Effect of Elevated Temperature on Deoxyribonucleic Acid Synthesis in Bacteriophage ϕ 29-Infected *Bacillus amyloliquefaciens*

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Deoxyribonucleic acid (DNA) synthesis in bacteriophage ϕ 29-infected *Bacillus amyloliquefaciens* was studied at 37 and 45 C. Infectious intracellular particles appear at the same time at both temperatures, but the average burst size is reduced 45 to 50% at 45 C. There is a transient inhibition of cellular mass increase at 45 C which is not observed at the lower temperature. In addition, the rate of host DNA synthesis is reduced and the onset of viral-specific DNA replication is delayed for 6 to 9 min at 45 C. These findings allowed us to screen phage ϕ 29 mutants which are sensitive to growth at 45 C for their ability to synthesize ϕ 29 DNA in the absence of host DNA replication. We obtained mutants which make no viral DNA, reduced levels of DNA, or normal quantities of DNA under nonpermissive conditions. Pulse-labeled viral DNA which sediments more rapidly than mature ϕ 29 DNA molecules was observed after gentle cell lysis and zone sedimentation. This DNA is not a precursor of normally sedimenting ϕ 29 DNA and apparently consists of mature ϕ 29 DNA molecules aggregated with large pieces of bacterial DNA.

The mature deoxyribonucleic acid (DNA) of bacteriophage ϕ 29 has been shown to be unique for a virulent virus in that it is nonpermuted and has exposed terminal repetitions (1, 20). These features, along with the fact that the ϕ 29 DNA molecule has a molecular weight of only 11×10^6 daltons (1), encouraged us to begin a systematic analysis of replication of the ϕ 29 genome. A series of temperature-sensitive mutants of phage ϕ 29 which were placed into 13 complementation groups (Hagen, Zeece, and Anderson, *submitted for publication*) allowed us to initiate studies on this particular aspect of phage ϕ 29 development. This report describes the effects of infection at the nonpermissive temperature on both wild-type and mutant phage DNA synthesis.

MATERIALS AND METHODS

Bacteria and phage. *Bacillus amyloliquefaciens* H (20) was employed in most of these studies. *B. subtilis* strain 12A (12) was used as the plating host for phage ϕ 29 because of the clearly defined plaques formed on this strain. The efficiency of plating was essentially the same with both strains.

Phage ϕ 29 was originally obtained from B. E. Reilly. Temperature-sensitive ϕ 29 phage mutants were kindly supplied by E. W. Hagen.

Growth conditions. Bacteria were grown with aera-

tion in Penassay Broth (Difco) as described by Reilly and Spizizen (12). The temperature of incubation was controlled within one-half degree of that designated for each experiment. Growth (increase in cell mass) was followed by optical density readings with a Klett-Summerson colorimeter (no. 66 filter) with Penassay Broth used as a blank. The rapid transfer of cells required during the pulse-chase experiment was performed as described previously (14).

Preparation and assay of phage. Purified stocks of wild-type phage ϕ 29 were prepared as described previously (2). High-titer mutant phage stocks were prepared by the agar layer method described by Swannstrom and Adams (18). Such stocks always showed a difference in titer at 37 and 45 C of at least 10^6 plaque-forming units (PFU) per ml. When phage were titrated during propagation, 0.2 ml of chloroform was added per ml of culture sample, and the cleared suspension was stored at 4 C. ^{32}P -labeled phage were prepared by infecting rapidly growing cells in Penassay Broth containing one-tenth the normal phosphate concentration and supplemented with $50 \mu\text{Ci}$ of $\text{H}_3\text{P}^{32}\text{O}_4$ per ml. After banding in CsCl , the phage had a specific activity of 1.5×10^6 counts per min per 10^{12} PFU.

Phage were assayed by the overlay technique as described by Reilly and Spizizen (12) with *B. subtilis* strain 12A as host. Burst size experiments were performed as described elsewhere (Hagen et al., *submitted for publication*).

Pulse-labeling and gentle lysis of infected cells.

