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Temperate *Bacillus subtilis* Bacteriophage ϕ3T: Chromosomal Attachment Site and Comparison with Temperate Bacteriophages ϕ105 and SPO2

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The temperate *Bacillus subtilis* bacteriophage ϕ3T contains within its genome a locus, designated *thyP3*, that encodes for a protein with thymidylate synthetase activity. Bacteriophage ϕ3T is different from the two previously characterized temperate phages, ϕ105 and SPO2, in: heteroimmunity, response to bacteriophage antisera, endonuclease digestion pattern, induction in the presence of 6-(*p*-hydroxyphenylazo)-uracil, and effect on the lytic cycle of bacteriophage ϕ1. The mean burst size of ϕ3T is 56. The dose response curve with bacteriophage ϕ3T DNA is linear for transfection and transformation to the Thy$^+$ phenotype. The inserted prophage has been mapped by PBS1 transduction; it is between chromosomal markers *ilvA8* and *gltA* in the terminus of the chromosome. Thus *thyP3* maps at a site separate from, but between, the bacterial markers *thyA* and *thyB* when *thyP3* is in the prophage state.

Infection of *Bacillus subtilis* usually results in: lysis with virulent bacteriophage such as SPO1, ϕ1, or ϕe; lysogeny in the case of bacteriophages ϕ105 and SPO2; the occurrence of generalized tranduction as observed with bacteriophages PBS1, SP10, or SPP1; or an apparent low frequency of specialized transduction as recently reported for bacteriophage ϕ105 (for review see reference 9). An unusual genetic modification was observed with phage ϕ3T, isolated by R. G. Tucker from a soil sample in 1964 (Biochem. J. 92:58p-59p, 1964). He noted that lysogeny with phage ϕ3T enabled thymine auxotrophs to grow in the absence of exogenous thymine (18). This phage differs from the other temperate phages in guanine plus cytosine content (18), heteroimmunity of mixed infections (20), and number of recognition sequences for the restriction endonuclease *Eco*RI (20). This study is aimed at further characterization of phage ϕ3T, exploration of the thymine "conversion" event, and establishment of the position of the attachment site for phage ϕ3T and the site of integration of the gene encoding thymidylate synthetase activity (*thyP3*) carried by phage ϕ3T. (Because the gene encoding *thyP3* can be integrated into the chromosome in the absence of the entire bacteriophage genome, we have used the term "transformation" to connote the process by which *thyA*$^-$ *thyB*$^-$ recipient cells acquire the capacity to grow in the absence of thymine after incubation with ϕ3T bacteriophage DNA.) Since our original description of *thyP3* (M. T. Williams and G. A. Wilson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, p. 110, H84; 27), Dean et al. have characterized a bacteriophage, ρ11, that also can convert thymine auxotrophs to prototrophy upon lysogenization (D. H. Dean, J. C. Orrego, K. W. Hutchison, and H. O. Halvorson, personal communication).

The data presented in this communication demonstrate that integration of *thyP3* occurs with a high frequency at a site near but distinct from the chromosomal genes that regulate thymine biosynthesis, *thyA* and *thyB*.

MATERIALS AND METHODS

Bacteria and bacteriophage. Phage ϕ3 was obtained from LuBelle Boice. To distinguish between the phage isolated by Tucker and a different phage designated ϕ3 by B. E. Reilly (Ph.D. thesis, Western Reserve University, Cleveland, Ohio, 1965), we designated Tucker's wild-type phage ϕ3T. The propagation and assay of phages ϕ1, ϕ105, and SPO2 were performed as described previously (23). Phage PBS1 was used in all transduction experiments (27). Our standard strain of *B. subtilis* 168 (BR 151) and its derivatives shown in Table 1 were used to assay these viruses. RUB 1617 is a ϕ3T lysogen that has nonglucosylated teichoic acid in the cell wall. The absence of glucose in teichoic acid results in a failure of adsorption of many of the viruses that infect *B. subtilis* (25). Therefore this deficiency prevents reattachment of ϕ3T to wall fragments after induction.

