 min before infection.

In agreement with Okubo et al. (26), no unusual bases were detected in formic acid digests of SP-10c phage DNA, but, in contrast to the earlier work, our digests always contained reduced molar yields of thymine relative to adenine (Table 4). The latter observation cannot be an artifact of the system, because equimolar amounts of thymine and adenine were routinely recovered from the DNAs of *Escherichia coli, B. subtilis,* and *Micrococcus luteus,* as

TABLE 3. *Transfer of nucleotides from bacterial to host DNA in the presence of FdUrd*[a]

Sample	cpm/ml		
	A	B	C
Before infection	281,612	722,799	
Lysate (before DNase I)	13,901	31,723	212,817
Lysate (after DNase I)	1,041	987	134,681

[a] Experimental protocol was identical to the one described for cultures A, B, and C under Table 1, except that FdUrd (300 μg/ml) and uridine (1,500 μg/ml) were added 5 min preinfection.

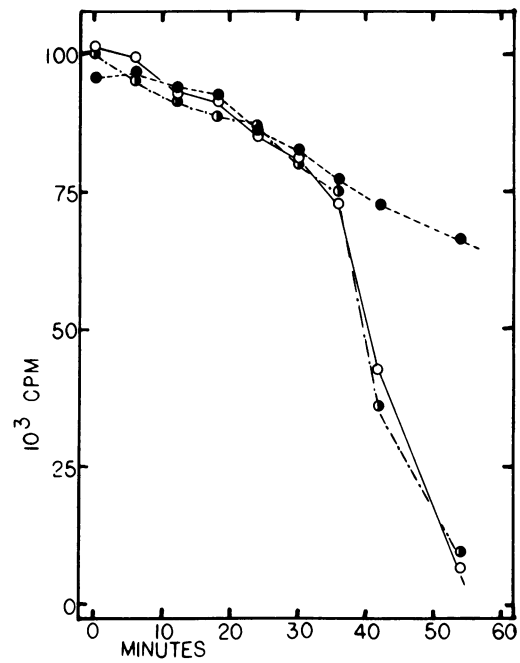

FIG. 5. *Hydrolysis of the host chromosome by SP-10c. Bacteria were grown for three generations in a medium that contained [methyl-³H]thymidine (10 μCi/ml). The cells were harvested by centrifugation, washed twice with 10 volumes of ice-cold medium, and resuspended to their original concentration in warm medium supplemented with 200 μM HPUra. Five minutes later, phage were added. Samples (0.5 ml) were removed, and the amount of label in DNA was measured. Symbols:* (○) *control,* (●) *chloramphenicol (50 μg/ml) added 5 min before infection,* (◑) *FdUrd (100 μg/ml), and uridine (500 μg/ml) added 5 min before infection.*

TABLE 4. *Bases in various DNAs*[a]

DNA	Relative molar yield of base[b]			
	Adenine	Guanine	Cytosine	Thymine
SP-10c	1.0	0.75	0.75	0.60
B. subtilis	1.0	0.78	0.78	0.99
E. coli	1.0	0.93	0.94	0.98
M. luteus	1.0	1.77	1.76	1.02
Poly[d(AT)]	1.0	None	None	1.09
Poly[d(GC)]	None	1.00	0.99	None

[a] One milligram of each DNA was precipitated with alcohol and redissolved into 1 ml of 88% formic acid. The samples were incubated at 195°C for 90 min. The samples were then evaporated to a final volume of about 50 μl. Duplicate 5-μl portions were spotted onto cellulose thin-layer plates (20 by 20 cm; no fluorescence indicator). The chromatograms were developed in the ascending mode with a mixture of *n*-butanol-acetic acid-water (4:1:1). Spots of the legitimate bases were run in parallel. Spots containing the bases were eluted with 1 M HCl and examined spectrophotometrically (26). In all cases, bases isolated from DNAs were identical in spectral properties to the legitimate bases. Controls showed that 1 M HCl eluted 99% of each base from the cellulose.

[b] Relative values were obtained by dividing the molar yield of a given base by the molar yield of adenine.

well as from the synthetic DNA, poly[d(AT)] (Table 4).