DNA in uninfected or phage-infected cells was normally pulse-labeled with ^3H -thymidine (0.1 or 0.01 mCi/ml of culture) for 3 min. In experiments requiring gentle cell lysis, the pulse was terminated by the addition of an equal volume of the lysis mixture described by Botstein (4), and the lysates were prepared for sucrose gradient analysis as described by Smith and Levine (16). When the rate of DNA synthesis was being determined, the pulse was terminated by the addition of an equal volume of iced 10% trichloroacetic acid. Duplicate samples were then washed through 25-mm membrane filters (0.45- μm pore size, HA type, Millipore Corp.) as described by Smith and Hanawalt (15). After drying, the filters were counted for radioactivity.

Sucrose gradient sedimentation. Zone sedimentation through sucrose gradients was carried out as described by Botstein (4) and Smith and Levine (16). Sedimentation was for 90 min at 35,000 rev/min in the SW39 swinging bucket rotor in a model L2-65 Spinco ultracentrifuge. The gradients were fractionated by puncturing the bottom of the tube and collecting the drops on Whatman 3MM filter discs. The discs were treated as described by Botstein (4), and the radioactivity was measured as described below.

DNA isolation. Phage DNA was quantitatively released from purified ϕ 29 particles by the addition of an equal volume of 4 M sodium perchlorate and incubation at 37 C for 60 min (1). After overnight dialysis against 0.01 standard saline citrate [(SSC) 0.15 M NaCl, 0.015 M sodium citrate] at 4 C, 0.1% sodium lauryl sulfate (final concentration), and 1 mg of Pronase per ml were added. After incubation at 37 C for 15 hr, the mixture was extracted twice with phenol (11). The DNA was dialyzed overnight against SSC and stored at 4 C.

B. amyloliquefaciens DNA was extracted, purified, and stored as described by Reilly and Spizizen (12).

DNA-DNA hybridization. The method of Denhardt (6) as modified by Kelly and Thomas (11) was used for DNA-DNA hybridization on membrane filters.

Radioisotopes and radioisotope counting procedures. Carrier-free $\text{H}_2\text{P}^{32}\text{O}_4$ and ^3H -thymidine (18.35 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass. Radioactive samples were counted with a Packard Tri-Carb liquid-scintillation spectrometer as described elsewhere (14).

RESULTS

Phage production at various temperatures. Table 1 shows the effect of temperature on the burst size of phage ϕ 29 in *B. amyloliquefaciens* H. The average burst size of the wild-type phage drops slightly as the temperature is raised from 37 to 45 C. The wild-type burst at 45 C was 45 to 50% of the burst at 37 C. Table 1 also presents burst size data obtained with three ϕ 29 temperature-sensitive mutants located in well-scattered cistrons (Hagen et al., submitted for publication). It is apparent that all of the mutants are sensitive to growth at 43 C and infectious particle development is completely blocked at 45 C. It is of in-

terest that two of the mutants are also somewhat temperature-sensitive at 37 C. Additional data on these mutants is presented elsewhere (Hagen et al., submitted for publication).

Before studying the growth of temperature-sensitive mutants under nonpermissive conditions, we determined whether there were any effects other than a reduction in burst size on the development of wild-type phage at 45 C. The ability of wild-type phage ϕ 29 to propagate at 37 C (Fig. 1A) was compared to development at 45 C (Fig. 1B). Adsorption of the phage was reduced slightly at 45 C, although the appearance of chloroform-resistant, infectious intracellular particles occurs at about 30 min in both cases. There is a reduction of 50 to 55% in the final yield of phage at the high temperature. Phage infection at 37 C has little effect on the increase in optical

TABLE 1. Effect of temperature on the average burst size of phage ϕ 29 and several temperature-sensitive mutants^a

Phage	Cistron	Temp of incubation			
		37 C	40 C	43 C	45 C
Wild-type		570	430	300	260
35	5	470	300	0	0
136	9	570	340	40	0
225	11	340	170	10	0

^a Burst size numbers represent the average of three different experiments done in triplicate.

FIG. 1. Effect of temperature on development of phage ϕ 29. Cultures were incubated at 37 C (A) and 45 C (B) to obtain 2×10^8 cells per ml and infected with phage at a multiplicity of 5 PFU per cell. Culture turbidity and phage production were determined. Symbols: ●, Klett₅₆₀ units; ○, PFU per ml of culture.

density of a culture (Fig. 1A). This was expected since previous studies (Schachtele, *unpublished observations*) had shown that phage $\phi 29$ has little effect on host macromolecular synthesis when infection is carried out at this temperature. However, as illustrated in Fig. 1B, phage infection at 45 C had a marked effect on the increase in optical density. There is an immediate inhibition of cell mass increase which lasts about 10 min. The optical density of the 45 C culture then increases for 30 to 40 min, reaching about 60% of the optical density of the 37 C culture prior to cell lysis.