TABLE 1. *Strains used*

Strain	Genotype	Source and comments
RUB 830	*trpC2 pheA thyA thyB*	R. E. Yasbin
RUB 830 (φ3T)	*trpC2 pheA thyA thyB* (φ3T)	φ3T lysogen of RUB 830[a]
RUB 1617	*gtaB* (φ3T)	See Materials and Methods
CU 405	*ilvA8 thyA*	S. Zahler
CU 438	*ilvA8 ilvD15 thyA thyB*	S. Zahler
CU 806	*ilvA8 thyB gltA1 citB*	S. Zahler
CU 809	*ilvA8 thyA thyB gltA1*	S. Zahler
BR 151	*trpC2 metB10 lys-3*	B. Reilly
BR 151 (φ3T)	*trpC2 metB10 lys-3* (φ3T)	φ3T lysogen
BR 151 (φ105)	*trpC2 metB10 lys-3* (φ105)	φ105 lysogen
BR 151 (SPO2)	*trpC2 metB10 lys-3* (SPO2)	SPO2 lysogen

[a] RUB 830 (φ3T) is phenotypically Thy^+ due to the presence of the functional *thyP3* locus.

Media and chemicals. M medium, Penassay broth (antibiotic medium no. 3; Difco), and tryptose blood agar base (Difco) were used as the basic media for propagation of bacteria and viruses (24). 6-(*p*-hydroxy-phenylazo)-uracil (HPUra) was obtained from B. W. Langley.

Propagation of phage φ3T. High titers of phage φ3T were obtained by growing strain RUB 1617 in M medium to a density of 50 Klett units (Klett-Summerson colorimeter, filter no. 66) and inducing with mitomycin C (final concentration, 0.5 μg/ml). To form lysogens by infection with φ3T, strains were grown to a density of 10^8 CFU/ml in Penassay broth, and φ3T was added at a multiplicity of infection (MOI) of 1. The plaque sizes of the infectious centers obtained from transfection were very small compared with those resulting from infection. They were best distinguished on tryptose blood agar base plates in an overlay containing the desired bacterial indicator, 2 ml of M semisolid agar, and 1 ml of tryptose broth (Difco). Infection with wild-type φ3T yielded turbid plaques the same size as SPO2 but with a very rough turbid zone.

Antisera. Antisera against φ3T and SPO2 were prepared by injecting rabbits with approximately 10^{11} PFU once a week for 3 weeks. After a 2-week period, the rabbits were given a fourth injection and bled 1 week later.

Single-step burst experiment. BR 151 (φ3T) was grown at 37°C in a shaking air bath to an optical density of 50 Klett units in M medium in the presence of φ3T antiserum. The cells were pelleted and washed twice in M medium and then resuspended in M broth, and mitomycin C was added. PFU were assayed at various times after induction (3). No more than 100 phage could be liberated by a single cell (Fig. 1). To confirm this result, an analysis of the burst size was performed as described by Adams (1).

Genetic analysis. The transformation and transfection procedures used in this study were described previously (4, 27). Standard methods for propagation of PBS1 transducing lysates and the transduction procedure were used (26). The donor lysates carried the *thyP3* and φ3T (except where noted in the text). Crosses were performed in the presence of φ3T antiserum to preclude infection. The position of the *att*φ3T locus was mapped by streaking transductants for the selected marker on a susceptible lawn in M agar and scoring the clones that gave rise to areas of lysis. Confirmation of the presence of the transduced prophage was made by streaking recipients onto a lawn of a clear-plaque mutant of φ3T and scoring the surviving colonies.

