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Temperature-Sensitive Mutants Affected in DNA Synthesis in Phage φ29 of *Bacillus subtilis*

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The DNA synthesis in *Bacillus amyloliquefaciens* infected with wild-type phage φ29 and with temperature-sensitive mutants has been studied in the presence of the drug 6-(*p*-hydroxyphenylazo)-uracil. The drug inhibits DNA synthesis in uninfected bacteria while it does not affect DNA synthesis and phage development in φ29-infected bacteria. DNA synthesis was blocked by infection at the restrictive temperature with the mutants F99 and K132, representatives of the two early functions identified so far, as well as with mutant C17, which affects a late function. The remaining mutants, belonging to eight complementation groups, synthesized DNA at the restrictive temperature. The proportion of the DNA resistant to DNAase was different, depending on the mutant.

Bacteriophage φ29 of *Bacillus subtilis* contains a double-stranded DNA molecule with a molecular weight of 11×10^6 [1] which can be isolated from the viral particle as a ring closed non-covalently by a protein [2].

For a study of the replication of φ29 DNA and the role played by the DNA-associated protein it would be desirable to shut-down bacterial DNA synthesis after infection and to have available phage mutants affected in DNA biosynthesis or encapsulation. For the first aim we took advantage of the results of Brown [3] who has shown that in *B. subtilis* and other gram-positive bacteria the synthesis of DNA is inhibited in the presence of the drug 6-(*p*-hydroxyphenylazo)-uracil while the synthesis of viral DNA and phage development are not affected in cells infected with some virulent phages, included φ29 (N. C. Brown, personal communication).

In this paper we show that the DNA synthesized in φ29-infected *B. amyloliquefaciens* in the presence of 6-(*p*-hydroxyphenylazo)-uracil hybridizes with viral but not with host DNA. On the other hand, an study of the DNA synthesized after infection with different temperature-sensitive mutants of φ29 [5, 6] has revealed the existence of three cistrons involved in DNA synthesis; two of the three cistrons (F99 and K132) are early whereas the third one (C17) is a late gene. DNA synthesis is a late function in the development of phage φ29. This probably means that gene C17 is directly involved in phage DNA synthesis. The study of the synthesis of φ29-specific DNA, not inhibited by the drug 6-(*p*-hydroxyphenylazo)-uracil, may throw further light on the understanding of the replication mechanism.

Abbreviations. Standard saline citrate, 0.15 M sodium chloride plus 0.015 M sodium citrate; butyl-PBD, 2-(4′-*t*-butylphenyl)-5-(4″-biphenyl) 1,3,4-oxadiazole; p.f.u., plaque-forming units.

Enzymes. Pancreatic deoxyribonuclease (EC 3.1.4.5); lysozyme or mucopeptide *N*-acetylmuramylhydrolase (EC 3.2.1.17).

MATERIALS AND METHODS

Chemicals

Cesium chloride, sequanal grade, was obtained from Pierce Chemical Co., sodium dodecylsulfate, lysozyme, bovine serum albumin, ficoll and polyvinylpirrolidone-360 from Sigma, pronase from Calbiochem, pancreatic DNAase I from Worthington, butyl-PBD from Ciba and [2-^{14}C]uracil from the Radiochemical Center (Amersham). 6-(*p*-Hydroxyphenylazo)-uracil was a gift from Dr N. C. Brown.

φ29 DNA was prepared from purified φ29 (supplied by V. Rubio) by treatment with autodigested pronase (1 mg/ml) [7] in the presence of 0.5% sodium dodecylsulfate and phenol extraction. *B. amyloliquefaciens* DNA was prepared as described by Okamoto *et al.* [8].

Bacteria and Phage

B. amyloliquefaciens, strain H, and phage φ29 were originally obtained from Dr B. E. Reilly. Phage φ29 was assayed as described before [5].

