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Differential Expression of Bacteriophage Genomes in Vegetative and Sporulating Cells of *Bacillus subtilis*

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Two antigenically distinct bacteriophages, β3 and β22, have been isolated and characterized with *Bacillus subtilis* strain W23 as a host. They differ in plaque morphology, single-step growth characteristics, host range, and thermal stability. The deoxyribonucleic acids isolated from β3 and β22 differ in base composition, density in CsCl and Cs_2SO_4, sedimentation coefficient, molecular weight, and thermal denaturation temperature. These phages have been used to analyze the ability of *B. subtilis* to sporulate despite infection by virulent phages. When development of phages β3 and β22 in sporulating cultures was compared with that in log cultures, an increase in the latent periods of infection and a decrease in the burst sizes for the two phages were observed. Sporulating cultures infected with β3 yielded the usual percentage (85%) of mature spores; 80% of these contained phage determinants and 20% were uninfected. However, cultures infected with β22 lysed. Of the small fraction (0.01%) which sporulated, 83% were uninfected and 17% were infected. Phage β3-infected and uninfected spores were examined to distinguish any chemical or physical differences. Preparations of both types of spore contained 81.4 μg of dipicolinic acid per mg (dry weight), and examination by phase-contrast microscopy gave no evidence of any difference in outward appearance. A 20% decrease in infected spore count was observed upon heating at 80 C for 10 min. Differences in the infection processes of the two phages prompted an analysis of the transcription process after infection. Deoxyribonucleic acid-ribonucleic acid hybrid analysis of relative amounts of phage-specific and host-specific messenger ribonucleic acid (mRNA) present in infected cells suggested that β3 was unable to repress the synthesis of host mRNA and that β3-specific mRNA synthesis was repressed in sporulation-phase cultures. Phage β22, in contrast, was able to repress host-specific mRNA synthesis in both log-infected and sporulation-infected cells. The results suggest that the differential expression of the phage genomes is due to the relative ability of the phages to repress the host genome.

Various *Bacillus* species are capable of sporulating despite infection by nonlysogenic phage (3, 13, 14, 21). When the spores produced under these conditions germinate, in many cases they lyse and release mature phage particles. In the case of phage PBS 1, it was shown (34) that infected *B. subtilis* spores contained PBS 1 deoxyribonucleic acid (DNA). These results demonstrate that the complete phage genome is incorporated into the mature spore and is expressed during germination and outgrowth.

How do cells infected with phage continue to perform the functions necessary for the production of mature spores? Two phages, β3 and β22, for *B. subtilis* have been isolated and selected to obtain information concerning this particular point. The two phages differ in that the β3 genome is incorporated into spores with high efficiency, whereas an insignificant incorporation of the β22 genome into spores is observed. The nucleic acid fractions of infected cells were studied to obtain some understanding of the expression of the phage and host genomes. It was found that during β3 infection of sporulating cells host messenger ribonucleic acid (mRNA) synthesis continued unabated, but only low levels of β3-specific mRNA were produced. In contrast in β22-infected cells, host mRNA synthesis was reduced drastically while transcription of β22 genome occurred efficiently. These results sug-

gest that repression of the host genome and the efficiency of transcription of the phage genome determine whether phage genomes are incorporated into spores.

MATERIALS AND METHODS

Bacterial strains and media. B. subtilis strains W23 and 168 were employed in the experiments described. The medium (M) used for the preparation of phage lysates contained 1.0% tryptone (Difco), 0.5% yeast extract (Difco), 0.17 M NaCl, and 5.5×10^{-3} M glucose. $CaCl_2$ (5×10^{-3} M) and $MnCl_2$ (10^{-4} M) were added separately after sterilization. Phage suspensions were assayed by the agar overlay technique of Adams (1). M medium containing 2.0% agar was used for the basal layer of the phage assay plates and 0.7% agar was used for soft agar. Other experiments were performed with SCM medium (7) supplemented with 0.3% casein hydrolysate, 5.5×10^{-3} M glucose, and 5×10^{-3} M $CaCl_2$, but minus PO_4^{-3} and sodium citrate.

Isolation of phages. Phages were obtained by heating soil samples suspended in M medium at 65 C for 30 min, and by shaking the preheated suspensions at 37 C in the presence of the added indicator strain, *B. subtilis* W23 (30). In some cases, β3-specific antiserum was added to the suspensions to select for phages serologically distinct from those isolated previously. Samples were plated for phage, and isolated plaques were selected for further studies.

Large quantities of phage were obtained by infecting 1-liter cultures of log-phase *B. subtilis* cells in 2,800-ml Fernbach flasks at 37 C. Cells were grown to an absorbancy at 660 mμ of 0.5 (2.5×10^8 cells/ml) as measured by a Gilford spectrophotometer. The cultures were then infected with phage multiplicites of infection (MOI) of 0.01 to 0.1. Lysates were made 5×10^{-3} M in $MgCl_2$ and treated with pancreatic deoxyribonuclease (5 μg/ml) and pancreatic ribonuclease (1 μg/ml) at 37 C for 60 min. The lysates were centrifuged at 10,400 $\times$ *g* for 10 min to remove cell debris. The supernatant fluid was filtered through a pad of kieselguhr (40 g) to remove any debris which had not spun down. The filtrate was centrifuged at 25,400 $\times$ *g* for 1 hr, and the pellets were suspended in 0.1 M tris(hydroxymethyl)aminomethane (Tris; *p*H 7.2)-5×10^{-3} M $MgCl_2$ (0.1 TM) buffer. Solid CsCl was then added to the phage suspensions to bring the final density to 1.5 g/cc. Density was determined from the refractive index of the solution by use of a Bausch & Lomb refractometer. The suspensions were centrifuged in the SW39 rotor in a Spinco L preparative ultracentrifuge at 33,000 rev/min for 16 to 20 hr. After centrifugation, the phage bands were collected by piercing the bottom of the tube and collecting the proper fractions. The suspensions were dialyzed against decreasing concentrations of NaCl in 0.1 TM buffer. The β3 phage preparations were stored at -20 C in the 0.1 TM buffer containing 10% dimethylsulfoxide (DMSO) (38). The β22 phage stocks were stored in the same solution at 4 C.

Single-step growth experiments were performed as described by Adams (1). Rabbit antiserum against the phages was prepared by subcutaneous injection of a homogenate of the phage and Freund adjuvant.

