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SYNTHESIS OF DNA CONTAINING URACIL DURING BACTERIOPHAGE INFECTION OF *Bacillus subtilis*

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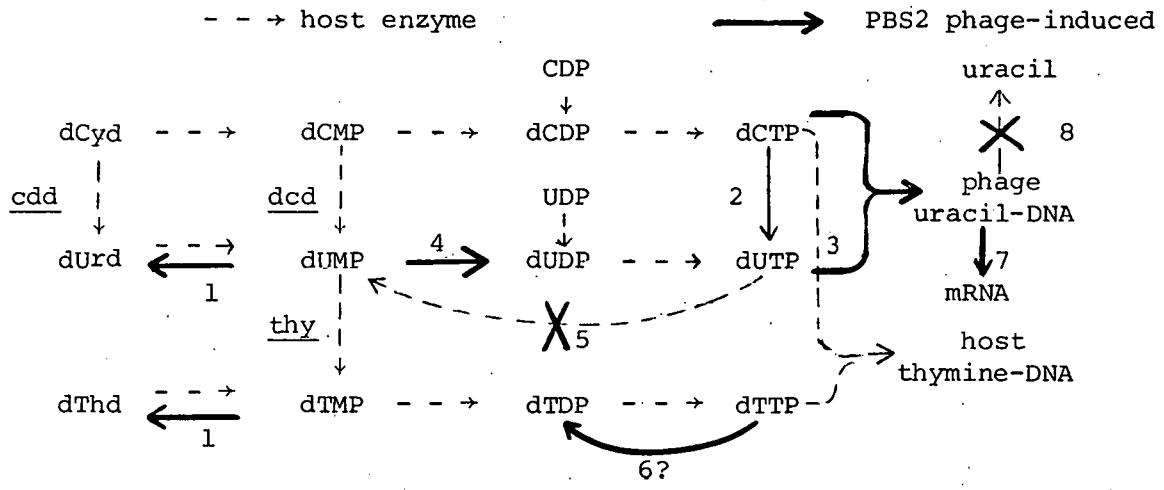
Title: Synthesis of DNA Containing Uracil during Bacteriophage Infection of Bacillus subtilis

Abstract:

We have studied the biosynthesis of enzymes and nucleotides to understand how the bacteriophages PBS1 and PBS2 can make uracil-containing DNA in a cell which normally makes thymine-containing Bacillus subtilis DNA. Using our newly developed chromatographic system, we have discovered dUTP in infected cells (the first demonstration ever of this compound in vivo). Labelling studies in mutant cells proved that dUTP was derived from phage-induced dCTP deamination as well as ribonucleotide reduction. We also discovered two dTMP synthetases in B. subtilis, both of which remain active after PBS1 phage infection (although dTTP disappears from infected cells). The purified PBS2-induced DNA polymerase has been characterized physically and kinetically; it has similar affinities for uracil- and thymine-containing DNAs and triphosphates as substrates.

Introduction

Bacteriophage PBS1 and its clear-plaque-variant PBS2 contain DNA with uracil instead of thymine, which is found in most DNAs (1,2). Based on the known Bacillus subtilis enzymes and by analogy to the well-studied T4 phage infection of Escherichia coli (3), we have proposed the following scheme for the modification of B. subtilis deoxyribonucleotide metabolism after PBS2 infection:



As indicated in the above scheme in solid lines, the infection of B. subtilis by phage PBS2 results in the induction of proteins believed to allow the phage to accumulate dUTP in a cell which normally makes dTTP for DNA synthesis. Those PBS2 proteins include:

	PBS2 protein induced	Product	Reference
1.	dTMPase (dUMPase	dThd + Pi dUrd + Pi at high K_m)	Kahan abstract (4); Price and Fogt (5)
2.	dCTP deaminase	dUTP + NH_3	Tomita and Takahashi (6); Price (7)
3.	DNA polymerase	DNA	Price and Cook (2)
4.	dUMP kinase (ATP)	dUDP + ADP	Kahan abstract (4)
5.	inhibitor of host's dUTPase	less dUMP made	Price and Frato (8,9)
6.	dTTPase (?)	dTDP	Tomita and Takahashi (10)
7.	RNA polymerase	mRNA	Price and Frabotta (11); Clark et al. (12); Price et al. (13)
8.	inhibitor of host's N-glycosidase	less uracil made	Tomita and Takahashi (14); Friedberg et al. (15,16); Katz, Price, and Pomerantz (17)

We have continued our investigations on these PBS2-induced functions and the host enzymes, to learn how this unique virus can make uracil-DNA in a cell which normally makes thymine-DNA. Our work for the past year is summarized below.