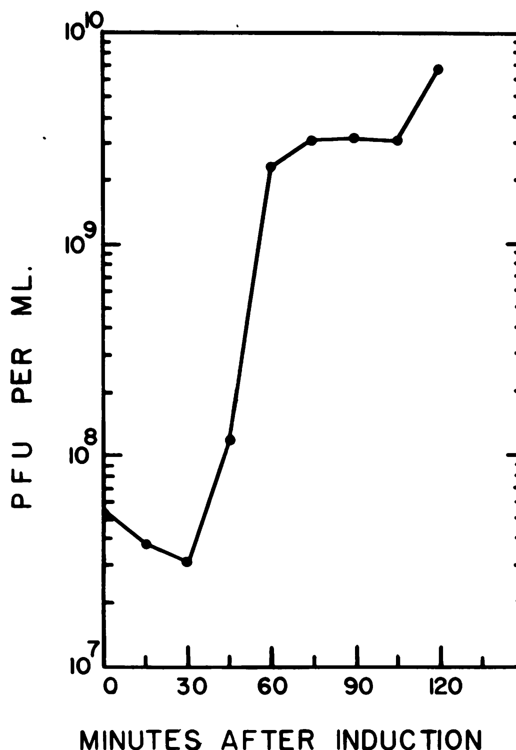

FIG. 1. *Burst size of bacteriophage φ3T. BR 151 carrying φ3T was grown at 37°C at 250 rpm to an optical density of 50 Klett units in M broth in the presence of phage φ3T antiserum. The cells were pelleted, washed twice, and resuspended in M broth, and mitomycin C (final concentration 50 μg/ml) was added. PFU were assayed at various times after induction.*

RESULTS

Comparison of phage ϕ3T to other temperate phages. (i) Burst size. After the addition of mitomycin C, there was approximately a 30-min lag before the onset of viral production. The average burst size did not exceed 100 viruses per lysogenic cell (Fig. 1) After induction of a ϕ3T lysogen with mitomycin C, serial dilutions were made as described by Adams (1). In a representative experiment consisting of an assay of 50 dilution tubes, 38 had no PFU and the remaining tubes had 21, 21, 21, 32, 34, 34, 83, 84, 85, 92, 132, 150 PFU, respectively, for a total of 789 PFU. The Poisson formula is: $P(r) = n^r e^{-n}/r!$, in which $P(r)$ is the proportion of plates containing r-induced lysogenic bacteria when the average number of induced lysogens per plate is n. Therefore:

$P(0) = 38/50$; thus $n = 0.274$ induced lysogens per plate
$P(1) = 10/50$ plates with one induced lysogen
$P(2) = 2/50$ plates with two induced lysogens

We must then have an average of 789 total PFU per 14 induced lysogens or 56 phage liberated per lysogen.

(ii) Phage neutralization. Because phages SPO2 and ϕ105 share antigenic components, it was necessary to determine whether there was cross-reaction among the structural components of phage ϕ3T and other characterized temperate viruses. Phage SPO2 antiserum neutralized phage ϕ105 but not ϕ3T, and phage ϕ3T antiserum did not neutralize either phage SPO2 or ϕ105 (Table 2).

TABLE 2. *Neutralization by specific phage antisera*

Antiserum	K value against:		
	ϕ3T	ϕ105	SPO2
ϕ3T	250	0	0
SPO2	0	250	250

(iii) Inhibition with HPUra. The inhibition of the host DNA polymerase III by HPUra led to marked reduction of virus production with phage ϕ105 but not phage SPO2 (14). To determine the effect of HPUra on phage ϕ3T, lysogenic cultures were induced by mitomycin C in the presence and absence of 250 μg of HPUra per ml. Phage ϕ3T, like phage SPO2, was not inhibited by HPUra (Table 3).

(iv) Exclusion of viral replication. The presence of lysogenic phages has been shown to result in the exclusion of other viral infections (9). The most carefully studied interactions in *B. subtilis* have been between cells carrying SPO2 and the virulent phage ϕ1 or ϕe. To determine whether phage ϕ1 is excluded by lysogeny with phage ϕ3T, various lysogens were infected by phage ϕ1. There was no significant exclusion of ϕ1 by phage ϕ3T (Table 4).

Integration of the gene encoding thymidylate synthetase (*thyP3*). (i) Requirement for integration of the complete phage genome. Three experiments were designed to determine whether the gene encoding *thyP3* could be integrated in the absence of the complete viral genome. First, we compared the efficiency of transfer of *thyP3* during infection and transfection with mature and prophage DNA at a final concentration of approximately 0.1 μg/ml. Surprisingly, the efficiency of cotransfer of *thyP3* with the phage genome varied with the mode of delivery of genetic information (Table 5). When cells were transformed with mature phage DNA and the Thy^+ recombinants were selected, none contained the complete prophage genome.