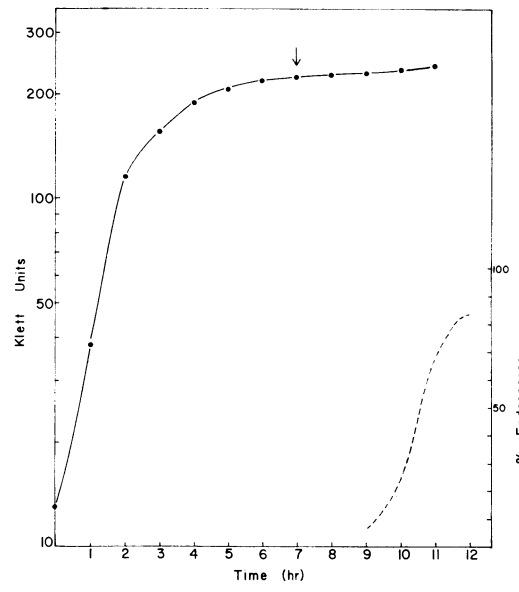

FIG. 1. *Growth curve and time of infection of Bacillus subtilis W23 in modified SCM medium. Turbidity measured in Klett units (red filter). Appearance of endospores (dash lines) was observed by phase-contrast microscopy. Arrow indicates the time at which sporulation-phase cultures were infected.*

Biology of infection during sporulation. As a result of the observations of McCloy (21, 22), experiments were undertaken to determine the extent of sporulation despite infection with β3 or β22. Growth of *B. subtilis* in the modified SCM medium (Fig. 1) was followed by use of a Klett-Summerson colorimeter equipped with a red filter. Sporulating cultures were infected, at a point indicated by the arrow, 2 hr prior to the appearance of endospores under phase-contrast microscopy, with phage MOI of 5. The infected cultures were then assayed for phage production and the degree of sporulation. Samples were taken at 30 min and 5 hr after infection to determine the extent of any increase in plaque-forming units (PFU). After the cultures had sporulated, the spores were harvested and washed three times with distilled water. The pellets were resuspended in the SCM salts solution. Total spores were determined by direct count by use of a Petroff-Hausser counting chamber. The suspensions were then treated with specific phage antisera sufficient to inactivate any external phage. A heat-shock treatment was eliminated, since some of the spores were heat-sensitive. The suspensions were then diluted and plated for infected and uninfected spores.

Since cultures of *B. subtilis* seemed to sporulate in spite of infection by β3, the spores of sporulation-infected and uninfected cultures were examined for any differences. Samples were analyzed for dipicolinic acid (DPA) by the method of Janssen, Lund, and Anderson (17).

Preparation and characterization of phage DNA. Phage DNA was extracted by lysing phage suspensions in saline-EDTA (0.15 M NaCl, 0.1 M ethylenediaminetetraacetate, *p*H 8.0) with 2% sodium dodecyl sulfate (SDS) and purified by the method of Marmur (23). DNA used for the determination of sedimentation coefficients was extracted from phage suspensions by the phenol method of Davison and Freifelder (5). Final preparations had 260/280 absorbance ratios of about 1.9, indicating removal of detectable phenol. Bacterial DNA was prepared as before (8).

The base compositions of the purified DNA preparations were determined by hydrolysis of the DNA in 98% formic acid (36) and separation of the resulting bases by paper chromatography (37). Descending chromatography was performed on Whatman no. 1 paper with a solvent system composed of 65% (v/v) isopropanol (Fischer, spectra-analyzed), water, and HCl, the final solution being 2.0 N in HCl. The bases were identified by comparing their absorption spectra, obtained by using a Cary model 14 recording spectrophotometer, with those of known purines and pyrimidines. The R_F values for the bases were compared with those of known bases.

The buoyant density of phage DNA in CsCl density gradient (44,770 rev/min 25 C) was determined by the method of Meselson, Stahl, and Vinograd (25) by banding phage and marker ^{15}N-*Pseudomonas aeruginosa* (ρ = 1.742 g/cc) DNA preparations at equilibrium with a Spinco E analytical ultracentrifuge. Ultraviolet absorption photographs were scanned with a Joyce-Loebl recording microdensitometer. The densities in CsCl and corresponding base compositions, determined according to the method of Schildkraut, Marmur, and Doty (33), were based on ρ = 1.710 g/cc for *Escherichia coli* DNA. Densities in Cs_2SO_4 gradients (44,770 rev/min 25 C) were determined as mentioned above and based on ρ = 1.426 g/cc for *E. coli* DNA (23). The value for $1/\beta$ for the Cs_2SO_4 gradient was determined to be 13.90×10^{-10} (cgs) from the work of Ludlum and Warner (20).

Sedimentation velocity studies were performed at 20 C with a Spinco E analytical ultracentrifuge. DNA solutions at a concentration of 20 μg/ml in standard saline citrate (SSC) buffer (24) were centrifuged at 27,690 rev/min, and sedimentation was followed by ultraviolet absorption optics. The resulting photographs were scanned as described above. All samples were run in a valve-type, synthetic-boundary cell rather than the standard 12-mm cell. This permitted loading of the cell through the cup opening with a wide-bore serological pipette, thus avoiding any shear degradation from the use of a needle and syringe. Values for the sedimentation coefficients were corrected for temperature and are reported as $S_{20,w}$.

The thermal denaturation temperatures (T_m) of the phage DNA in SSC buffer were determined by use of a heated cell compartment attached to a Gilford spectrophotometer and equipped with a thermister probe and direct temperature readout apparatus. Base compositions were determined from the T_m by the relationship shown by Marmur and Doty (24).

Preparation of pulse-labeled RNA preparations. Phage-infected cells were pulse-labeled with ^{3}H-uridine or $^{32}PO_4^{-3}$ to detect the presence of host-specific and phage-specific RNA. For pulse-labeling of infected log-phase cells, cells were grown in SCM medium to a density of 2.5×10^8 cells/ml; the cells were collected by centrifugation and resuspended in one-tenth the original volume of SCM medium minus glucose. A MOI of 5 was added to the suspension, and 5 min was allowed for phage adsorption. The suspension was then diluted to its original volume with SCM medium plus glucose, and the culture was pulse-labeled at various 5-min intervals with ^{3}H-uridine (5 μc/ml, 18.2 μc/μg) after suspension in the glucose-containing medium. Cells infected in the sporulation phase were pulse-labeled in a similar manner with the exception that the cells were resuspended in the "spent" medium instead of fresh medium after centrifugation. Sporulation-infected cultures were pulse-labeled with ^{3}H-uridine (20 μc/ml, 18.2 μc/μg).

The labeled cells were chilled quickly by pouring the culture over frozen 0.01 M Tris (*p*H 7.2)–5×10^{-3} M $MgCl_2$ (5×10^{-3} TM) buffer containing 0.01 M NaN_3 and 15% sucrose. The cells were harvested, and the RNA was extracted by grinding the frozen cell pellets in a prechilled mortar (−20 C). RNA was purified by the method of Gierer and Schramm (11).