DNA Synthesis in φ29-Infected Bacteria in the Presence of 6-(p-Hydroxyphenylazo)-uracil

The bacteria were grown at 37 °C in a defined medium containing the salts indicated by Anagnostopoulos and Spizizen [9] supplemented with 0.1 M NaCl, 0.01 mM $MnCl_2$, 20 mM D-glucose and a mixture of the 20 natural L-amino acids at a final concentration of 0.1 mM each. When the cell concentration was 10^8/ml the bacteria were concentrated ten-fold by centrifugation and resuspension in the same medium, lacking glucose and amino acids, in the presence of 10 mM KCN. After 5 min at 37 °C the cells were infected with phage φ29 at a multiplicity of 20 and kept at 37 °C for 10 min without shaking. The cells were then centrifuged and resuspended in the same volume of complete medium containing [^{14}C]uracil (0.5 μCi/ml; 4 μCi/μmol) with or without 0.4 mM 6-(*p*-hydroxyphenylazo)-uracil. The infected culture was shaken at 42 °C till lysis. At different times aliquots were removed from the culture to assay for plaques and alkali-resistant and acid-insoluble (cold 5% trichloroacetic) radioactivity, before or after treatment with DNAase.

Isolation of Radioactive DNA from φ29-Infected Bacteria

Bacteria were grown, infected and labelled as indicated above. After 50 min incubation the cells (1 ml) were collected by centrifugation, resuspended in 2 ml 0.1 M Tris-HCl pH 8.0 at 0 °C and treated with lysozyme (500 μg/ml) for 5 min at 0 °C. After this treatment, 0.5% sodium dodecylsulfate and autodigested pronase (1 mg/ml) [7] were added and the mixture was incubated for 3 h at 37 °C; pronase was again added at the same concentration and the incubation was continued for 3 h. The mixture was extracted three times with one volume of phenol. To the aqueous phases NaCl to a concentration of 0.5 M and three volumes of absolute ethanol were added and the mixture was kept overnight at −20 °C. The precipitate was collected by centrifugation for 15 min at 27000 × *g*, washed once with cold 67% ethanol, resuspended in 1 ml 1 M KOH and kept overnight at 37 °C to degrade the RNA present. The mixture was neutralized, precipitated again with ethanol and the precipitate was washed as before. The final precipitate was dissolved in 1 ml of 6× standard saline citrate.

Hybridization-Competition Experiments

Increasing amounts of denatured φ29 or *B. amyloliquefaciens* DNA in 2.5 ml of 6× standard saline citrate were filtered through nitrocellulose filters (Millipore HAWP). The DNA-containing filters were placed in vials, kept overnight under vacuum at room temperature and then for 2 h at 80 °C. 0.7 ml of a mixture containing 0.02% ficoll, 0.02% polyvinylpirrolidone, 0.02% bovine serum albumin and 0.05% sodium dodecylsulfate were added to each vial and the vials were incubated for 6 h at 65 °C [10]. After this time 0.5 μg denatured [^{14}C]DNA, isolated from φ29-infected bacteria in the presence of 6-(*p*-hydroxyphenylazo)-uracil, was added to each vial. For competition experiments the filters contained a constant amount of denatured φ29 DNA and as competitor increasing quantities of either viral or host DNA. The vials were incubated for 16 h at 65 °C. After incubation, each side of the filter was washed with 40 ml standard saline citrate, the filters were dried and the radioactivity counted in a Packard TriCarb spectrometer using as scintillation liquid a solution containing 4 g butyl-PBD per liter toluene.