Since the results presented in Fig. 2 to 4 indicated that any thymine in SP-10c DNA was not synthesized via the conventional pathways, attempts were made to determine the precursor to thymine in formic acid digests of phage DNA. Accordingly, phage DNA was labeled with [5-^{3}H]uridine, [6-^{3}H]uridine, and [G-^{3}H]cytidine. DNA was isolated and treated with formic acid, and the distribution of label in the four bases was determined. All of the label originally in [G-^{3}H]cytidine and [5-^{3}H]uridine was recovered from SP-10c DNA as cytosine (Table 5). On the other hand, label originally in [6-^{3}H]uridine was recovered as thymine and cytosine (Table 5). Thus, the thymine recovered from SP-10c DNA does, in fact, appear to be a 5-substituted derivative of uracil.

RNA synthesis. Upon infection of *B. subtilis* W23 by SP-10c, the instantaneous rate of RNA synthesis (as measured by the uptake of [8-^{3}H]adenine or [5-^{3}H]uridine in a series of 1-min "pulses") decreased for the initial 4 min (Fig. 6). At later times, two waves of RNA synthesis were observed. The first wave was prereplicative, whereas the second, less conspicuous, wave was coincident with viral DNA replication.

DNA-RNA hybridization experiments. These experiments were performed to gain some idea about the relative abundance of host and viral transcripts in [^{3}H]RNA isolated from phage-infected cells. A constant concentration of "pulse-labeled" RNA was incubated with filters that contained varying concentrations of heat-denatured host or SP-10c DNA, and the amount of RNA hybridized was recorded.

TABLE 5. *Distribution of label among pyrimidines in SP-10c DNA*[a]

Precursor	cpm	
	Cytosine	Thymine
[G-^{3}H]cytidine	692,467	406
[6-^{3}H]uridine	334,091	352,216
[5-^{3}H]uridine	587,043	1,277

[a] Three 2-liter cultures of *B. subtilis* W23 were infected with SP-10c. At 2 min, each of the cultures received (per ml) 2 μCi of one of the precursors indicated above. After lysis, the viruses were purified by differential and buoyant-density centrifugation (4, 26). DNA was isolated and hydrolyzed, and the component bases were resolved by chromatography (Table 4). The spots were eluted and the amount of label present was determined. Spots corresponding to adenine and guanine contained only 112 to 235 cpm.

RNA labeled at preinfection times or during the first 4 min postinfection failed to anneal to filters that contained heat-denatured SP-10c phage DNA, whereas 60% of the same RNAs hybridized to filters that contained saturating concentrations of denatured host DNA (Fig. 7). Thereafter, the abundance of host-specific RNA declined until, by 36 to 40 min, none was detectable in "pulse-labeled" RNA.

Hybridization competition among phage RNAs. Two discrete waves of RNA synthesis were evident in SP-10c phage-infected bacteria (Fig. 6). DNA-RNA hybridization competition experiments were performed to see whether these waves represented synthesis of unique or overlapping sequences. Although valuable, the results of such studies must be treated with caution (6).

For the first experiments, RNA was labeled with [5-^{3}H]uridine from 0 until 12 min postinfection. This labeled RNA was annealed to filters containing heat-denatured SP-10c phage DNA, in either the absence or presence of unlabeled RNAs extracted 4, 6, 8, 10, 12, 20, and 40 min postinfection. Few, if any, sequences in labeled RNA were competable by unlabeled 4-min RNA (Fig. 8), an observation consistent with the hyridization data given above (Fig. 7). Unlabeled RNAs isolated 6 to 10 min postinfection competed against a progressively higher fraction of the material in labeled RNA. The 12- and 20-min competitors contained all sequences