Phage-infected cells were pulse-labeled with $^{32}PO_4^{-3}$ to determine the base composition of phage-specific RNA. Cells were grown in SCM medium to a density of 2.5×10^8 cells/ml, collected by centrifugation, and resuspended in one-tenth the original volume minus glucose. A MOI of 5 was added to the suspension, and 5 min was allowed for phage adsorption. The suspension was then diluted to its original volume with SCM plus glucose, and the culture was pulse-labeled for 5 min during the latter half of the latent period with $^{32}PO_4^{-3}$ (20 μc/ml). These cells were treated as described above, and the RNA was purified by the same method.

Isolation and base composition of phage-specific messenger RNA. Phage-specific mRNA-^{32}P was isolated by the formation of DNA-RNA hybrids by means of the membrane-filter technique of Nygaard and Hall (26). Annealing mixtures were incubated at 46 C for 16 to 18 hr. The mixtures were then diluted to 0.25 M KCl and treated with pancreatic ribonuclease (20 μg/ml) at room temperature for 1 hr before filtration. Base ratio analyses were performed as described by Hayashi and Spiegelman (16). Samples were counted in a Packard Tri-Carb liquid scintillation spectrometer.

DNA-RNA hybrid assay. Hybrid formation was detected by the membrane-filter technique of Nygaard and Hall (26) as modified by Gillespie and Spiegelman (12). Annealing mixtures were incubated at 66 C in a sixfold concentration of standard saline citrate (6 × SSC) buffer (23) for 24 hr. [Standard saline citrate (1 × SSC) buffer (*p*H 7.0) consists of 0.15 M NaCl and 0.015 M sodium citrate.] After washing, the membrane filters were treated with 20 μg/ml of pancreatic ribonuclease in 2 × SSC buffer at room temperature for 1 hr. Filters were dried at 110 C for 30 min and counted in a Packard Tri-Carb liquid scintillation spectrometer.

TABLE 1. *Properties of Bacillus subtilis phages β3 and β22*

Phage properties	Phage	
	β3	β22
Plaque size (mm)	0.5-1.0	0.5-1.0
Plaque morphology	Clear center, turbid edge	Clear center, sharp edge
Latent period (min)	35	50
Burst size	25	115
Host specificity	W23	W23, 168
β3 antiserum	+	−
β22 antiserum	−	+
SP10 antiserum	+	−
Heat stability[a] (65 C)	1.205 min^{-1}	0.191 min^{-1}
Storage	Labile, 4 C Stable, -20 C	Stable, 4C
UV spectrum (peak λ)	260 mμ	260 mμ

[a] Reported as inactivation constant, k, of first-order reaction, $ln\ N/N_0 = -kt$.

Labeling of DNA with ^3H-thymidine. Cultures infected in the sporulation phase of growth were labeled with ^3H-thymidine to detect the synthesis of phage DNA. Cells infected in the same manner as in the ^3H-uridine pulses were labeled with ^3H-thymidine or uridine-6-^3H (1 μc/ml, 0.2 μg/ml). Cells were exposed to ^3H-thymidine or uridine-6-^3H from 49 to 90 min after infection and were harvested as above. The pellets were suspended in 5×10^{-3} TM plus 15% sucrose, and the suspensions were made 1 mg/ml in lysozyme. The suspensions were quick-frozen and thawed twice in an acetone-dry ice bath, and were incubated at 37 C until spheroplasts appeared. The cells were disrupted with 2% SDS and the DNA was purified by the method of Marmur (23).

Detection of phage DNA synthesis in sporulation-infected cultures. The presence of phage DNA synthesis was detected by a method, devised by Denhardt (6), which is based on the DNA-RNA hybrid assay technique using nitrocellulose membranes. Labeled DNA solutions in 3 × SSC were exposed to the maximal output of a Biosonik ultrasonic disintegrator for a period of 30 sec. The DNA was then denatured by heating the solutions at 100 C for 15 min and quick cooling in an ice bath. Solutions of denatured DNA (1 × DNA) were incubated at 65 C for 12 hr with membranes containing 100 μg of host or phage DNA. The hybridized DNA was assayed, after washing each side of the membrane with 40 ml of SSC buffer, by counting the dried filters in a liquid scintillation counter.

Materials. The reagents were obtained from the following sources: New England Nuclear Corp., Boston, Mass. [uridine-5-^3H (18.2 μc/μg), uridine-6-^3H (38.1 μc/μg), and ^3H-thymidine (27.8 μc/μg)], Worthington Biochemical Corp., Freehold, N.J. (pancreatic ribonuclease, pancreatic deoxyribonuclease, and lysozyme), and Aldrich Chemical Co., Inc., Milwaukee, Wis. (DPA).

RESULTS

Properties of β3 and β22 phages. From 70 independent phage isolates, β3 and β22 were selected, since they had biological and chemical properties suitable for our studies. Some of the characteristics of phages β3 and β22 are shown in Table 1. Phage β3 formed plaques on strain W23, but not on 168, whereas β22 was able to form plaques with equal efficiency on both strains. Plaque morphology varied, depending on the age and dryness of the assay plates. New plates containing the 2.0% agar base layer were incubated for 24 hr at 37 C before use for phage assays. When tested with phage-specific antisera, β3 and β22 were found to be antigenically distinct. Phage β3 was inactivated at a rate similar to that of SP10 with SP10 antiserum. When thermal inactivation of the phages was carried out in the modified SCM medium at 65 C, the β3 phage was much more sensitive to heat inactivation, and this property may be correlated to its instability upon storage. Phage β3 is labile when stored at 4 C in 0.1 M TM buffer containing 10% dimethyl sulfoxide (DMSO). However, the phage is quite stable when stored in the same medium at −20 C. (38). Phage β22 is stable at 4 C in the TM buffer containing 10% DMSO. Single-step growth experiments with the two phages revealed minimal latent periods of 35 min for β3 and 50 min for β22 (Fig. 2). These experiments showed an extended rise period of

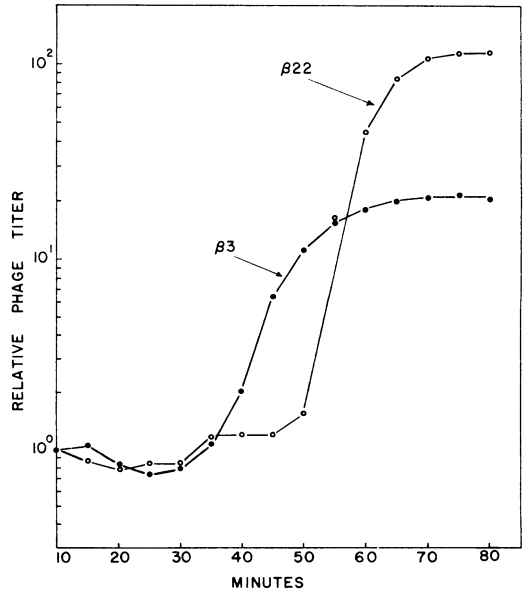

FIG. 2. *Single-step growth curves of β3 and β22 on Bacillus subtilis W23.*

TABLE 2. *Properties of the DNA of Bacillus subtilis phages β3 and β22*[a]

DNA properties	Phage	
	β3	β22
G + C (%)	42	36
ρ in CsCl	1.706 (47)	1.697 (38)
ρ in Cs_2SO_4	1.436	1.422
$S_{20,w}$	48	38
Molecular weight[b]	5.9×10^7	3.3×10^7
T_m in SSC buffer	80.5 (27)	85.5 (35)
Unidentified component	+	−
Ultraviolet maximum (mμ)	257	260

[a] Values in parentheses are base compositions, as per cent G + C, as calculated from the buoyant density and T_m by use of the formulas of Schildkraut et al. (33) and Marmur and Doty (24).