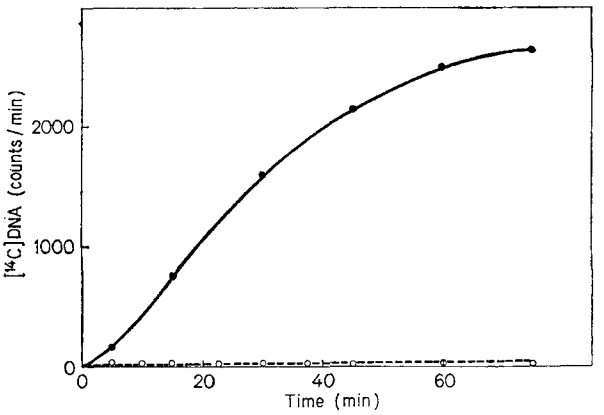

Fig. 1. *DNA synthesis in* Bacillus amyloliquefaciens *in the presence and in the absence of 6-(p-hydroxyphenylazo)-uracil.* The bacteria were grown in synthetic medium and processed as described in Materials and Methods, except that phage was not added. [^{14}C]Uracil (0.5 μCi/ml, 4 μCi/μmol) was added in the presence (O----O) or in the absence (●——●) of 0.4 mM 6-(*p*-hydroxyphenylazo)-uracil and the culture was incubated at 42 °C. At the times indicated, aliquots of 20 μl were removed, incubated with 1 M KOH overnight at 37 °C, neutralized and the acid-insoluble (cold 5% trichloroacetic) radioactivity determined

RESULTS AND DISCUSSION

DNA Synthesis in φ29-Infected Bacteria in the Presence of 6-(p-Hydroxyphenylazo)-uracil

The synthesis of DNA in *B. amyloliquefaciens* is completely blocked in the presence of 6-(*p*-hydroxyphenylazo)-uracil (Fig. 1). However, DNA synthesis and phage development are not impaired in φ29-infected bacteria in the presence of the drug (Fig. 2). As can be seen in Fig. 2B, DNA synthesis is a late function in the development of phage φ29. This

figure also shows that approximately 70% of the DNA synthesized in the presence of 6-(*p*-hydroxyphenylazo)-uracil is resistant to DNAase, suggesting that the DNA is encapsulated or is present in a form resistant to the enzyme.

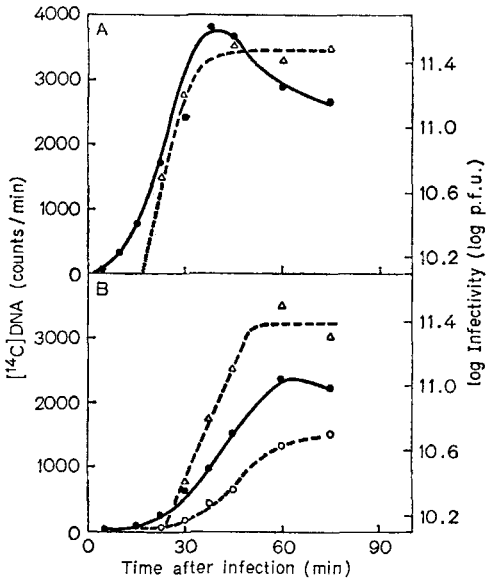

Fig. 2. *DNA synthesis and phage development in φ29-infected bacteria in the presence (B) and in the absence (A) of 6-(*p*-hydroxyphenylazo)-uracil.* The bacteria were grown and infected as described in Materials and Methods. At the times indicated aliquots were removed to assay infectivity after lysis with lysozyme, and acid-insoluble radioactivity after treatment with alkali as indicated in Fig. 1, before or after incubation with DNAase (2 μg/ml) for 30 min at 37 °C. △----△, Infectivity (plaque-forming units); ●——●, total DNA; O----O, DNAase-resistant DNA

To study whether or not the DNA synthesized in the presence of the drug was phage specific, the DNA was isolated as described in Materials and Methods and subjected to hybridization with either φ29 DNA or DNA from *B. amyloliquefaciens*. As shown in Fig. 3A, there was about 75% hybridization with φ29 DNA and essentially none with *B. amyloliquefaciens* DNA. The DNA from phage-infected bacteria which hybridized with φ29 DNA was competed to an extent of about 90% by φ29 DNA but less than 10% by *B. amyloliquefaciens* DNA (Fig. 3B). On the other hand, 72% of the total radioactivity moved in CsCl at a position which overlapped with the peak of infectivity (Fig. 4), which has a density of 1.45 g × cm^{-3} [11]. This corresponds with the amount of DNA resistant to DNAase obtained in that experiment. In agreement with this, the DNA present in the peak of infectivity was DNAase-resistant while the rest of the radioactivity in the gradient was sensitive to DNAase. The above results indicate that most of the DNA synthesized in φ29-infected bacteria in the presence of 6-(*p*-hydroxyphenylazo)-uracil is phage-specific.