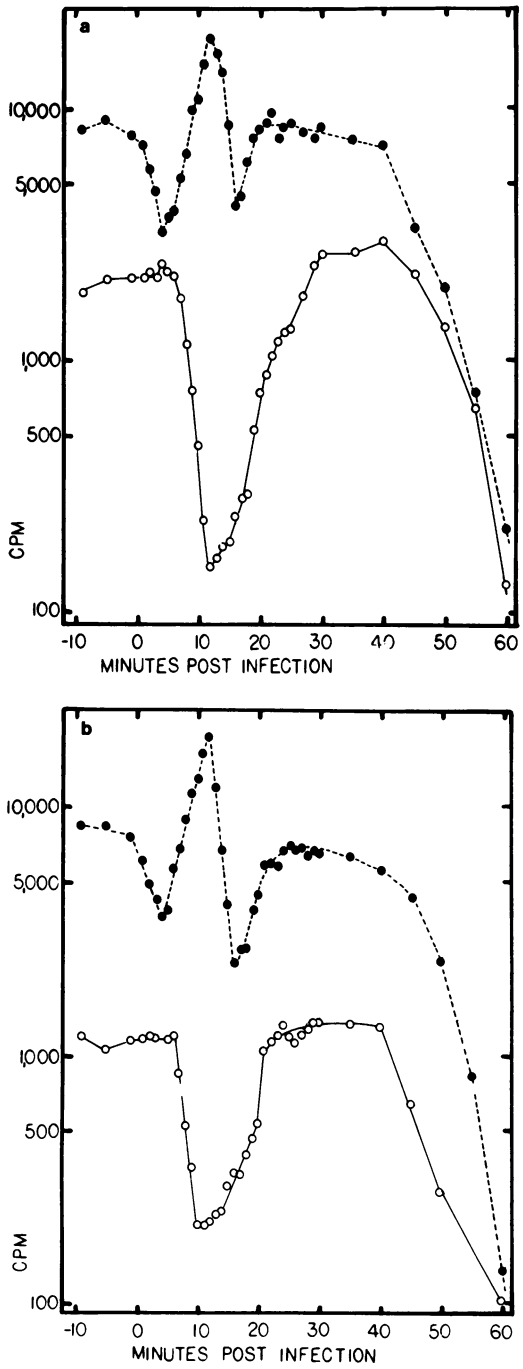

FIG. 6. *Instantaneous rate of RNA and DNA synthesis in SP-10c phage-infected cells. A large culture of bacteria was infected in the absence of HPUra. At the times specified, 1-ml portions were transferred to fresh tubes that contained either [8-^{3}H]adenine (a) or [5-^{3}H]uridine (b). The final precursor concentration was 10 μCi/ml. After 1 min with label, the portions were removed and placed in an acetone-solid CO_2 bath. At the end of the experiment, the samples were thawed, and the distribution of incorporated label into RNA and DNA was determined (18). Symbols are placed at the midpoint of each pulse. Symbols: (●) RNA, (○) DNA.*

in [^{3}H]RNA. However, comparing the initial slopes of the curves obtained with 12- and 20-min competitors implies that many sequences underwent a severalfold reduction in relative abundance during the time interval in question. Unlabeled 40-min RNA was a poor competitor of 0- to 12-min [^{3}H]RNA.

In the second series of experiments, RNA was labeled from 15 until 30 min postinfection, and competitors were extracted at 12, 15, 20, 30, and 40 min. Sequences competable by 12- and 15-min unlabeled RNA comprised only 12 to 18% of the material in [^{3}H]RNA, implying that sequences made at early times of the program represent relatively little of the RNA made at late times (Fig. 9). Unlabeled RNAs isolated at 20, 30, and 40 min were all potent competitors of 15- to 30-min [^{3}H]RNA.

Enzyme synthesis. To date, four different phage-specific enzymes have been identified. These are deoxynucleoside di- and triphosphatase, DNA-dependent DNA polymerase, lysozyme, and a nonspecific nuclease that hydrolyzes native DNA.

The di- and triphosphatase appeared 6 to 8 min after infection and was maximal by 12 min (Fig. 10). Di- and triphosphates of deoxythymidine and deoxyuridine were the best substrates, but measurable activity was seen with dCDP and dCTP (Table 6). The enzyme had no measurable activity on the di- and triphosphates of deoxyadenosine or deoxyguanosine. Bacterial DNA synthesis, as measured by the incorporation of exogenous thymidine, stopped 12 min postinfection (Fig. 1), the time at which dTDPase-dTTPase activity reached its maximum levels (Fig. 10); it is possible that cessation of host DNA synthesis resulted from depletion of dTTP by the phage-induced triphosphatase.