[b] Calculated from the $S_{20,w}$ by the equation of Eigner and Doty (9).

20 min, similar to that found with the RNA phages (19).

Table 2 contains some properties of the phage DNA preparations. The guanine plus cytosine (G + C) content listed was determined chemically. Chromatographic separation of formic acid hydrolysates of β3 and β22 DNA revealed the presence of only the four usual bases. The R_F values of the spots and the spectral characteristics of the eluted compounds compared with those of standard solutions of adenine, guanine, cytosine, and thymine. The density of the DNA of β3 in CsCl corresponds to a base composition of 46.9% G + C, whereas the T_m corresponds to 27% G + C. The density of β22 DNA in CsCl corresponds to 37.8% G + C and the T_m corresponds to 35% G + C, which is in good agreement with the chemical analysis. The density of β3 DNA in Cs_2SO_4 equal to 1.436 g/cc, differs greatly from that of the host, which equals 1.424 g/cc. The discrepancies between the values for the density and T_m of β3 DNA and those values predicted for DNA with a base composition of 42% G + C suggest that the DNA of β3 may be modified, e.g., by glycosylation or methylation. Both β3 and β22 DNA produced unimodal distributions when denatured by heat or alkali and banded in CsCl density gradients. The sedimentation coefficients ($S_{20,w}$) of β3 and β22 DNA preparations were 48 and 38, respectively. The molecular weights were calculated by use of the relationship of Eigner and Doty (9), $S_{20,w} = 0.034 M^{0.405}$.

The base composition of RNA hybridizing with the phage and host DNA preparations is presented in Table 3. The base ratios of the mRNA are similar to those of the respective DNA preparations.

Biology of the infection process of β3 and B22 in sporulating cells. After characterization of the phages and their DNA, experiments were undertaken to determine the degree of sporulation despite infection with β3 and β22. Sporulating cultures infected with β3 showed a 2.5-fold increase in PFU between 0.5 and 5 hr after infection, whereas cultures infected with β22 showed a 12-fold increase. The latent periods for both phages were extended to 90 to 120 min. The extent of sporulation of cells infected by each of the two phages is compared in Table 4. The normal percentage of sporulation of uninfected cultures in this medium was approximately 85%. If cultures infected with β3 were allowed to sporulate, it was found that the normal percentage of cells yielded mature spores. Of the resulting spores, 80% were infected and 20% were uninfected. The cultures infected with β22, on the other hand, sporulated very poorly. Sporulating cultures supported the multiplication of β22, and essentially all of the cells lysed. Of the small fraction (0.01%) which sporulated, 83% were uninfected and 17% were infected.

TABLE 3. *Base compositions of messenger RNA for β3, β22, and Bacillus subtilis W23*

Organism	Moles per cent[a]				Per cent G + C	$\frac{A+G}{U+C}$	A/U	G/C
	Cytidylate (C)	Adenylate (A)	Uridylate (U)	Guanylate (G)				
β3	17.6	33.2	26.4	22.8	40.4	1.27	1.26	1.30
β22	16.5	29.1	31.8	22.6	39.1	1.07	0.92	1.37
B. subtilis	21.2	30.2	28.1	20.5	41.7	1.03	1.08	0.97

[a] This is an average of four analyses for each type of RNA. The hybridization mixtures included 50 μg of heat-denatured DNA and 5×10^5 counts/min of pulse-labeled RNA-^{32}P. The hybrids were collected on filters, the RNA preparations hydrolyzed with alkali, and the base compositions were determined as described in Materials and Methods.

TABLE 4. *Biology of the infection of sporulating cells*[a]

Phage	Sporulating cells/ml	Total spores	Uninfected spores	Infected spores	Per cent sporulation
β3.....	8×10^8	6.9×10^8	1.4×10^8 (20)	5.5×10^8 (80)	86.00
β22.....	8×10^8	1.0×10^5	8.3×10^4 (83)	1.7×10^4 (17)	0.01

[a] Values in parentheses are percentages of total spores.

Attempts were made to distinguish a chemical or physical difference between the spores resulting from β3-infected cultures and uninfected spores. Samples of β3-infected and uninfected spores were assayed for DPA by the method of Janssen et al. (17). Calculations based on 2.2×10^9 *B. subtilis* spores per mg (dry weight) resulted in a value of 81.4 μg of DPA/mg (dry weight) for both the uninfected and infected spore preparation. This value is in close agreement with the value of 85 μg of DPA per mg (dry weight) obtained by Sadoff (31) for *B. cereus* spores. Examination of the spore suspensions by phase-contrast microscopy gave no evidence of any difference in outward appearance or refractility. Further examination of the heat sensitivity of infected spores revealed that approximately a 20% decrease in recovery of infective centers was produced when β3-infected spore suspensions, in addition to being treated with antiserum, were heated at 80 C for 10 min (Table 5). The heat-shock treatment had no effect on the recovery of uninfected spores from these suspensions nor on the recovery of viable spores from uninfected control cultures. From this limited study, there appear to be no differences in the gross structure and composition of β3-infected spores other than the fact that they contain phage genetic determinants. However, there is some decrease in the heat resistance of the host spore as a result of phage infection.

Analysis of mRNA synthesis after infection of log phase cells by β3 and β22. The difference in the infective processes of the two phages prompted an analysis of the transcription process after infection. A procedure was designed with which a quantitative estimation of the relative amounts of phage-specific and host-specific complementary RNA present in infected cells could be obtained. Preliminary studies were made to determine the optimal conditions for the formation of DNA-RNA hybrids for the assay system used. Hybrid assays were carried out with three different concentrations of SSC buffer and two different incubation temperatures, as shown in Table 6. The optimal condition for hybrid formation was incubation in 6 × SSC buffer at 66 C. Next, the rate of hybrid formation was followed to determine the optimal time for incubation of hybridization mixtures. As shown in Fig. 3, the rate of formation of DNA-RNA complexes declined after 15 hr of incubation in 6 × SSC buffer at 66 C. Therefore, the condition used for the remainder of the study was incubation for 24 hr in 6 × SSC buffer at 66 C.