Brown has recently reported that the process inhibited by this drug is the semiconservative replication and not the DNA repair mechanism [12]. The fact that φ29-specific DNA synthesis is not blocked by the drug while host DNA synthesis is completely inhibited implies that the phage has its own replication mechanism independent, at least in part, of that present in the host.

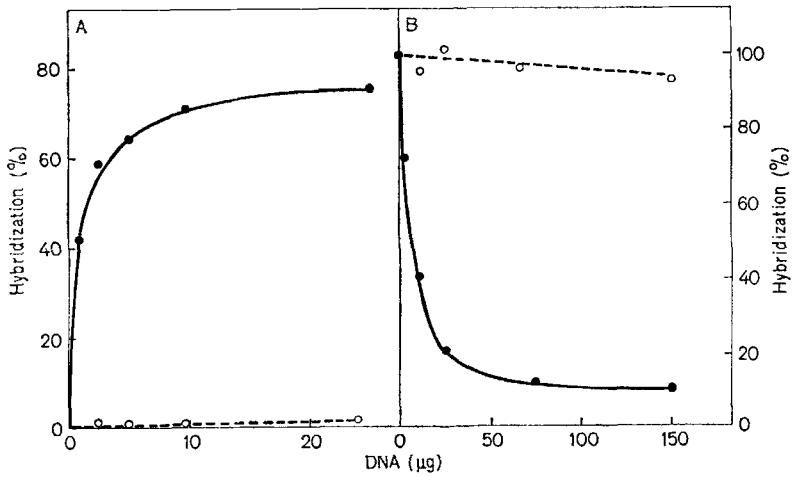

Fig. 3. *Hybridization-competition of the DNA synthesized in φ29-infected bacteria with φ29 or* B. amyloliquefaciens *DNA.* [14C]DNA was isolated from φ29-infected bacteria in the presence of 6-(*p*-hydroxyphenylazo)-uracil as described in Materials and Methods. (A) The indicated amounts of φ29 DNA or *B. amyloliquefaciens* DNA were adsorbed to nitrocellulose filters. 0.5 μg denatured [14C]DNA (1500 counts/min) were added to each vial and the hybridization was carried out as described in Materials and Methods. (B) 3 μg φ29 DNA was adsorbed to each nitrocellulose filter. For the hybridization, 0.5 μg [14C]DNA (1500 counts/min) was added to each vial in the absence or in the presence of the indicated amounts of either φ29 DNA (●——●) or *B. amyloliquefaciens* DNA (O----O)