Nuclease activity likewise appeared 6 to 8 min postinfection but, in this case, maximum enzyme levels were not reached until 20 min (Fig. 10). The enzyme had equivalent activity when tested against all of the naturally occurring DNAs mentioned in Table 4 (data not shown).

DNA-dependent DNA polymerase activity first appeared 10 to 12 min after infection and was maximal by 20 min (Fig. 10). Price and Fogt (28) have suggested that those subtilis phages whose DNA synthesis is HPUra refractile code for their own replicational machinery; this hypothesis is supported by our findings

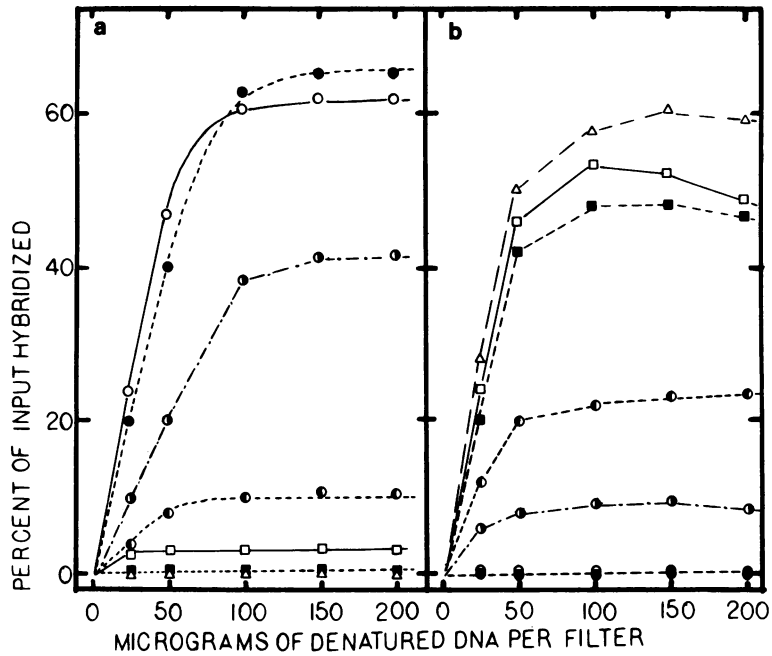

FIG. 7. *DNA-RNA hybridization. A large culture of W23 was infected with SP-10c phage in the absence of HPUra. At the times indicated below, 50-ml portions were removed and transferred to fresh vessels that contained [5-^{3}H]uridine. The cells were labeled for 4 min and RNA was isolated. [^{3}H]RNA (1 μg) was annealed to filters that contained the indicated concentrations of heat-denatured bacterial DNA (a) or phage DNA (b). Symbols: (○) RNA labeled from 5 min before until 1 min before infection (specific activity = 18,680 cpm/μg), (●) RNA labeled from 0 until 4 min postinfection (specific activity = 9,125 cpm/μg), (◑) RNA labeled from 6 until 10 min postinfection (specific activity = 29,815 cpm/μg), (◐) RNA labeled from 12 until 16 min postinfection (specific activity = 8,235 cpm/μg), (□) RNA labeled from 18 to 22 min postinfection (specific activity = 18,588 cpm/μg), (■) RNA labeled from 32 until 36 min postinfection (specific activity = 16,052 cpm/μg), and (△) RNA labeled from 38 to 42 min postinfection (specific activity = 11,142 cpm/μg).*

that SP-10c codes for its own DNA polymerase and that SP-10c DNA synthesis is HPUra refractile (Fig. 2 and 10). Phage-coded lysozyme was not evident earlier than 16 min and was present at maximum levels by 30 min (Fig. 10).

Effect of antibiotics on enzyme synthesis. The results shown in Fig. 10 indicated that different phage-coded enzymes appeared at different times postinfection which, in turn, was consistent with the observation that different "classes" of phage-specific RNA appeared at different times (Fig. 8 and 9). To determine the earliest time at which select functional messengers were present in cells infected with SP-10c phage, the dependence of enzyme synthesis on the time of addition of various antibiotic inhibitors of RNA synthesis was studied. The antibiotics employed were rifampin, an inhibitor of initiation (39), and streptolydigin, an inhibitor of chain elongation (31). Controls showed that, throughout the SP-10c program, RNA synthesis decreased >99% within 1 min of the addition of either drug (data not shown).