PBS2-induced inhibitor of host's N-glycosidase

Last year's Progress Report contained our data on an enzymatic activity in B. subtilis extracts which specifically degraded PBS2 uracil-DNA. We tried to resolve conflicting reports by Tomita and Takahashi (14) of a Ca^{++}-activated endonuclease releasing deoxyuridine and by Friedberg et al. (15,16) of an EDTA-stable N-glycosidase releasing uracil. Our data support most of Friedberg's observations and have now been published (17). Tomita and Takahashi (personal communication, 1976) have as a result reexamined their spectral data and now withdraw their claim for deoxyuridine as the product.

Thus B. subtilis contains an N-glycosidase which clips uracil residues out of uracil-DNA. It must be important for the phage to induce an inhibitor (protein #8 above) of this enzyme, in order to allow the stable replication of PBS2 uracil-DNA. Indeed, such a PBS2 protein is induced in excess to prevent uracil-DNA degradation (14-17).

An interesting observation in this regard with another system has been made jointly by the groups of Lehman and Weiss (18). E. coli sof mutants (dnaS), first isolated as hyper-recombinatory and accumulating large amounts of 4S "Okazaki fragments", are now shown to be really dut mutants (lacking dUTPase activity). Apparently such mutants accumulate some dUTP and incorporate it into replicating DNA in place of dTTP; these uracil residues would then be attacked by the E. coli N-glycosidase and finally the "short Okazaki fragments" would be released. Indeed it seems plausible that a significant proportion of the Okazaki fragments in wild-type cells may be an "artifact" resulting from the excision of uracil in DNA and may not be true intermediates in so-called "discontinuous" DNA replication (18). [One of our papers on the B. subtilis dUTPase and a PBS2 inhibitor has now been published (9)].

Discovery of dUTP in PBS1-infected cells

Our model for PBS1-induced proteins altering B. subtilis deoxyribonucleotide metabolism (see scheme above) predicts that: (a) the dTTP in uninfected cells will disappear after phage infection; and (b) dUTP, which has never been detected before in vivo (19), should accumulate after PBS1 infection for uracil-DNA synthesis. Last year we described in the Progress Report a new two-dimensional thin-layer chromatographic system which we had devised to separate dUTP from dTTP and other pyrimidine nucleoside triphosphates.

This year Jan Neuhard and I are collaborating on investigations using his regular system (which does not resolve dUTP from dTTP; Ref. 20) and our new system. By labelling a uracil-requiring (pyr) strain in minimal medium with [6-^{3}H]uracil and extracting the pools with formic acid for chromatography in our new system, we were able to quantitate the levels of pyrimidine nucleoside triphosphates in uninfected and PBS1 phage-infected B. subtilis. The concentration of dTTP and the other triphosphates dropped dramatically within 3 to 5 min after infection, and then UTP, CTP, and dCTP quickly rose again to high levels. The dTTP concentration, however, remained low (less than 10% of its original value), and dUTP appeared and accumulated from 10 to 20 min after infection as phage DNA synthesis was beginning. Our demonstration [confirmed

with $^{32}P_i$ and [5-^{3}H]uracil labelling in the regular system (20) as well] represents the first time that dUTP has ever been detected *in vivo*, and it confirms our prediction that dUTP is the probable substrate for the replication of uracil-containing PBS1 DNA. This accomplishes a major goal originally set for the research under this contract.