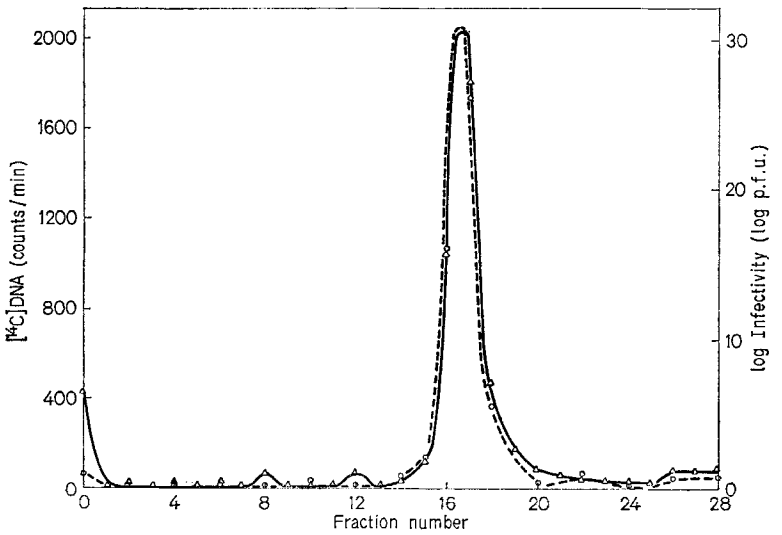

Fig. 4. *Centrifugation in CsCl of a lysate of φ29-infected bacteria labelled with [^{14}C]uracil in the presence of 6-(p-hydroxyphenylazo)-uracil.* φ29-infected bacteria were labelled with [^{14}C]uracil in the presence of 6-(*p*-hydroxyphenylazo)-uracil as indicated in Materials and Methods. The lysate was centrifuged in a CsCl gradient, containing 40% CsCl (w/w), for 17 h at 45000 rev./min at 15 °C in a 50-Ti rotor of an L2-50 ultracentrifuge. Fractions were collected and removed to determine the alkali-resistant, acid-precipitable material was determined in aliquots of 50 µl (△——△). Aliquots of 10 µl were infectivity (O----O)

DNA Synthesis in Bacteria Infected with Temperature-Sensitive Mutants of Phage φ29

Temperature shift-up experiments carried out with mutants of phage φ29 belonging to 11 complementation groups have indicated the existence of two early functions clustered at the left end of the genetic map [6], whereas the mutants in the remaining nine complementation groups affect late functions [5]. When DNA synthesis was studied at 42 °C after infection of bacteria in the presence of 6-(*p*-hydroxyphenylazo)-uracil with mutants affecting the two early functions, F99 or K132, DNA synthesis was almost completely blocked (Fig. 5A and Table 1). As a control, DNA synthesis was essentially normal after infection with these mutants at 30 °C (Fig. 5B).

As indicated before, DNA synthesis is a late function in the development of phage φ29. The above results suggest that the blockage in DNA synthesis at 42 °C by the two early mutants can be an indirect effect and that the gene(s) directly responsible for the DNA synthesis is expressed late after infection. Fig. 5A shows that DNA synthesis was completely blocked after infection at 42 °C with mutant C17, which affects a late function [5] and maps towards the right end of the genetic map [6]. DNA synthesis was normal after infection with this mutant at 30 °C (Fig. 5B).

All the remaining mutants synthesized DNA at 42 °C; some of them, as mutant J116, I81 and D46, produced essentially normal levels of DNA; other mutants, as mutant G93, A18 and B108,

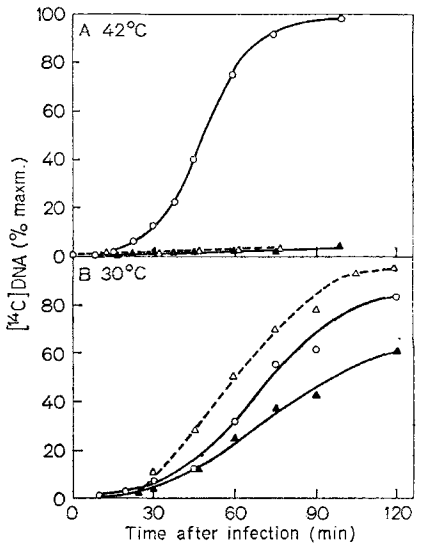

Fig. 5. *DNA synthesis in* B. amyloliquefaciens *infected with mutants F99 or C17 in the presence of 6-(p-hydroxyphenylazo)-uracil.* The bacteria were grown and infected with the wild-type phage or with mutants F99 or C17 as indicated in Materials and Methods. 0.5 µCi/ml of [^{14}C]uracil (4 µCi/µmol) was added in the presence of 0.4 mM 6-(*p*-hydroxyphenylazo)-uracil and the infected bacteria were incubated at (A) 42 °C or (B) at 30 °C. At different times aliquots of 20 µl were removed and the alkali-resistant, acid-precipitable radioactivity determined. O——O, Wild-type phage; ▲——▲, mutant F99; △----△, mutant C17

synthesized higher levels of DNA than the wild-type phage; finally, mutants E54 and H119 produced lower levels of DNA than normal (Fig. 6 and