TABLE 4. *Efficiency of plating of bacteriophage ϕ1 on lysogens of BR 151*

Host	PFU/ml
BR 151	5.9×10^9
BR 151 (ϕ3T)	4.7×10^9
BR 151 (ϕ105)	5.4×10^9
BR 151 (SPO2)	$<10^3$

TABLE 3. *Induction of bacteriophage in the presence of HPUra*[a]

Bacteriophage	PFU at:			
	Control (h)[b]		Experimental (h)[c]	
	0	3[d]	0	3
ϕ3T	6.2×10^5	2.2×10^9	2.8×10^5	1.1×10^9
ϕ105	5.6×10^4	1.6×10^9	3.1×10^4	3.6×10^4
SPO2	2.2×10^5	4.4×10^9	4.1×10^4	1.4×10^9

[a] Lysogens of ϕ3T, ϕ105, and SPO2 were grown to a cell density of 10^8 cells/ml and induced by transfer into medium containing mitomycin C with or without HPUra.
[b] Contained 2.5 mM NaOH for pH equilibration.
[c] HPUra at final concentration of 250 μg/ml.
[d] PFU were measured immediately after transfer and after 3 h of incubation as described in Materials and Methods.

TABLE 5. *Cotransfer of φ3T and thyP3*

Selected trait	% of unselected trait on:	
	Infection	Transfection[b]
Thy+[a]	98 (MOI = 1.0)	56 (prophage DNA)
	69 (MOI = 0.1)	0 (mature DNA)
PFU[c]	100	100[d]

[a] RUB 830 was incubated with either intact phage, prophage DNA, or mature DNA and selected for Thy+ CFU. These CFU were then tested for the presence of the phage genome by their ability to lyse a nonlysogen.

[b] DNA concentration was 0.1 μg/ml.

[c] RUB 830 lysogens from infection or transfection were assayed for Thy+.

[d] For both prophage and mature DNA.

In contrast, 56% of the Thy+ recombinants obtained with prophage DNA yielded phage. Although 98% of the Thy+ recombinants after infection at an MOI of 1.0 yielded phage, this number was decreased significantly at a lower MOI. On the other hand, when survivors in plaques were picked, all of the lysogens carried *thyP3*. Second, we treated the mature phage DNA with the site-specific endonuclease *Bam*HI. *thyP3* activity was still present in 5-h digests even though plaque-forming activity had been destroyed (Table 6). Finally, the fragment carrying the *thyP3* gene has been purified by agarose gel electrophoresis (G. A. Wilson and F. E. Young, unpublished observations). Under these conditions it is possible to introduce the *thyP3* gene with fragment A obtained after digestion with the site-specific endonuclease *Bam*HI (17).

(ii) Efficiency of transformation for *thyP3*. Previous studies in our laboratory have demonstrated that heterologous DNA is incorporated at a low frequency as compared with intergenotic DNA (21). To compare the efficiency of transformation of *thyP3* and *trpC2*, DNA was isolated from a prototrophic lysogen carrying phage φ3T and incubated with competent cells of RUB 830. The slope of the dose response curve for infectious centers was approximately one (Fig. 2), as in the case of φ105 prophage DNA (15). Spatz and Trautner have shown that there is a linear relationship between DNA concentration and transfection with DNA isolated from lysogens of φ105 or SPO2. They concluded that cooperative interaction of molecules was not required to produce an infective center. Our observations with phage φ3T support the hypothesis that molecules from temperate phages do not undergo a type of inactivation that necessitates cooperative interaction between molecules of DNA (16). The slope of the curves for Thy+ and Trp+ transformants were similar. Only at high concentrations of DNA did the numbers of Thy+ transformants and PFU approach each other. On the other hand, the frequency of transformation for Trp+ was always 10- to 20-fold greater than that of Thy+.

(iii) Comparison of Thy+ transformants and PFU after transfection with mature phage DNA. Previous experiments with phage infection (11) and transfection (19) have indicated a unique order of penetration of phage DNA. To determine the efficiency of Thy+ transformation as compared with transfection and to establish the kinetics of these events, mature phage DNA was incubated with competent cells from strain RUB 830, and the reaction was

TABLE 6. *Survival of Thy+ transforming activity[a]*

Experimental conditions	Transformants/ml	
	Thy+	PFU
φ3T DNA	9.8×10^4	50
φ3T DNA + *Bam*HI	9.4×10^4	<10^1
φ3T DNA + DNase	<10^1	<10^1

[a] Bacteriophage DNA was treated with *Bam*HI (22). After 5 h of digestion, samples were removed for assay of Thy+ transformants and PFU as described in Materials and Methods.