By the criterion applied, funtional messengers for deoxynucleoside di- and triphosphatase, nuclease, DNA polymerase, and lysozyme were first evident 6, 6, 8, and 14 min postinfection, respectively (Fig. 11). In all cases, appearance of functional messengers preceded appearance of enzyme by 2 to 4 min, which is consistent with the lag between transcription and translation in other procaryotic systems (see [15] and [23] for references and discussion). More importantly, synthesis of the enzymes became rifampin refractile at different times postinfection, thereby indicating the existence of multiple classes of promoters.

DISCUSSION

Formic acid digests of SP-10c DNA contained what seemed to be the four conventional bases (Table 4). Notwithstanding, several lines of evidence support the interpretation that thymidine nucleotides are excluded from SP-10c DNA. (i) Exogenous thymidine was not incorporated into viral DNA, even in the presence of deoxyadenosine (Fig. 1 to 3). In this context, it is relevant to note that thymidine phosphoryl-

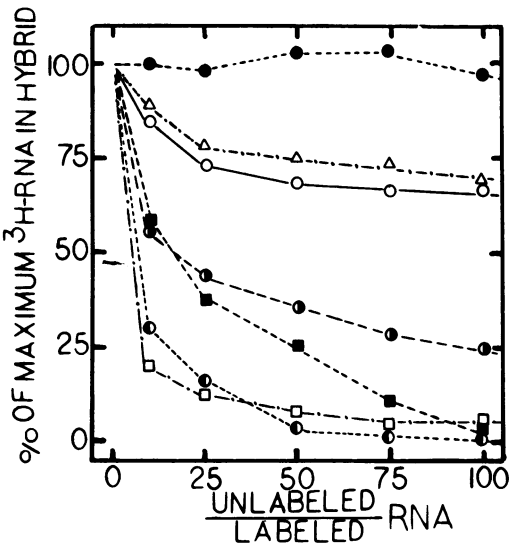

FIG. 8. *Hybridization competition between early RNAs of SP-10c. [³H]RNA was labeled from 0 until 12 min postinfection, and 1 μg (ca. 46,000 cpm) was annealed to filters that contained 20 μg of heat-denatured phage DNA in the absence and presence of unlabeled RNAs. In the absence of unlabeled RNA, ca. 13% of the input was retained by the filter. Symbols: unlabeled RNA isolated (●) 4, (○) 6, (◑) 8, (◐) 10, (□) 12, (■) 20, and (△) 40 min postinfection.*

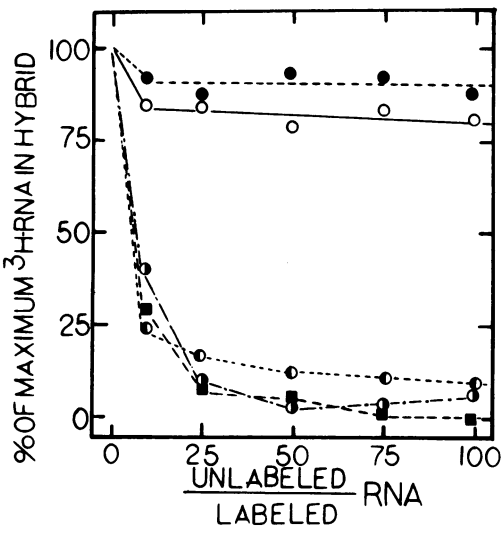

FIG. 9. *Hybridization competition between early and late RNAs of bacteriophage SP-10c. [³H]RNA was labeled from 15 until 27 min postinfection. It had a specific activity of 30,298 cpm/μg. The remainder of the procedure is described under Fig. 6. In the absence of unlabeled RNA, 42% of the input hybridized, whereas only 0.9% of the input was retained by DNA-less filters. Symbols: unlabeled RNAs isolated (●) 12, (○) 15, (◑) 20, (◐) 30, and (■) 40 min postinfection.*

