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Mutants of *Bacillus subtilis* Bacteriophage φe Defective in dTTP-dUTP Nucleotidohydrolase

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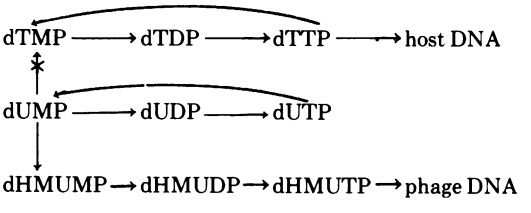
Mutants of bacteriophage φe inducing only 1 to 5% of wild-type levels of dTTP-dUTP nucleotidohydrolase give normal bursts of viable progeny phage whose DNA contains 5 to 10% thymine (but no uracil) in place of 5-hydroxymethyluracil. The relative heat lability of one phage mutant enzyme solubilized from the membrane fraction of infected cells suggests that a φe gene codes for the induced dTTPase-dUTPase.

φe is one of a group of *Bacillus subtilis* bacteriophages, unique because its DNA contains the unusual base 5-hydroxymethyluracil (HMU) rather than thymine (10). The HMU-DNA *B. subtilis* phages are similar in morphology, size, and DNA content (12) to the *Escherichia coli* T-even phages whose DNA contains 5-hydroxymethylcytosine rather than cytosine (14). Like the T-even phages, phage φe induces several proteins early after infection, apparently to alter the host's deoxyribonucleotide metabolism to allow the synthesis of φe HMU-containing DNA and to prevent the synthesis of DNA containing thymine or uracil. One of these proteins, dTTPase-dUTPase, catalyzes the hydrolysis of dTTP and dUTP to their respective monophosphates and pyrophosphates (L. T. Dunham, Ph.D. thesis, Univ. of Michigan, Ann Arbor, 1973; 8). This enzyme is presumably important for the operation of the following pathways in φe-infected cells (8, 10):



This report (taken in part from a Ph.D. thesis submitted by L.T.D. to the Faculty of the Rackham Graduate School of The University of Michigan, Ann Arbor, 1973) extends the characterization of three φe mutants defective in the dTTPase-dUTPase in an attempt to define the role of this enzyme during phage infection.

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Mutant φm3 was isolated (8) as a survivor of hydroxylamine mutagenesis; φm3 induces only 35% of the normal dTTPase-dUTPase level during infection at 37 C. In agreement with the results of Roscoe (8), we find that the φm3 enzyme loses activity during incubation of crude extracts at 50 C in the absence of substrate, whereas the wild-type enzyme is stable (Dunham, Ph.D. thesis). However, we have also shown that the ϕe^+ enzyme is membrane associated (Dunham, Ph.D. thesis). To eliminate the possibility that the φm3 enzyme appeared more heat labile because of an altered interaction with the membrane, we solubilized the φm3 enzyme from the membrane fraction (Fig. 1). The partially purified φm3 dTTPase-dUTPase was unstable to preincubation at 50 C (Dunham, Ph.D. thesis) and showed a temperature optimum of 30 to 35 C, considerably lower than that of the ϕe^+ enzyme (Fig. 1). This supports Roscoe's conclusion (8) that a structural gene for the φe nucleotidohydrolase is located on the phage genome, although the presence of host-coded subunits has not been excluded.

Mutant φm2 was isolated (8) from the same screening as φm3. In confirmation of Roscoe's results (8), we found that φm2 induces 35% of normal dTTPase-dUTPase activity during infection at 37 C, but less than 5% at 45 C (Table 1). The φm2 and ϕe^+ enzyme are both stable during preincubation of extracts at 50 C (Dunham, Ph.D. thesis; 8) or solubilized fractions (Dunham, Ph.D. thesis), and both enzymes have a temperature optimum of 40 to 50 C (Fig. 1). Furthermore, the low residual activity induced by φm2 at 45 C has the same purification properties, molecular weight on Bio Gel A5m (100,000), and substrate concentration depend-