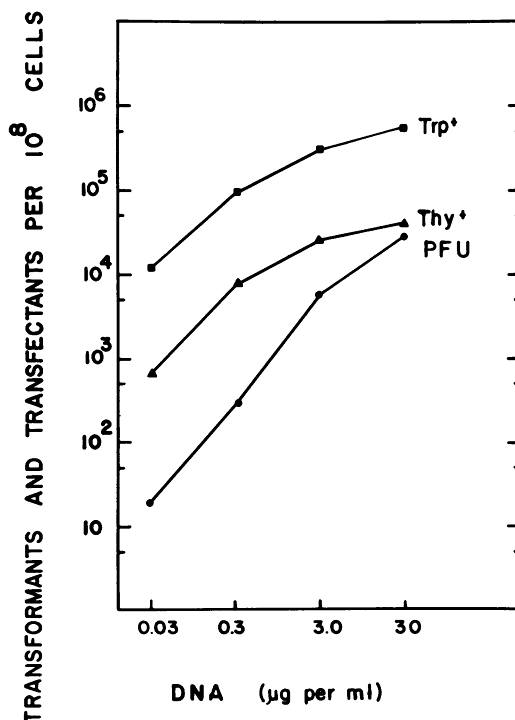

FIG. 2. *Dose response curve for transformation with φ3T prophage DNA. DNA from strain CU 809 (φ3T) was used to transform competent RUB 830. Thy+ and Trp+ CFU and φ3T PFU were assayed.*

terminated with DNase as a function of time (Fig. 3). The efficiency of Thy+ transformants was significantly greater than PFU at all points examined. Most of the Thy+ transformants did not contain the complete prophage genome. Furthermore, Thy+ transformants appeared after a lag of 3 min, whereas PFU were not detected until after 5 min of incubation with

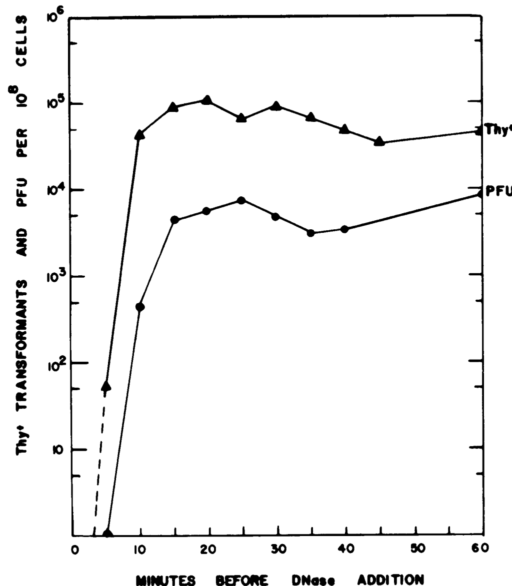

FIG. 3. *Rate of entry of thyP3 versus PFU. Mature bacteriophage ϕ3T DNA (0.1 μg/ml) was incubated with competent cells of strain RUB 830. As a function of time, the reaction was terminated by DNase (50 μg/ml), and Thy+ transformants and PFU were determined.*

competent cells. These data, in concert with the physical mapping data (17), indicate that the gene encoding thymidylate synthetase activity is not at one end of the molecule. Alternatively, there may be only a short lag between the uptake of the *thyP3* gene and the entire molecule of phage ϕ3T DNA. Because the mature ϕ3T DNA migrates as a single band in agarose-ethidium bromide gel electrophoresis, it is unlikely that Thy+ transformants occur solely from fragmented DNA. Experiments are in progress to analyze the rate of entry with mutants of phage ϕ3T.

Chromosomal location of ϕ3T prophage. Although the *thyP3* gene encoding thymidylate synthetase activity is apparently integrated into the chromosome, its relationship to *thyA* and *thyB* remains to be established. It is doubtful that ϕ3T codes for an amber suppressor function, since lysogenization with ϕ3T does suppress the *hisB2* mutation or allow infection of known *sus* phage (M. T. Williams, unpublished observations).