After it was learned from preliminary hybridization studies that both phage-specific and host-specific mRNA were being synthesized in phage-infected cells, it became important to have quantitative rather than qualitative estimations of each type of RNA present. The experimental method devised to ascertain the relative amounts of phage-specific and host-specific mRNA present

TABLE 5. *Effect of heat treatment on recovery of β3-infected spores*

Spore prepn	Assay	Treatment[a]	
		None	80 C for 10 min
Uninfected control	Viable count	6.9×10^8	6.6×10^8
β3-infected	Infective centers	6.1×10^8	4.9×10^8
	Viable	7.5×10^7	7.3×10^7

[a] Treatment in addition to incubation in the presence of phage-specific antiserum.

TABLE 6. *Optimal temperature and SSC buffer concentration for DNA-RNA hybrid formation*[a]

DNA	SSC concn	Hybridization temp	
		46 C	66 C
β3	2×	11.1	12.9
	4×	8.5	15.3
	6×	6.7	18.7
β22	2×	19.5	26.6
	4×	16.2	40.6
	6×	14.3	40.4

[a] Values are expressed as percentage of input cpm of pulse-labeled RNA-^{3}H forming ribonuclease-stable hybrids.

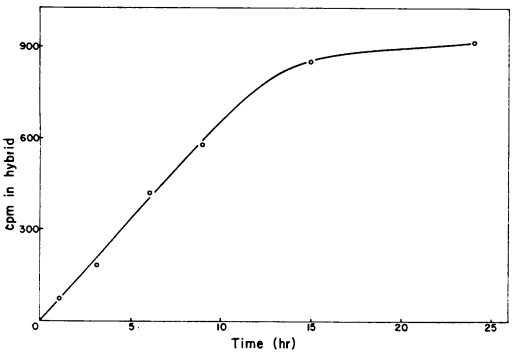

FIG. 3. *Kinetics of DNA-RNA hybrid formation. Hybridization solutions each containing 0.5 μg (5,600 counts per min per μg) of RNA extracted from pulse-labeled, uninfected cells were incubated for the indicated periods in the presence of filters containing 50 μg of Bacillus subtilis 1 × DNA.*

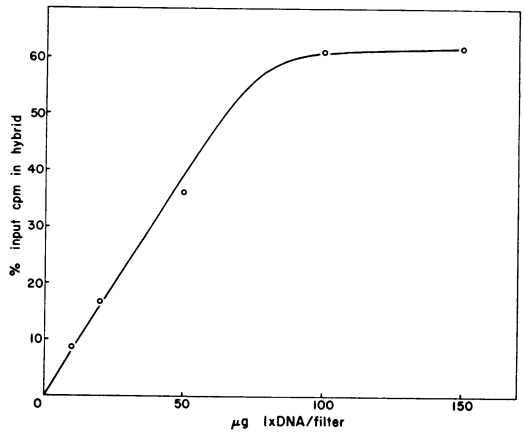

FIG. 4. *Titration of log uninfected RNA-^{3}H with Bacillus subtilis 1 × DNA. Hybridization solutions each containing 0.5 μg (5,600 counts per min per μg) of RNA extracted from logarithmically growing, pulse-labeled, uninfected cells were incubated in the presence of filters containing increasing amounts of B. subtilis 1 × DNA. The pulse labeling was for 4 min. Blank filters served as controls. Hybrid formation using filters containing β3 and β22 1 × DNA preparations (100 μg/filter) was less than 0.1 and 1.1% of the input counts per minute, respectively (Table 4).*

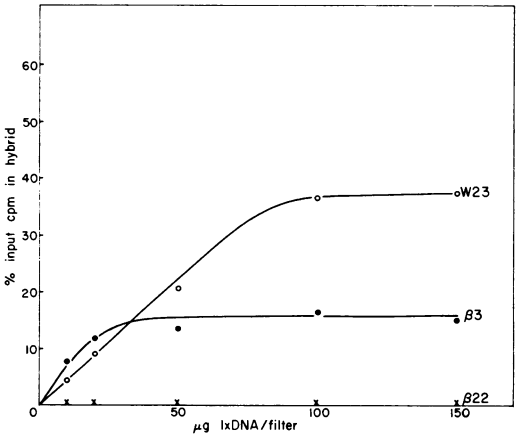

FIG. 5. *Titration of log β3-infected RNA-^{3}H with Bacillus subtilis, β3, and β22 1 × DNA preparations. Hybridization solutions each containing 0.9 μg (5,780 counts per min per μg) of RNA extracted from logarithmically growing, pulse-labeled, β3-infected cells were incubated in the presence of filters containing increasing amounts of B. subtilis, β3, or β22 1 × DNA preparations, respectively. The pulse labeling occurred between 20 and 25 min after infection. Blank filters served as controls.*

was to incubate fractions of a minimal amount of pulse-labeled RNA with a series of filters, each containing increasing amounts of the respective 1 × DNA. The rationale behind this procedure was that the amount of RNA hybridizing with the DNA would remain constant beyond a certain level of DNA per filter. At a point where there was an excess of complementary DNA "sites" for each RNA species present, all of the RNA capable of hybridization with a given DNA preparation would become complexed. The next six figures represent the results of such "titration" experiments with the various pulse-labeled RNA preparations obtained from infected and uninfected cultures.

Figure 4 shows the formation of DNA-RNA hybrids between pulse-labeled RNA extracted from uninfected logarithmically growing cells and increasing amounts of *B. subtilis* 1 × DNA; 62% of the input counts were maximally incorporated into the hybrids. Controls were used to detect any cross-hybridization between the host-cell RNA and the phage DNA preparations. Less than 0.1% of the input host-cell RNA counts complexed with β3 1 × DNA (100 μg/filter) and 1.1% complexed with β22 1 × DNA (100 μg/filter).

Figure 5 is a set of titration curves of pulse-labeled RNA extracted from β3-infected, logarithmically growing cells, and increasing amounts of *B. subtilis*, β3, or β22 1 × DNA preparations, respectively. The level of host-specific RNA dropped to about two-thirds of the level observed in uninfected cells. Phage β3-specific RNA accounted for about one-third of the total counts hybridized, and the amount of cross-hybridization with β22 DNA was negligible.