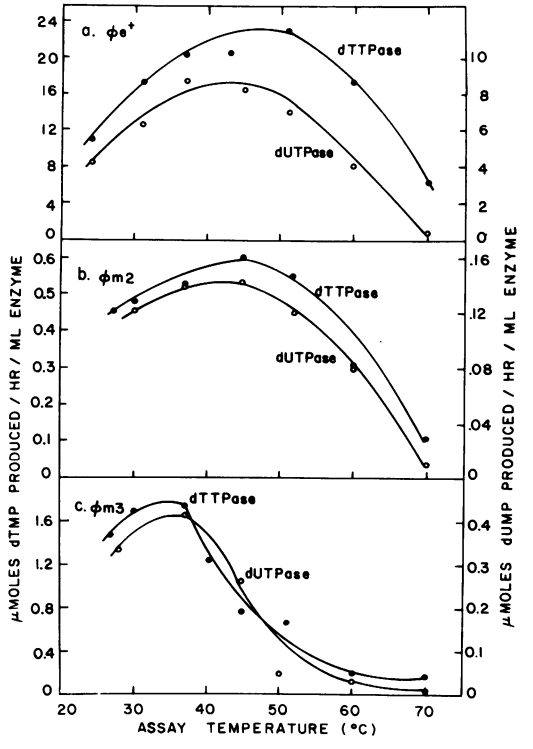

FIG. 1. *Dependence on assay temperature of dTTPase-dUTPase activities induced by φe⁺ at 37 C, φm2 at 45 C, and φm3 at 37 C. The substrates were present at concentrations (2 mM for dTTP and 0.034 mM for dUTP) equal to three to five times their K_m values to avoid substrate-induced stabilization of dUTPase activity at high dUTP concentrations (Dunham, Ph.D. thesis). All reaction components (Dunham, Ph.D. thesis) except the enzyme were incubated for 2 min at the indicated temperatures; then the enzyme was added and incubation continued for an additional 15 min. Partially purified (about 10-fold) enzymes were utilized; they were obtained from crude extracts of B. subtilis 3610 thy⁺-infected cells by centrifugation at 100,000 × g, solubilization with 5% deoxycholate, and Bio Gel A5m chromatography in the presence of 1% Triton X-100 (Dunham, Ph.D. thesis). In contrast to the statement of Roscoe (8) we observed no activity (<0.1%) at 0 C.*

ence curves for dTTP and dUTP (K_m = 400 and 11 μM, respectively) as the φe⁺ and φm3 enzymes (Dunham, Ph.D. thesis). Thus, the φm2 mutation appears to greatly reduce the synthesis of dTTPase-dUTPase without affecting the enzyme's properties (at least those tested).

In an attempt to distinguish a possible *trans*-acting control gene mutation (φm2) from a structural gene mutation (φm3), a complementation test was done by mixed infection at 37 and 45 C with a multiplicity of five phage each. The levels of activity induced and the percentage of heat-stable (φm2) versus heat-labile (φm3) enzymes were those predicted if each mutant were acting independently (Dunham, Ph.D. thesis); φm2 and φm3 are codominant to one another. (We have not attempted the tedious task of genetically mapping these mutations, since the only phenotype which we can test is dTTPase-dUTPase activity. Such mapping would therefore involve growing each progeny phage at two temperatures and preparing extracts for assay under two conditions.) Thus, φm2 may have a mutation in a regulatory gene for dTTPase-dUTPase synthesis which acts only in *cis* (although a possible structural gene mutation has not been rigorously excluded).

Mutant *sus*5-18R was isolated (M. C. Newlon, Ph.D. thesis, Massachusetts Institute of Technology, Cambridge; 2) as a revertant of a second gene-suppressible mutation, and it induces only 1% of normal dTTPase-dUTPase activity on infection at 37 or 45 C (Table 1). Like φm2 and φm3, *sus*5-18R is not a lethal mutation. All three mutants induce normal levels of the essential φe dUMP hydroxymethylase (8) and of dCMP deaminase (Dunham, Ph.D. thesis). No reduction in burst size was seen for the three mutants relative to φe⁺ at 37 or 45 C at high multiplicities of infection (Table 1). Similar but more variable results have been obtained (Dunham, Ph.D. thesis) using a multiplicity of 0.1 and diluting the infected culture by 10^6 to estimate the one-step growth rate and burst size (80 to 250 for φe⁺, φm2, and *sus*5-18R at 45 C).

The φe-induced dTTPase-dUTPase seems to function in the exclusion of thymine from phage DNA. Others (2, 4, 9) have shown that some radioactive thymine was incorporated into phage DNA when the dTTPase-deficient mutants φm2 and *sus*5-18R were grown on a thymine-requiring host in the presence of labeled thymine. This system bypassed the phage-induced dTMP synthetase inhibitor, allowing maximal thymine incorporation into mutant phage DNA. However, neither the uracil content nor the homogeneity of this DNA was reported. In view of the finding that the K_m for dUTP is 35-fold lower than that for dTTP, we suggest that the dTTPase-dUTPase may also function to prevent uracil incorporation into phage DNA. This possibility is supported by the recent observation (15) that an HMU-phage DNA polymerase can utilize dHMUTP, dTTP, and dUTP at equal rates in vitro.