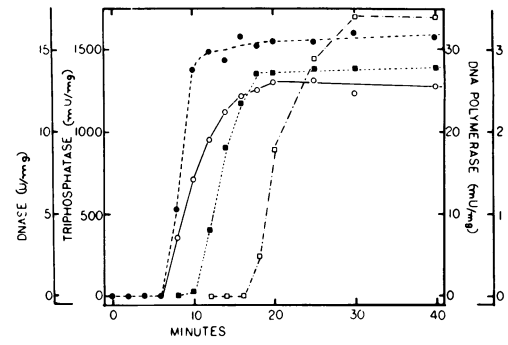

FIG. 10. *Enzyme synthesis in SP-10c phage-infected cells. Large cultures were infected and harvested, and extracts were assayed for enzyme activity as described under Materials and Methods. Symbols: (●) deoxynucleoside di- and triphosphatase (measured here as its deoxythymidine triphosphatase activity), (○) DNase, (■) DNA-dependent DNA polymerase, and (□) lysozyme.*

TABLE 6. *Activity of SP-10c di- and triphosphatase on different substrates*[a]

Substrate	Apparent activity (mU/mg of protein)
[*methyl*-³H]dTDP	1,926
[*methyl*-³H]dTTP	2,212
[5-³H]dUDP	1,758
[5-³H]dUTP	1,896
[5-³H]dCDP	117
[5-³H]dCTP	112
[G-³H]dADP	≤0.1
[G-³H]dATP	≤0.1
[G-³H]dGDP	≤0.1
[G-³H]dGTP	≤0.1

[a] Reaction mixtures were prepared as described under Materials and Methods. Each precursor was present at a specific activity of 45,000 cpm/μmol. At the end of the reaction, the appropriate deoxynucleoside monophosphate was added to a final concentration of 5 mM. Product and unreacted substrate were separated by thin-layer chromatography (2). Spots corresponding to the mono- and triphosphate (which remains at the origin) were eluted, and the amount of label in each was estimated. In all experiments, label in the mono-, di-, and triphosphate spots accounts for >99% of the input label. Significant amounts of label were never observed in the regions of the chromatograms corresponding to the nucleoside or free base.

ase and thymidine kinase levels remain constant throughout the SP-10c program (data not shown), implying that the salvage pathway remains operational during the entire infection process. Furthermore, SP-10c fails to code for any 5'-nucleotidase activity (data not presented). Consequently, nonincorporation of exogenous thymidine into viral DNA is not due to a viral-induced "turnoff" of the salvage path-

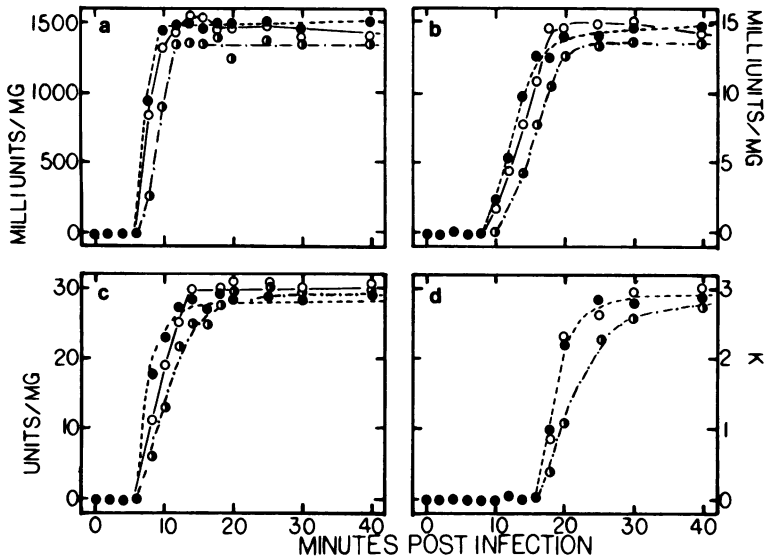

FIG. 11. *Dependence of enzyme synthesis on the time of addition of rifampin and streptolydigin. Two-liter cultures of W23 were infected with SP-10c. At the times specified, 150-ml portions were removed and split into three equal portions. One portion was transferred to a fresh vessel that contained rifampin to give a final drug concentration of 10 μg/ml. The second portion was transferred to a vessel that contained streptolydigin to give a final drug concentration of 10 μg/ml. The third portion was harvested immediately and served to measure the amount of enzyme present at each addition of drug. Those portions that received antibiotic were incubated until a total of 41 min postinfection. (a) Deoxynucleoside di- and triphosphatase (measured as its deoxyuridine triphosphatase activity); (b) DNase; (c) DNA-dependent DNA polymerase; (d) lysozyme. Symbols: (●) rifampin, (○) streptolydigin, and (◑) enzyme level at time of drug addition.*