A similar set of curves showing the hybridization of pulse-labeled RNA extracted from β22-

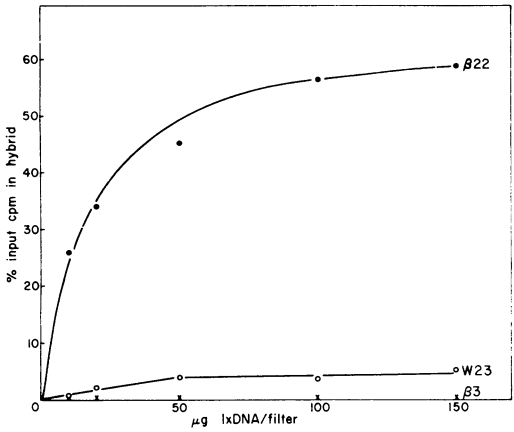

FIG. 6. *Titration of log β22-infected RNA-³H with Bacillus subtilis, β3, and β22 1 × DNA preparations. Hybridization solutions each containing 0.6 μg (2,750 counts per min per μg) of RNA extracted from logarithmically growing, pulse-labeled, β22-infected cells were incubated in the presence of filters containing increasing amounts of B. subtilis, β3, or β22 1 × DNA, respectively. The pulse labeling occurred 31 to 36 min after infection. Blank filters served as controls.*

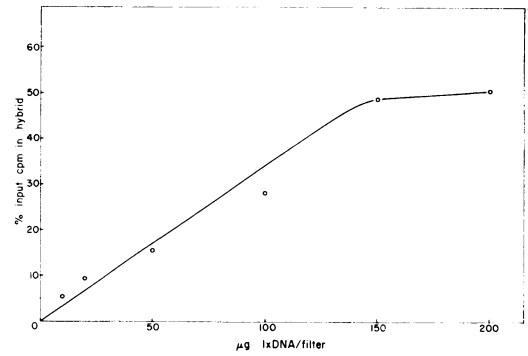

FIG. 7. *Titration of sporulation uninfected RNA-³H with Bacillus subtilis 1 × DNA. Hybridization solutions each containing 0.8 μg (1,090 counts per min per μg) of RNA extracted from pulse-labeled, sporulation-phase cultures were incubated in the presence of filters containing increasing amounts of B. subtilis 1 × DNA. The pulse labeling was for 5 min. Blank filters served as controls. Hybrid formation using filters containing β3 and β22 1 × DNA (100 μg/filter) was less than 0.1 and 1.4% of the input counts, respectively (Table 4).*

infected, logarithmically growing cells and increasing amounts of *B. subtilis*, β3, and β22 1 × DNA preparations, respectively, is presented in Fig. 6. The predominant species of RNA, about 59% of the input counts, are complementary to the DNA of β22. In this case, the maximal amount of RNA hybridizing with host DNA decreased to less than 5% of the input counts as compared to 62% hybridization with the uninfected cell RNA. No cross-hybridization was observed with β3 DNA.

Analysis of mRNA synthesis after infection of sporulating cells by β3 and β22. When a similar series of experiments were performed with pulse-labeled RNA isolated from uninfected cultures and cultures infected during the sporulation phase of the growth cycle, an interesting comparison could be made with the exponential-phase experiments just described. A titration curve for DNA-RNA hybrid formation between pulse-labeled RNA extracted from uninfected sporulation-phase cells and increasing amounts of *B. subtilis* 1 × DNA is shown in Fig. 7. Maximally, 51% of the input counts were incorporated into the hybrids. Controls set up to detect any hybridization between heterologous RNA and DNA species showed that less than 0.1% of the input counts complexed with β3 1 × DNA (100 μg/filter) and 1.4% complexed with β22 1 × DNA (100 μg/filter).

Figure 8 is a set of similar hybridization curves of pulse-labeled RNA extracted from β3-infected,

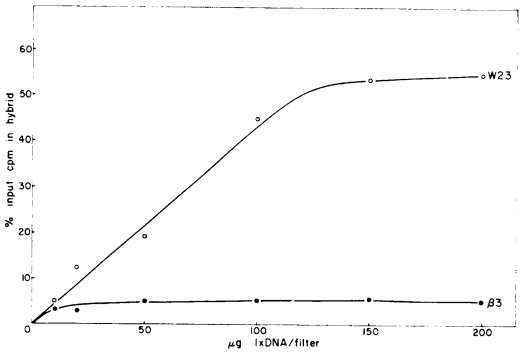

FIG. 8. *Titration of sporulation β3-infected RNA-³H with Bacillus subtilis and β3 1 × DNA. Hybridization solutions each containing 1.1 μg (3,680 counts per min per μg) of RNA extracted from pulse-labeled, β3-infected, sporulation-phase cultures were incubated in the presence of filters containing increasing amount of B subtilis or β3 1 × DNA, respectively. The pulse labeling occurred between 50 and 55 min after infection. Blank filters served as controls.*

sporulation-phase cultures and increasing amounts of *B. subtilis* or β3 1 × DNA preparations, respectively. The curve representing the titration of host-specific RNA was identical to that of the uninfected control curve (cf. Fig. 7). This indicates that no alteration of the host transcription process has occurred as a result of the infection by β3. The β3-specific RNA accounted for about one-tenth of the total counts hybridized, which, when compared with the curves for log β3-infected RNA, suggests that

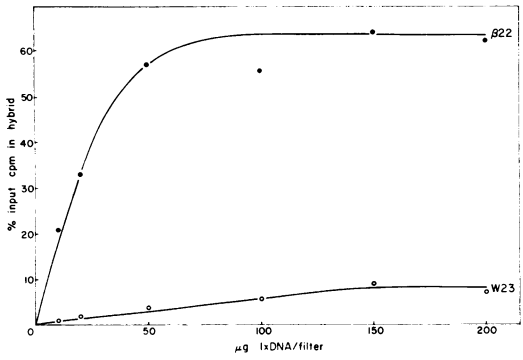

FIG. 9. *Titration of sporulation β22-infected RNA-³H with Bacillus subtilis and β22 1 × DNA. Hybridization solutions each containing 2.7 μg (6,880 counts per min per μg) of RNA extracted from pulse-labeled, β22-infected, sporulation-phase cultures were incubated in the presence of filters containing increasing amounts of B. subtilis or β22 1 × DNA, respectively. The pulse labeling occurred between 50 and 55 min after infection. Blank filters served as controls.*

the synthesis of β3-specific mRNA is being inhibited.

Finally, the hybridization of pulse-labeled RNA isolated from β22-infected sporulation-phase cultures with increasing amounts of *B. subtilis* or β22 1 × DNA preparations, respectively, is represented by the results in Fig. 9 Again, the predominant species of RNA, about 63% of the input counts, are complementary to the β22 DNA. Also, the amount of RNA which hybridized maximally with the host DNA was greatly decreased as compared with the maximal hybridization of the uninfected culture RNA (cf. Fig. 7). These results suggest that very little, if any, host mRNA is synthesized in cells infected with β22.

Table 7 summarizes the results of Fig. 4 through 9 by tabulating the maximal percentages of input counts of pulse-labeled RNA hybridizing with the given DNA species.