To investigate the metabolic origin of dUTP in PBS1-infected *B. subtilis*, a similar experiment was performed using: (a) a *pyr cdd* strain (21) which lacks cytidine/deoxycytidine deaminase so it cannot degrade these compounds; and (b) our isolate of a derivative *pyr cdd dcd* strain which also lacks dCMP deaminase and thus has no pathways for converting cytosine- to uracil-containing compounds (see scheme above). Cultures were labelled with [5-^{3}H]-uracil in addition to $^{32}P_i$ and unlabelled cytidine (to reduce the amount of tritium entering cytosine-containing nucleotides). Uninfected and 30-min PBS1-infected samples were extracted as before for resolution of all 8 common ribo- and deoxyribonucleoside triphosphates in the regular system (20) followed by scintillation counting of both isotopes in each nucleotide. The uninfected cell's dTTP (and also the 4 purine compounds) contained no tritium, as expected since the 5-tritium from uracil in dUMP is lost on methylation to dTMP (22). From the $^3H/^{32}P$ ratio in dUTP (0.51) relative to that in UTP (0.88) and in CTP or dCTP (0.25), one can calculate that [^{3}H]dUTP in infected cells was derived 42% from reduction of uridine nucleotides versus 58% from deamination of cytidine nucleotides in the *cdd* strain (which has host dCMP deaminase and phage dCTP deaminase activities available; see above scheme). An identical experiment in the *cdd dcd* strain lacking the host dCMP deaminase gave similar results (46% versus 54% respectively), suggesting that the phage-induced dCTP deaminase is sufficient to make the necessary dUTP and that the host dCMP deaminase may not contribute much to dUTP production (or that activity of the phage enzyme increases to compensate for the absence of the host enzyme). A parallel experiment using uninfected cells labelled with [6-^{3}H]-uracil and $^{32}P_i$ showed that dTTP was derived 55% from uridine nucleotides and 45% from deoxycytidine compounds in the *cdd* strain, but 100% from uridine nucleotide reduction in the *cdd dcd* strain as expected (and proving that the *dcd* strain has no dCMP deaminase activity *in vivo*).

B. subtilis dTMP synthetases and PBS1 phage infection

We next considered whether the *B. subtilis* dTMP synthetase activity was inhibited after PBS1 infection, since infected cells contained little or no dTTP (see above) and the uracil-DNA phage would presumably have no need for dTMP. However, we found about 80% of normal dTMP synthetase activity [assayed by the release of tritium from [5-^{3}H]dUMP (22)] in either sonic extracts or toluenized cells.

Then in further collaborative efforts with Jan Neuhard, I investigated and helped to solve a "classical" problem in *Bacillus* genetics: in contrast to one *thy* gene in *Escherichia* and *Salmonella*, it takes *two* mutations (*thyA* and *thyB*) in *B. subtilis* to produce a growth requirement for thymine (23). The reason becomes apparent in our discovery that uninfected wild-type *B. subtilis* extracts contained two dTMP synthetases resolvable on a DEAE-cellulose column. Both of these peaks remained in PBS1-infected cells. The major peak A is controlled by the *thyA* gene, the minor peak B by the *thyB* gene, and either enzyme is sufficient alone to support normal growth rates without thymine. The two dTMP synthetases differ in their heat-stability (B is very labile), their K_M for dUMP (4 μM for A versus 20 μM for B), and the inhibition caused by dTMP and 5-F-dUMP (A is 10-fold more sensitive).

The dTMP synthetase A and B activities are also measurable in vivo, by growth in minimal or rich medium in the presence of [5-^{3}H]uracil, whose tritium is lost on conversion of [^{3}H]dUMP to dTMP in the cells (24). We demonstrated that the growth rate and the amount of tritium released per cell was the same in thyA$^+$thyB$^+$, thyA$^-$thyB$^+$, and thyA$^+$thyB$^-$ cells at 37° (and that thyA$^-$thyB$^-$ cells have very little activity and require thymine for growth). Furthermore, the rate of tritium release remained unchanged (60 to 100% of uninfected cells) for at least 30 min after PBS1 infection of each strain.