Therefore, phages φe⁺, φm2, and *sus*5-18R were grown on *B. subtilis thy⁻ tlr* (9), and the buoyant density of extracted phage DNA was

TABLE 1. *Nucleotidohydrolase induction, burst size, and DNA buoyant density of wild-type and mutant φe phages*

Phage[a]	Temp of infection (C)	Sp act (μmol/h/mg)		Burst size	Phage DNA[b]	
		dTTPase	dUTPase		Density (g/cm³)	Thymine for HMU (%)
Uninfected	37	0.2	2			
φe⁺	37	113	71	210	1.740	<2.5
φe⁺	45	102	63	110	1.739	<2.5
φm2	37	35	27	320	1.738	5
φm2	45	4	5	180	1.736	10
*sus*5-18R	45	1	2	170	1.737	7.5

[a] *B. subtilis thy⁻ tlr* was grown at 37 C in the presence of thymine (2 μg/ml) to a density of 10^8 colony-forming units per ml. Since cells grown at 45 C for several generations become resistant to phage (1, 8), they were shifted from 37 to 45 C when necessary at 10 min before infection. Using lysates previously prepared under the same conditions of infection, we added phage at a multiplicity of five per cell. The cultures were then shaken vigorously at the indicated temperature. After 25 min of infection, extracts were prepared with lysozyme and assayed at 37 C with tritium-labeled substrates (Dunham, Ph.D. thesis). When lysis of the remaining infected cultures was complete (2 h at 37 C or 1.5 h at 45 C), the lysates were freed of debris by centrifugation and were titered for phage produced per original cell (burst size). Then the phage were concentrated by polyethylene glycol precipitation, purified through a CsCl block gradient (4), and treated with sodium dodecyl sulfate and phenol to extract the DNA (Dunham, Ph.D. thesis).

[b] The DNA buoyant densities were determined in a Beckman model E ultracentrifuge by using CsCl gradients with an internal marker of *Micrococcus lysodeikticus* DNA of density 1.731 g/cm³. The relative substitution of thymine for HMU was calculated from the difference in density (3) of 0.040 g/cm³ between φe DNA (28.5% HMU) and *B. subtilis* DNA (28.5% thymine). Thus, a 10% substitution of thymine for HMU would result in a decrease in φe DNA density of 0.004 g/cm³.

determined (Table 1). Each DNA gave a single sharp UV-absorbing peak in the analytical ultracentrifuge. The substitution of thymine for HMU was calculated from the shift in buoyant density to be 10% for φm2 DNA and 7.5% for *sus*5-18R DNA from infections at 45 C. Enzymatic hydrolysis of the DNAs confirmed the level of thymidine substitution calculated from the density determinations, although the detection of low levels of thymidine is difficult (Dunham, Ph.D. thesis). Acid hydrolysis to the free bases cannot be used since acid degrades some HMU to thymine (11). The uracil content of the phage DNAs was examined directly by acid and enzymatic hydrolysis (Dunham, Ph.D. thesis). Neither uracil nor deoxyuridine (less than 3% of the HMU content) was detected in any of the DNAs, although it was found in the PBS2-uracil-DNA control (6). Therefore, the density shifts observed above can be attributed to the presence of thymine alone.

The absence of uracil in phage DNA synthesized by the dUTPase-deficient mutants φm2 and *sus*5-18R suggests that either the residual 1 to 5% of phage-induced dUTPase activity is sufficient or that some other activity prevents dUTP accumulation and uracil incorporation into phage DNA by the phage DNA polymerase activity (15). This may reflect the action of the host dUTPase activity (Table 1); unfortunately no bacterial dUTPase mutants have yet been isolated to test this possibility. In addition, the φe dUMP hydroxymethylase presumably diverts dUMP from dUTP synthesis. No φe dUMP hydroxymethylase mutants have yet been found (Newlon, Ph.D. thesis) to test this hypothesis, although such mutants have been isolated in other HMU-DNA phages (5, 7). Of course the accumulation of uracil-containing φe DNA requires that *B. subtilis* does not have mechanisms of its own which might degrade uracil-DNA, such as the 5′ → 3′ exonuclease activity associated with DNA polymerase I in *E. coli* (13). However, it seems likely that uracil-containing φe DNA would be stable, since phage PBS2, whose DNA contains uracil instead of thymine, does replicate in *B. subtilis* (6). The biological significance of the unusual base HMU in bacteriophage φe DNA remains undefined.

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