DNA synthesis in infected cultures at 37 and 45 C. To find an explanation for the inhibitory effect of infection at 45 C, pulse-labeling with ^3H -thymidine was used to determine the rate of DNA synthesis during wild-type phage development at 37 and 45 C. Figure 2 illustrates that the rate of synthesis at 37 C remains almost constant for about 15 min and then rapidly increases to a peak at about 30 min. In contrast, the rate of DNA synthesis at 45 C drops for about 22 min and then increases to a maximum at about 45 min. Thus, the pattern of DNA synthesis at the two temperatures is different, and the drop in rate after infection at 45 C indicated that both host and viral DNA synthesis might be effected.

Appearance of viral DNA at 37 and 45 C. Figure 3A illustrates the separation of host DNA and $\phi 29$ DNA which can be obtained by gentle cell lysis and sedimentation through a 5 to 20% sucrose gradient. Since the viral DNA is small (11×10^6 daltons) compared to the gently extracted host DNA (about 120×10^6 daltons), the two species can be readily separated and identified by this procedure. Samples of a culture infected with phage $\phi 29$ at 37 C were pulse-labeled with ^3H -thymidine for 3 min, gently lysed, and analyzed by zone sedimentation. At 37 C, viral DNA first begins to appear between the 9th and 12th min, and is synthesized in large quantities by the 30th min (Fig. 3 B-E). In samples from cultures grown at this temperature, there was always label which sedimented where host DNA was normally found, although the shape of the peak was variable.

Figure 4 presents a similar experiment except that the infection was carried out at 45 C. In this case, viral DNA could not be detected before the 18- to 21-min pulse. It is important that, in these samples, there was a time interval (18 to 21 min) when no label could be found in the area of the gradient where host DNA normally banded. Later in infection (Fig. 4E), radioactive DNA could be found near this region, and its location and size were variable. The nature of this DNA will be discussed later. It appears that the differences in the rate and quantity of DNA synthesis in infected cultures incubated at the two temperatures are

FIG. 2. Rate of DNA synthesis in cultures infected with phage $\phi 29$ at 37 and 45 C. Bacteria were grown at each temperature to a density of 2×10^8 cells per ml and infected with phage at a multiplicity of 10 PFU per cell. Samples of each culture were pulse-labeled with ^3H -thymidine, and the amount of label in DNA was determined. Symbols: $\circ$, infection at 37 C; $\bullet$, infection at 45 C.

the consequence of inhibition of host DNA synthesis and a later onset of phage DNA replication at 45 C.

Figure 5 presents an experiment in which the synthesis of viral-specific DNA at 37 and 45 C was monitored by DNA-DNA hybridization. Phage $\phi 29$ DNA could be detected in the 37 C culture in a sample pulse-labeled with ^3H -thymidine between the 12th and 15th min after infection. In contrast, $\phi 29$ DNA could not be detected before the 18- to 21-min pulse in the samples from a 45 C culture. These findings support the observation that incubation at 45 C has an effect on the onset of viral DNA synthesis which is not apparent at 37 C.

Analysis of DNA synthesis by temperature-sensitive mutants at 45 C. The preceding experiments indicated that infection with phage $\phi 29$ at 45 C presented conditions in which the replication of viral DNA could be studied in the absence of host DNA synthesis. Analysis of the ability of selected temperature-sensitive mutants to induce

FIG. 3. Sucrose gradient analysis of pulse-labeled DNA from cells infected with phage $\phi 29$ at 37 C. Uninfected (A) or infected (B-E) bacteria were pulsed with ^3H -thymidine, and the nascent DNA was analyzed by zone sedimentation. Symbols: $\circ$, ^3H -thymidine-labeled DNA (counts per minute per milliliter); $\bullet$, ^{32}P -labeled DNA from phage added to an uninfected cell extract before gentle lysis (counts per minute per milliliter).