Table 1. *DNA synthesis at 42 °C in* B. amyloliquefaciens *infected with temperature-sensitive mutants of phage φ29*
The mutants are ordered from top to bottom according to their position in the genetic map [6]. Values for DNAase-resistant DNA are given as a percentage of the total DNA synthesized in each case. The values in parenthesis represent the percentage with respect to the DNA synthesized by the wild-type phage

Mutant	DNA synthesis	DNAase-resistant DNA
	%	%
ts+	100	66
F99	3	—
K132	2	—
J116	70	7 (5)
G93	160	15 (23)
A18	152	45 (69)
I81	105	20 (21)
E54	53	30 (16)
H119	53	26 (14)
C17	3	— —
B108	131	26 (34)
D46	84	25 (21)

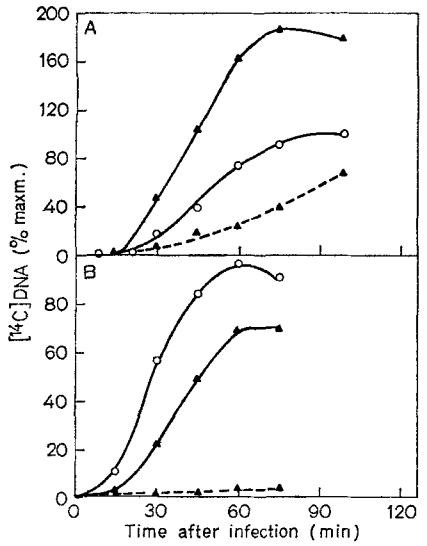

Fig. 6. *DNA synthesis at 42 °C in bacteria infected with mutant A18 or J116 in the presence of 6-(*p*-hydroxyphenylazo)-uracil.* The bacteria were grown and infected with wild-type phage or with mutants A18 (A) or J116 (B) as indicated in Materials and Methods. 0.5 μCi/ml [14C]uracil (4 μCi/μmol) was added in the presence of 0.4 mM 6-(*p*-hydroxyphenylazo)-uracil and the infected bacteria were incubated at 42 °C. At different times aliquots of 20 μl were removed to determine alkali-resistant, acid-precipitable radioactivity, before and after treatment with DNAase. O——O, Total DNA after infection with wild-type phage; ▲——▲, total DNA after infection with mutant A18 (A) or J116 (B); ▲----▲, DNAase-resistant DNA after infection with mutant A18 or J116

Table 1). Only the DNA produced by mutant A18 was resistant to DNAase in the same proportion as in the lysates infected with the wild-type phage (Fig. 6A). The DNA synthesized by the other mutants was more sensitive to DNAase than the DNA made after infection with wild-type phage (Fig. 6B and Table 1). The mutants whose DNA is sensitive to DNAase could be defective in DNA encapsulation either due to a mutation in some of the structural proteins needed for phage assembly or in some other function directly involved in the process of DNA encapsulation such as the DNA-associated protein [2]. The study of the structural proteins present in the phage-related particles produced by each of the mutants under non-permisive conditions is underway.

φ29 DNA synthesis has been studied by Schachtele *et al.* [13] after infection of *B. amyloliquefaciens* at 45 °C with wild-type phage and with temperature-sensitive mutants belonging to three complementation groups. Under these conditions, only 8% of the DNA synthesized after phage infection was shown to hybridize with φ29 DNA. From these studies it was found that one of the mutants does not synthesize phage DNA. It would be interesting to compare this DNA-negative mutant with the three complementation groups unable to synthesize viral DNA at 42 °C reported in this paper.

We are presently studying the intermediates in φ29 DNA replication after infection with wild-type phage and with different temperature-sensitive [5, 6] and suppressor-sensitive mutants (F. Moreno, unpublished results).

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