Analysis of DNA synthesis after infection of sporulating cells by β3 and β22. Sporulating cells infected with the two phages were labeled with ³H-thymidine or uridine-*6-³H* to detect any synthesis of phage DNA. Attempts were made to distinguish the host DNA from phage DNA on the basis of their differences in buoyant density. However, experiments involving CsCl and Cs_2SO_4 density gradient centrifugation in the SW39 rotor in a Spinco L preparative ultracentrifuge were fruitless. Fortunately, a timely article appeared describing a method for the detection of complementary DNA (6). Results of experiments using this method as described in Materials and Methods are presented in Table 8.

TABLE 7. *Maximal percentage of input counts hybridizing to DNA*

Growth phase of cells	Source of DNA on Filter	Source of RNA-³H		
		Uninfected control	β3-infected	β22-infected
Log	*Bacillus subtilis*	% 61.8	% 37.6	% 4.6
	β3	<0.1	16.0	0
	β22	1.1	<0.7	58.8
Sporulation	*B. subtilis*	50.6	54.4	7.1
	β3	<0.1	5.5	—
	β22	1.4	—	63.0

TABLE 8. *Percentage of counts renaturing with host or phage DNA*[a]

DNA on filter	Source of DNA-³H	
	Sporulating β3-infected cells	Sporulating β22-infected cells
Bacillus subtilis	0.4	0.8
β3	15.0	—
β22	—	19.2

[a] Values presented as net average percentage of input counts per minute. Blank filters used as controls.

In both cases, the labeled DNA preparation isolated from infected cells was complementary to the DNA of the phage which had been used to infect the culture. These results led to the conclusion that phage DNA is synthesized in cultures infected in the sporulation phase of growth with either β3 or β22.

DISCUSSION

Of the many phages isolated, β3 and β22 were selected for further study because they were the most stable and most virulent phages isolated. Of the two, β22 is more stable under a variety of conditions than β3. The unstable nature of the *Bacillus* phages has been reported in several investigations (4, 30). These phages are unstable when stored in the culture medium and are sensitive to osmotic shock and chloroform treatment. The best method we have found for maintaining the stability of the phages has been storage in 10% DMSO (38).

The isolation procedure was designed to select for phages which were contained in bacterial spores and had, in this manner, obtained the heat resistance of the spore. The presence of antiserum during the isolation process was very

useful for eliminating phages serologically similar to those previously isolated.

A single-step growth curve with an extended rise period is characteristic of a number of *Bacillus* phages. Similar curves have been obtained for the transducing phage SP10 (3), phage SP82 (14), and the group 1 phages (4) for *B. subtilis*. Saunders and Campbell (32) have also shown similar results with phage TP-84 for *B. stearothermophilus*.

Hydrolysis of the DNA of $\beta 3$ or $\beta 22$ and separation of the resulting bases indicated that the DNA preparations did not contain an unusual purine or pyrimidine. Ultraviolet spectra of these bases were identical to those of the four common bases. The molar ratios of guanine and cytosine, as well as adenine and thymine, were equivalent. None of the physical characteristics examined suggested that the DNA of $\beta 22$ was altered in any way. However, the discrepancies between the densities and thermal denaturation temperatures of $\beta 3$ and host DNA preparations, which have identical base compositions, led to the conclusion that the DNA of $\beta 3$ contains some component other than the four common deoxyribonucleotides. Similar observations have been made for SP10 DNA (3, 28), thus providing additional evidence of the chemical relationship between the two phages. Although only the four usual bases were found in $\beta 3$ DNA, it is possible that the DNA contains some base which is converted to one of the common bases during the hydrolysis procedure. Enzymatic digestion of the DNA and separation of the resulting nucleotides might indicate the presence of such a base. Several phages have been found in which thymine has been replaced by another pyrimidine in the phage DNA (14, 18, 29, 35).

Bacteriophages $\beta 3$ and $\beta 22$ can be distinguished from each other on the basis of several properties of the phages and their DNA. The phages themselves differed in plaque morphology, one-step growth characteristics, host specificity, serum neutralization, thermal stability, and stability upon storage (Table 1; Fig. 2). The phage DNA preparations were distinguished on the basis of base composition, densities in CsCl and Cs_2SO_4, sedimentation coefficient, molecular weight, and thermal denaturation temperature (Table 2). DNA-RNA hybridization experiments disclosed, by the lack of cross-hybridization between heterologous phage DNA and RNA, that the nucleotide sequences on the phage chromosomes were not homologous.

Examination of the biology of the infection processes in sporulating cultures of *B. subtilis* revealed two similarities between that of $\beta 3$ and $\beta 22$. The increases in PFU during sporulation infection were decreased to one-tenth of the burst sizes determined in one-step growth experiments of log-infected cultures. Also, the latent period of each phage was two to four times the latent period observed in log-infected cultures. Reasons for these observations have not been elucidated. It can only be postulated that shifts in cellular metabolism during sporulation have an adverse effect on the synthesis of phage components. Exhaustion of the glucose and available nitrogen source which is accompanied by the induction of sporogenesis (15) appears to give rise to conditions less favorable to the production of phage particles.

On the other hand, the end results of infection of sporulating cells by $\beta 3$ and $\beta 22$ were extremely different. The results (Table 4) show that cultures infected with $\beta 3$ in the sporulating phase of growth are capable of sporulating and incorporating the phage genome into the mature spore. Sporulation and phage development occurred concomitantly with no apparent interference in sporulation by the infection process. These results are similar to the results obtained with SP10 infection of *B. subtilis* (3). Cultures infected with $\beta 3$ yielded the same percentage of spores as the uninfected cultures. In direct contrast to the $\beta 3$ situation, infection of sporulating cultures with $\beta 22$ resulted in lysis of most of the cells. The small fraction of those surviving (Table 4) consisted mainly of uninfected spores. The small portion of surviving spores containing phage determinants was possibly due to the direct inclusion of the phage genome before it had a chance to express itself.

Assay of the $\beta 3$-infected sporulating cultures for survivors soon after infection revealed that almost 100% of the cells were infected and contained phage genetic material. Less than 2% survivors were found in each case. Yet 20% of the spores resulting from such cultures were uninfected (Table 4). This suggested that several situations occurred in these cultures. A small fraction (less than 2%) of the cells were uninfected. Of the infected cells, three types are postulated. Phage genomes may be present only in the developing spore but not the sporangium (type 1) or in both the developing spore and the sporangium (type 2). These cell types (ca. 80%) will sporulate to yield all phage-infected spores. Other cells (ca. 20%) may contain phage determinants only in the sporangium and none in the developing spore (type 3). These cells will not be counted as survivors in the ordinary test for survivors because plating them on fresh medium would cause them to be lysed by the phage when the sporulation process was reversed. Therefore, less than 2% survivors are observed.