FIG. 4. Sucrose gradient analysis of pulse-labeled DNA from cells infected with phage $\phi 29$ at 45 C. Infected bacteria were pulsed with ^3H -thymidine, and the DNA was analyzed by zone sedimentation.

FIG. 5. Effect of temperature on production of viral-specific DNA. Pulse-labeled DNA was isolated from phage ϕ 29-infected cells and hybridized to cold ϕ 29 DNA. Symbols: ○, samples from culture infected at 37°C; ●, samples from culture infected at 45°C.

viral DNA synthesis at 45°C was performed by pulse-labeling infected cultures for 3 min with ^3H -thymidine at 30 min after infection, gently lysing the cells, and sedimenting the lysates through sucrose gradients. Figure 6A presents a control gradient in which the sample was from cells infected with wild-type phage. Both normally and heavily sedimenting DNA can be seen. Figure 6B illustrates results obtained with mutant 35 from cistron 5. This mutant appears to make little if any viral DNA at 45°C, although there is a small amount of rapidly sedimenting DNA present toward the bottom of the gradient. Figure 6C shows results obtained with mutant 136 from cistron 9. This mutant appears to make an intermediate level of viral DNA when infection is carried out at the nonpermissive temperature. Although the DNA which is found toward the bottom of the gradient has not been analyzed, it would be of interest to determine whether it is viral or host DNA. Figure 6D represents results obtained with mutant 225 from cistron 11. This mutant produces normal or elevated levels of viral DNA at 45°C.

Since the above experiments analyzed the ability of mutants to induce DNA synthesis at only one specific time after infection, it was possible in the case of mutant 35 that replication occurred earlier in infection and then ceased or was delayed until later in the lytic cycle. In addition, it was of interest to determine the time-course of synthesis of the DNA produced by mutant 136. Table 2 presents the results of an experiment in which DNA-DNA hybridization was used to

FIG. 6. Zone sedimentation of pulse-labeled DNA from cells infected with temperature-sensitive mutants under nonpermissive conditions. Bacteria were grown at 45°C and infected with wild-type or mutant phage at an input multiplicity of 10 PFU per cell. Samples of each culture were labeled with ^3H -thymidine at 30 min after infection for a total of 3 min. After gentle lysis, the labeled DNA was analyzed. A, Sample from wild-type phage ϕ 29-infected culture; B, sample from mutant 35-infected culture; C, sample from mutant 136-infected culture; D, sample from mutant 225-infected culture.

follow the production of viral-specific DNA by mutants 35, 136, and 225 at 45°C. The results with mutant 35 indicate that this mutant does not induce viral DNA synthesis for at least 57 min after infection. In contrast to this, mutant 225 makes viral DNA at 45°C in a manner almost identical to that of wild-type phage. Mutant 136 is of particular interest since the onset of viral

DNA synthesis is essentially normal (21 to 24 min), but replication is retarded and never reaches the wild-type rate. The mechanism for this inhibition of replication has not been clarified.

Rapidly sedimenting ϕ 29 DNA. The nature of the pulse-labeled DNA which sedimented more rapidly than mature ϕ 29 DNA molecules in several of our experiments was investigated. If this DNA is a precursor to mature DNA molecules, one should be able to chase the label from the lower portion of the gradient into the normal phage DNA band by incubating infected pulse-labeled cells in a medium containing an excess of nonradioactive thymidine. Table 3 presents an experiment in which cells infected with wild-type

phage ϕ 29 were incubated at 45 C to allow inhibition of host DNA synthesis, and 30 min after infection were pulse-labeled for 3 min with 3 H-thymidine. The pulse was terminated by rapidly transferring the cells to fresh prewarmed medium containing excess thymidine. Samples of the culture were removed at various intervals and their labeled DNA was analyzed by sucrose gradient sedimentation. It is apparent that the amount of DNA in both the rapidly sedimenting and normal ϕ 29 DNA bands remained almost constant even when the chase was continued for 15 min. Thus, it appears unlikely that the fast-sedimenting DNA is a precursor of mature ϕ 29 DNA molecules.

The rapidly sedimenting DNA and the normally sedimenting mature ϕ 29 DNA observed in the above experiment were collected from a set of tubes centrifuged under identical conditions, and the pooled samples were analyzed by DNA-DNA hybridization. Table 4 presents the results of this study and clearly shows that essentially all of the rapidly sedimenting label is in ϕ 29 DNA and that there is only a small amount of host DNA present. Table 4 also shows that there is no labeled host DNA present in the band representing mature ϕ 29 DNA molecules.