way or to degradation of deoxythymidylate by viral-coded enzymes. (ii) Replication of the phage genome was FdUrd refractile (Fig. 4). In many other experiments, we have found that FdUrd had no obvious effect on the eclipse or latent periods, burst size, and phage-coded enzyme production (data not shown). (iii) There was no transfer of thymidine nucleotides from host to phage DNA (Tables 2 and 3). (iv) SP-10c codes for a potent dTDPase-dTTPase activity (Fig. 10; Table 6). Typically, di- and triphosphatases occur only in those situations in which a normal nucleotide is excluded from phage DNA (see [12] and [21] for references).

These apparently conflicting results can be reconciled, at first glance, in several ways. First, the base identified as thymine may, in fact, be some other 5-substituted derivative of uracil. This possibility is deemed somewhat unlikely because (i) the base isolated from SP-10c DNA can support the growth of *E. coli* BIOT⁻ (a thymine auxotroph isolated by Donald Morrison of this department), (ii) the base isolated from phage DNA is chromatographically and spectrophotometrically indistinguishable from thymine in several solvent systems, and (iii) thymine and the base isolated from phage DNA have identical nuclear magnetic resonance spectra (data not shown). Second, it is possible that the thymine present in SP-10c DNA represents a postreplicational modification of uracil. However, this hypothesis implies that dUTP is a substrate for phage DNA synthesis, which does not square with the 10- to 15-fold increase in dUDPase-dUTPase activity observed during the SP-10c program (Fig. 11; Table 6). For the sake of comparison, it is worth noting that dUTP is probably a substrate for SP-15 phage DNA synthesis (24) and this virus does not seem to code for a dUDPase-dUTPase (Boghosian and Witmer, unpublished data). Third, the thymine present in formic acid digests of SP-10c DNA may be a breakdown product of an unusual base; the dihydroxypentyl uracil in SP-15 DNA is degraded to thymine by perchloric acid but not by formic acid (20). Obviously, much more work is required to clarify this situation.

None of the hypotheses considered above adequately accounts for the reduced yield of thymine in acid digests of phage DNA (Table 4). Offhand, such results indicate that SP-10c DNA contains a base (with the H-bonding properties of thymine) that has thus far escaped detection. We have eliminated the possibility that an unusual base co-chromatographs with adenine, guanine, or cytosine, because the latter

three bases isolated from SP-10c DNA are identical to the legitimate bases in both spectrophotometric and nuclear magnetic resonance properties (data not shown). There are other possibilities to account for the data in question, but in the absence of more specific information, it seems best to defer further discussion.

Bott and Strauss (4) reported that extracts of W23 infected with wild-type virus contain a nuclease that hydrolyzes bacterial DNA in vitro, but these authors failed to detect extensive degradation of the host genome in wild-type-infected cells. We observed a dramatic rise in nuclease activity upon infection by SP-10c (Fig. 10), but extensive degradation of host DNA was also evident at late times of the program (Fig. 5). If the nuclease assayed in extracts is responsible for the observed hydrolysis of the host chromosome in vivo, then some mechanism must exist to minimize the impact of this enzyme in wild-type-infected cells.

Hybridization competition experiments suggested the existence of discrete early and late classes of SP-10c RNA (Fig. 8 and 9). This conclusion is substantiated by the observation that the four enzyme activities studied represent transcription from three distinct classes of promoters (Fig. 11). Multiple promoter classes imply positive control of phage transcription (8–10, 22). A more definitive answer to this problem will require more information concerning the biochemistry and genetics of SP-10. While on this subject, it is worth noting that PBS2, another pseudolysogenic phage that infects *B. subtilis*, does not seem to have discrete early and late subclasses of RNA, because phage-coded enzymes, DNA, and progeny viruses all appear within a few minutes of each other (27).

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