The *thyP* locus and attachment site of ϕ3T integration were mapped by PBS1-mediated transduction (Table 7). All transduction crosses were performed in the presence of ϕ3T antisera. The relative positions of *thyP* and *att* ϕ3T are displayed in Fig. 4. It can be seen that the integration site for phage ϕ3T appears in the terminal region of the chromosome, separate from *thyA* and *thyB* but between these loci. Unfortunately, there are not numerous auxotrophic markers in this region. Therefore, fine-structure analysis must await the isolation of more mutants in the terminus of the chromosome.

TABLE 7. *Linkage of ϕ3T to chromosomal markers*[a]

Selected marker	Cotransfer of unselected markers							
	metB	*ilvD*	*thyB*	*ilvA*	*att*ϕ3T	*thyP*	*gltA*	*thyA*
metB				857/1,049 (18.3)	49/689 (92.9)	8/193 (95.9)	0/293	
ilvD			374/385 (2.9)	1,416/1,484 (4.6)		15/339 (95.6)		0/189
thyB	186/200 (7.0)			188/200 (6.0)			0/200	
ilvA	144/157 (8.3)		444/449 (1.1)		97/510 (81.0)	32/284 (88.7)	11/465 (97.6)	
thyP	2/58 (96.6)	1/16 (93.8)		7/110 (93.6)	100/116 (13.8)		6/94 (93.6)	
gltA				0/407	5/100 (95.0)	8/567 (98.6)		82/143 (42.7)

[a] Frequency of linkage of unselected donor marker found in selected markers in various recipients. The rates show the actual number of donor-type recombinants (numerator) and the total number of transductants examined (denominator). The data in the parentheses are the recombination frequencies (1 − percentage of cotransfer).

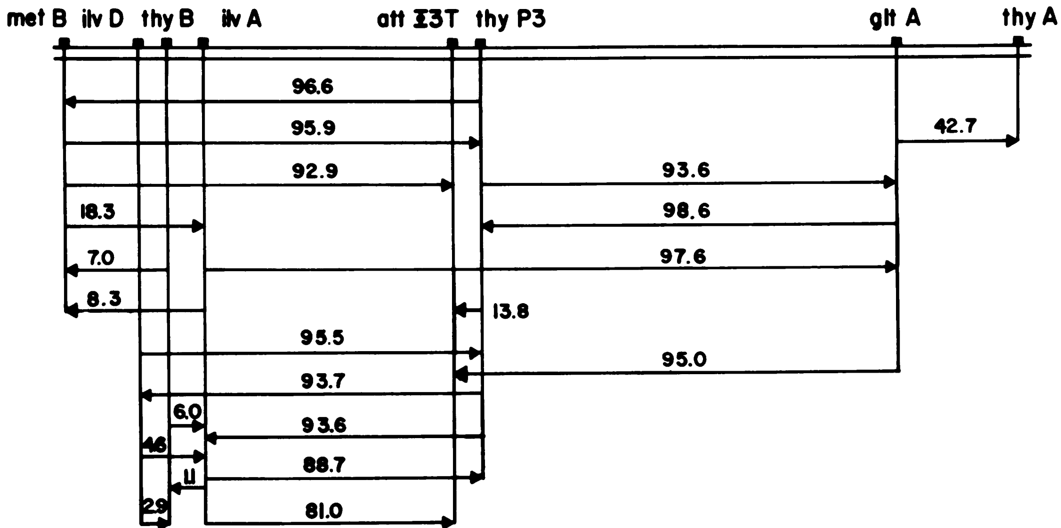

FIG. 4. *Genetic map of the terminal region of the B. subtilis chromosome showing the attachment site of the ϕ3T genome and thyP3 locus. Map distances are based on the PBS1 transduction analysis shown in Table 7.*