Various attempts were made to convert the radioactive rapidly sedimenting DNA to molecules which would sediment with normal ϕ 29 DNA in sucrose gradients. Two techniques were immediately successful. First, shearing the sample, by drawing it through a small bore pipette several times before layering onto the gradient, caused the heavily sedimenting DNA to band at

TABLE 2. *Production of viral-specific DNA by temperature-sensitive mutants at 45 C*

Phage	Cistron	Per cent hybridization at ^a					
		12-15 min ^b	21-24 min	27-30 min	36-39 min	45-48 min	54-57 min
Wild-type		0.0	0.8	4.4	8.0	7.0	6.3
35	5	0.0	0.0	0.0	0.0	0.0	0.0
136	9	0.0	0.7	2.7	4.9	4.0	2.2
225	11	0.0	0.6	5.4	6.4	7.5	3.0

^a Pulse-labeled DNA was isolated and hybridized to nonradioactive viral DNA baked on membrane filters.

^b Time of pulse.

TABLE 3. *Effect of chase on pulse-labeled rapidly sedimenting DNA*

Time after transfer to chase medium ^a	Total counts/min ^b	
	Normally sedimenting DNA (fractions 24-30)	Rapidly sedimenting DNA (fractions 10-20)
min		
0	3,719	2,185
3	3,752	2,044
6	4,465	3,251
9	4,021	4,049
12	4,304	2,703
15	4,213	3,720

^a Cells infected with wild-type phage ϕ 29 were incubated at 45 C for 30 min and then pulse-labeled with 3 H-thymidine for 3 min. After rapid transfer to fresh medium containing an excess of nonradioactive thymidine (1 mg/ml), samples were removed and their DNA content was analyzed by extraction and zone sedimentation.

^b The total counts per minute represents the amount of radioactivity in each of the indicated fractions determined as described in Materials and Methods.

TABLE 4. *Binding of rapidly sedimenting and normally sedimenting DNA to host and ϕ 29 DNA^a*

Sample	Total counts/min bound to filter	
	Host DNA	ϕ 29 DNA
Rapidly sedimenting DNA (1,075 counts/min) ^b	86	1,025
Normally sedimenting mature ϕ 29 DNA (2,920 counts/min) ^b	34	2,900

^a DNA was labeled and analyzed as described in Table 3. Pooled samples obtained from sucrose gradients were dialyzed against SSC for 18 hr and incubated on membrane filters containing an excess of nonradioactive host or phage DNA (10 μ g/filter).

^b These numbers represent the average counts per minute added before DNA-DNA hybridization.

the position of mature $\phi 29$ DNA molecules (Table 5). Whatever the structure of the rapidly sedimenting DNA, it could be altered to a given size by relatively gentle shearing. Second, allowing the sample to sit at room temperature for an additional 4 to 5 days prior to centrifugation caused a reduction in amount or a loss of the rapidly sedimenting radioactivity and an increase in radioactivity in the $\phi 29$ DNA band (Table 5).

DISCUSSION

The effect of elevated temperature (from 37 to 45 C) on the inhibition by phage SP82 of protein and DNA synthesis in *B. subtilis* was studied by Hayward (10). It was demonstrated that a short incubation of the cells at 45 C before infection resulted in interference with the ability of the phage to inhibit host protein and DNA synthesis as it normally did at 37 C. Prolonged pregrowth at 45 C eliminated the interference. It was suggested that the inhibition of host DNA synthesis at either temperature was the result of reduction of the thymidine triphosphate pool after infection.

Our work with phage $\phi 29$ presented in this paper gave a very different result when infection was studied at 45 C. As illustrated in Fig. 1, infection with phage $\phi 29$ at 45 C had an immediate reproducible effect on growth of the host cells accompanied by inhibition of host DNA synthesis (see Fig. 4). In addition, the onset of viral-specific DNA synthesis at the elevated temperature is delayed for 6 to 9 min. Although we have no immediate explanation for these inhibitory effects, it is possible that at 45 C an infected cell is

unable to repair some damage normally caused by phage infection. It is hoped that studies with phage $\phi 29$ mutants will reveal whether it is a viral function that is being expressed at 45 C and not at 37 C. Since phage $\phi 29$ DNA does not contain any unusual bases (Anderson and Warner, *unpublished observation*), it is unlikely that the inhibitory effects on DNA synthesis are due to depletion or alteration of the nucleoside triphosphate pools.