However, if the cells are allowed to sporulate, the spores resulting from this situation will be uninfected. The apparent discrepancy between the number of survivors (less than 2%) and the number of uninfected spores (20%) can thus be resolved by this reasoning.

Attempts to find a difference between β3-infected and uninfected spores yielded one distinction. Although there was no difference in DPA content or phase brightness, there was some increase in the heat sensitivity of the infected spores (Table 5). A similar decrease in heat resistance was observed by Goldberg and Gollakota (13) with phage-infected *B. cereus*. This effect could be due to the sensitivity of either the host or phage genomes since infected spores were enumerated from plaques formed by plating samples for infective centers. Production of a plaque from an infected spore requires both the germination and outgrowth of the spore, and multiplication of the virus particle followed by lysis of the infected bacterium. Heat inactivation of the phage without a similar inactivation of the spore would result in an increase in the uninfected cell count of an infected spore suspension. Since there was no increase in the uninfected spore count following heat shock, we concluded that the decrease in infected spore count was due to heat inactivation of the host. The phage determinant could be inactivated as well, but would not be expressed in any case unless the host remained viable.

Armstrong and Boezi (2), using the liquid annealing procedure of Nygaard and Hall (26, 27), reported that only 25% of the pulse-labled RNA isolated from *E. coli* was capable of forming a ribonuclease-stable complex. Using the Gillespie-Spiegelman modification (12), we obtained from 50 to 70% of the acid-precipitable counts in the form of the ribonuclease-resistant hybrids (Table 7). Therefore, this method was the best for detecting quantitative changes in the amount of RNA hybridizable with a given DNA species as a measure of repression of host or phage genetic expression after infection. Assuming equal rates of transcription, a decrease in the level of hybrid formation would indicate that repression of synthesis of that RNA species had occurred. Optimal conditions for hybrid formation were obtained (Table 6). Under these conditions, maximal levels of RNA complexed at DNA to RNA ratios of 200:1 to 400:1 (Fig. 4–9). Similar observations were made by Pigott and Midgley (Biochem. J. **101**:9P, 1966), who reported a maximum of 80% hybridization between *E. coli* pulse-labeled RNA and 1 × DNA using the Gillespie-Spiegelman technique.

Hybridization assays of RNA extracted from logarithmically growing β3-infected cultures (Table 7) indicate that both synthesis of phage-specific and of host-specific mRNA occurred concomitantly in infected cells. A problem arises when mRNA complementary to two heterologous genomes occurs in the same preparation. All of the mRNA present in uninfected cells is specific for the host chromosome, whereas both host-specific and phage-specific mRNA occur in infected cells. This must be considered when comparing the amount of hybrid formed between host DNA and RNA from infected and uninfected cells. Taking this into account, there appears to be little or no decrease in the level of host-specific mRNA as a result of infection by β3, but a substantial decrease occurs after β22 infection.

The analyses of mRNA from log-infected and sporulation-infected cultures have provided the distinguishing characteristics of the infection processes of the two phage systems. When the hybridization of β3 DNA with log-infected and sporulation-infected mRNA is compared, it is evident that the expression of the phage genome is being repressed in sporulating cultures. It seems that infection by β3 does not result in the inhibition of host mRNA synthesis in either the log or sporulation phases of the growth cycle. Although the percentage of hybridizable host mRNA has remained at the same level as that of the uninfected sporulation-phase control, the level of phage-specific mRNA has decreased several-fold from that found in log-infected cultures. The remaining amount of β3-specific mRNA present in sporulation-infected cultures can be explained in terms of the heterogeneity of the culture with respect to the growth stage of individual cells and the percentage of sporulation. This will be discussed further. Alternatively, it is evident that the synthesis of host-specific mRNA is being repressed in cells infected with β22 (Table 7). The situation is nearly identical in log-infected and sporulation-infected cultures. These results suggest that the difference in the infection process of these phages is a function of their relative ability to repress the host genome.

The results have shown that phage DNA synthesis occurs in both β3-infected and β22-infected sporulating cells at a time when essentially no bacterial DNA synthesis is taking place (Table 8). These results are only qualitative, and no estimation of the relative amounts of DNA synthesis can be made. The failure of the host and phage DNA preparations to separate in the density gradients is due possibly to aggregation of the DNA species as a result of the relatively high DNA concentrations required to determine the distribution of the unlabeled

marker DNA by measurements of optical density at 260 mμ. The DNA bands also adhere to the sides of the nitrocellulose tubes used in the swinging bucket rotor, causing the DNA species to mix when the fractions are collected. Another reason is that the newly synthesized β3 DNA may not have been modified as yet to band at a greater density. Since its base composition is identical to that of the host DNA, it would band in the same position in the gradient as the host DNA.

The characteristics of a sporulating culture of *B. subtilis* present several problems which complicate the present type of analysis. First, the failure of the culture to sporulate synchronously introduces a morphological and physiological heterogeneity into the culture. In a population of sporulating cells, there are cells at several stages of spore development. It is thus possible that a portion of the population may not have reached the stage at which the expression of the β3 genome was depressed. Secondly, only 85% of the cells in the sporulation phase of growth are capable of forming mature spores in the SCM medium used in these experiments. What is happening in the nonsporulating fraction of the culture infected with β3? Are these cells still capable of repressing the phage genome? There is no way of determining whether β3 phage mRNA and DNA synthesis takes place in a small fraction of the culture or in the total cell population. In fact, it is possible that the small amount of β3 mRNA and DNA synthesized during the sporulation phase takes place in the nonsporulating fraction of the population.

Finally, during the early stages of sporogenesis, the cell becomes compartmentalized (15). The forespore septum divides the cell into two compartments. one containing the developing "forespore," and the other the larger portion of the cell, the "sporangium." Components specific for the spore are synthesized in the forespore while the sporangium continues to produce vegetative-cell materials (15). Is it possible for phage to develop vegetatively within the sporangium yet be repressed in the forespore? If the metabolism of the forespore is different from that of the sporangium, there could be some substance synthesized within the forespore which is capable of inhibiting phage multiplication. These points would have to be considered in future analyses. However, the present results still indicate a significant difference between the log-infected and sporulation-infected cultures.

In conclusion, the results suggest that the differential expression of the phage genomes is due to the relative ability of the phages to repress the host genome. Phage β22 is capable of repressing host mRNA synthesis in both logarithmically growing and sporulating cells of *B. subtilis*. The ability of the infection process of β22 to interrupt host metabolism results, during the sporulation phase, in phage maturation and lysis of the host cell before necessary spore constituents can be synthesized to form a resistant organelle. On the other hand, β3 appears to be incapable of repressing host mRNA synthesis. Instead, phage mRNA synthesis seems to be sufficiently repressed in sporulating cells to allow the host to synthesize and organize the various spore components into a mature spore.

ACKNOWLEDGMENTS

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