DISCUSSION

During lysogeny, the viral genome is integrated into the bacterial chromosome (2). The maintenance of the integrated (or prophage) state usually involves repression of certain regions of the viral genome. During lysogenization by some viruses, a portion of the viral genome may continue to function. This results in the acquisition of a new property (lysogenic conversion), as in the conversion of nontoxinogenic strains of *Corynebacterium diphtheriae* to lysogenic, toxinogenic strains by exposure to the temperate phage β^{tox+} (7). A particular locus on the phage genome, designated *tox*, is the structural gene for the diphtherial toxin. The *tox* gene can be expressed in three different states of the phage β^{tox+} genome: (i) as a nonreplicating, nonintegrated intracellular phage; (ii) as a replicating vegetative phage; and (iii) as a repressed, integrated prophage. A similar situation appears to exist in *Bacillus cereus* strain w (β,*wx*). When the bacteria are cured of prophage *wx*, the ability to produce the bacteriocin phospholipase A is also lost. The capacity is restored by infection with bacteriophage *wx* (10).

The relationship between certain viral infections and thymidylate synthetase activity has been noted in various systems (6). The effects range from a rapid decrease in thymidylate synthetase activity during phage ϕe infection of *B. subtilis* to the appearance of phage-determined thymidylate synthetase activity in *Escherichia coli* infections with phages T2, T4, T5, and T6. The lytic phage ϕe contains 5-hydroxymethyluracil in its DNA in place of thymine. Upon infection of *B. subtilis*, ϕe not only induces the new enzyme dUMP hydroxymethylase, but also gives rise to an inhibitor of host thymidylate synthetase. The effect of this inhibition is to preclude the incorporation of thymine into ϕe DNA by preventing the thymidylate synthetase from competing with dUMP hydroxymethylase for their common substrates (8).

Although thymidylate synthetase is present in uninfected *E. coli*, Matthews (12) has shown that the presence of a phage gene for thymidylate synthetase is necessary for normal burst size of phage T4. The thymidylate synthetase molecules produced by phages T2, T4, T5, and T6 are not identical, however, and in the case of T4 the molecule also becomes a structural element of the capsid protein (5). It must be emphasized that all these infections are lytic.

We agree with Tucker (18) that the *B. subtilis* phage ϕ3T is indeed temperate as evidenced by the following: (i) despite repeated subculture, the lysogen remains stable; (ii) free phage may be liberated upon induction of the lysogen by mitomycin C; and (iii) cultivation in specific phage antiserum has no effect on the lysogenic state. We have confirmed Tucker's observation that formation of a stable prophage in a thymine-requiring host results in restoration of thymidylate synthetase activity to wild-type levels, and we are currently working on further enzyme purification and interaction with antifolates. Integration takes place near

the terminus of the *B. subtilis* chromosome, and every stable lysogen thus produced is Thy$^+$. Furthermore, integration of *thyP* can occur in the absence of the complete phage genome.

At present we do not know how much of the viral genome is incorporated with the gene encoding thymidylate synthetase activity (*thyP3*). Recent studies from our laboratory (R. S. Graham and G. A. Wilson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, p. 97) have demonstrated that *thyP* activity remains after treatment of the mature phage DNA with endonucleases *Bam*HI, *Bgl* II, *Pst*, and even *Eco*RI (22). Only DNase I completely inactivates the *thyP* gene (Table 3). Recent analysis of this viral genome by a rapid procedure of physical mapping has established that *thyP3* resides on fragment A (approximately 36.6 megadaltons) of this 74.8-megadalton virus (17). This study also determined that the phage is linear and not circularly permuted. Dean and co-workers (D. N. Dean, J. C. Orrego, K. W. Hutchinson, and H. O. Halvorson personal communication), using a closely related phage isolated by J. Hoch (ρ11), noted numerous similarities between ρ11 and φ3T, but demonstrated that there were minor differences in *Eco*RI digests of the two phage genomes. More recently we have established major differences in gel patterns with a variety of restriction nucleases (R. S. Graham, personal communication).

The regulation of thymidylate synthetase activity and the mechanism of integration of the *thyP3* gene in the chromosome of *B. subtilis* are some of the more intriguing questions. If *thyP3* was originally a bacteriophage gene, then it is to our knowledge the only example of a high-frequency integration of a bacteriophage gene into the chromosome of the host. We are currently exploring the amount of bacteriophage genetic information integrated with the *thyP3* gene and attempting to elucidate the regulation of this phage gene in the bacterial host.

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