Conditional-lethal mutants have been utilized by several investigators to explore the replication of double-stranded phage DNA. For example, amber mutants have allowed detection of intermediates in the replication of T4 DNA (8), and temperature-sensitive mutants have been used to analyze the synthesis and maturation of P22 DNA (5). In this report we present some preliminary work with several temperature-sensitive mutants of phage $\phi 29$ and demonstrate that mutants are available which (i) make no viral DNA under nonpermissive conditions (mutant 35), (ii) make an intermediate level of viral DNA (mutant 136), or (iii) make normal quantities of phage DNA (mutant 225). No mutants have been detected which show abnormal sedimentation characteristics when their replicating DNA is analyzed by zone sedimentation. Additional work with these and other mutants (Hagen et al., *submitted for publication*) at 45 C will hopefully allow us to investigate in detail the replication of $\phi 29$ DNA.

Rapidly sedimenting intracellular forms of phage DNA have been reported by several investigators. Probably the best characterized form is the superhelix found in cells infected with the temperate phage lambda (3, 21) and P22 (13, 19). As discussed by Thomas, Kelly, and Rhoades (19), this entity is probably not an intermediate in replication but a product of the replicative process. Other fast-sedimenting molecules have been detected during the development of T4 (7, 8), T5 (17), T7 (11), P22 (4), and lambda (22). All of these forms appear to be intermediates in the formation of mature viral DNA molecules, and as demonstrated by Kelly and Thomas (11) with electron microscopy, these molecules probably represent concatemers. In this report we have presented evidence that the rapidly sedimenting pulse-labeled DNA found in phage $\phi 29$ -infected cells is almost exclusively viral (Table 4), but that it is probably not an intermediate in the formation of mature $\phi 29$ DNA molecules since it cannot be chased into the latter form (Table 3). We feel that the rapidly sedimenting $\phi 29$ DNA represents viral DNA molecules which are trapped by the large pieces of host DNA present in the gradient. Our evidence for this is the conversion of the labeled DNA to material that sediments

TABLE 5. Sedimentation of gently extracted DNA after shearing and storage^a

Sample	Total counts/min	
	Rapidly sedimenting DNA (fractions 10-20)	Normally sedimenting mature $\phi 29$ DNA (fractions 24-30)
Untreated	2,241	6,320
Gently sheared ^b . . .	112	7,967
Stored at 24 C for 5 days ^c	510	8,245

^a Infected cells were pulse-labeled with ³H-thymidine for 30 min after infection at 45 C and were gently lysed. Zone sedimentation was performed as described in Materials and Methods.

^b The labeled sample was gently sheared by drawing the lysate three times through a small-bore pipette before layering on the gradient.

^c The sample was stored at 24 C for 5 additional days prior to sedimentation. The untreated sample was centrifuged 48 hr after preparation.

with mature ϕ 29 DNA molecules by allowing the samples to remain at 24 C for 5 to 7 days before gradient analysis or by gently shearing the DNA to reduce the viscosity of the sample (Table 5). As discussed by Smith and Levine (16), allowing the samples extracted by gentle lysis procedures to remain at room temperature causes disaggregation of DNA molecules. If samples from phage ϕ 29-infected cells are analyzed on gradients the same day that they are prepared, the bulk of the mature DNA cosediments with host DNA.

We made attempts to isolate rapidly sedimenting forms of ϕ 29 DNA employing very short pulses with ^3H -thymidine and DNA extraction and analysis as described by Kelly and Thomas (11) and others. Although we detected small quantities of label which sediment faster than the mature DNA peak, chase experiments have not been successful. We were recently able to detect attachment of ^{32}P -labeled parental DNA to a fast-sedimenting component, and this work will be presented elsewhere. Additional work with temperature-sensitive mutants of phage ϕ 29 are in progress, and we hope that they will allow us to uncover the mode of replication of the phage ϕ 29 genome.

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