Different evidence to support this conclusion was derived by labelling in broth cultures with [5-^{3}H]deoxycytidine using the pyr cdd dcd strain. It can only convert the label to deoxycytidine compounds, since it lacks deoxycytidine deaminase (21) and dCMP deaminase; thus no deoxyuridine nucleotides can be formed so no tritium can be released by its dTMP synthetase reaction (less than 3% of the rate of tritium release of its parent cdd strain). However, PBS1 infection of labelled cdd dcd cells gave a rapid rate of tritium release from [5-^{3}H]deoxycytidine for over 20 min, presumably because the phage-induced dCTP deminase produced [^{3}H]dUTP which went to [^{3}H]dUMP to form dTMP and [^{3}H]H_2O (see above scheme). Indeed, this experiment proves that the PBS1-induced dCTP deaminase (enzyme #2) is functioning in vivo.

The experiments just described using [5-^{3}H]uracil or [5-^{3}H]deoxycytidine in vivo demonstrate that the activities of dTMP synthetases A and B continue after PBS1 phage infection. Thus the phage-induced dUMP kinase (enzyme #4) and the induced dUMPase [a high-K_M activity of the phage dTMPase, enzyme #1] do not seem to severely deplete the dUMP pool, necessary as substrate for the dTMP synthetases. And the "unneeded" dTMP which would then be formed in infected cells is presumably degraded by the phage-induced dTMPase (enzyme #1) to thymidine.

PBS2 phage-induced DNA polymerase

The dUTP and other triphosphates found in phage-infected cells are presumably polymerized enzymatically to form phage uracil-DNA. We observed a large increase in the specific activity of DNA polymerase after PBS2 infection of B. subtilis (2). This enzyme has now been purified 900-fold in 22% yield, as described in last year's Progress Report. The protein appears to form unstable aggregates and even precipitates in low salt solution, but in high salt it behaves on Sephadex columns and sucrose gradients like a stable enzyme of 142,000 daltons. When subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate, the preparation shows equimolar amounts of 2 subunits of 68,000 and 76,000 daltons. Studies of suppressor-sensitive PBS2 mutants have also indicated that 2 genes control the PBS2 DNA polymerase (25).

The purified phage DNA polymerase requires a high ionic strength for optimal activity (300 mM NaCl optimum at 14 mM $MgCl_2$, or 60 mM $MgCl_2$ optimum at 60 mM NaCl). The activity is completely dependent upon added DNA, using denatured DNA (uracil or thymine-containing DNA) much better than native DNA, and being most active on "activated" DNA (DNase-digested to fragments with single-stranded gaps). The apparent K_M for DNA decreases with decreasing DNA size, from 500 µg/ml for 10^7-dalton DNA to 20 µg/ml for 10^5-dalton denatured PBS2 DNA.

The PBS2 DNA polymerase requires all 4 common deoxyribonucleoside triphosphates for maximal activity on natural DNA templates. The enzyme displays competitive kinetics for dUTP ($K_M = K_i = 16$ µM) versus dTTP ($K_M = K_i = 7$ µM) with V_{max} values similar on single-stranded PBS2 uracil-DNA. Likewise the K_M

for dUTP (4 μM) and dTTP (3 μM) were low with thymine-containing salmon sperm DNA as the template. Thus the PBS2 polymerase like many other known polymerases, is rather non-selective in its substrate specificity, using uracil or thymine-containing DNAs and triphosphates at similar rates.

A nuclease activity is associated with the PBS2 DNA polymerase, as suggested by parallel purification, stabilization by albumin or salt solutions, effects of high NaCl concentrations on activity and molecular size, heat inactivation, inhibition by dextran sulfate (50% at 0.5 μg/ml), and preference for denatured DNA. Preliminary studies using ^{32}P-5'-labelled DNA containing [^{3}H]thymidine residues suggests that the attack is exonucleolytic from the 3'-end to produce 5'-monophosphate products. The nuclease is inhibited by the addition of the 4 triphosphates (polymerizing conditions); likewise, the production of mononucleotides from the triphosphates during polymerase assays is dependent upon added DNA. Thus these activities all appear to reside in the PBS2 DNA polymerase protein. Some of this information has been recently presented (26).

The evidence indicates that the PBS2 DNA polymerase is a different protein from the 3 B. subtilis DNA polymerases (27-29). We eliminate the host's polymerase I by a polA mutation. Polymerases II and III elute earlier off the phosphocellulose column than the phage enzyme. The phage polymerase is not active under the routine host polymerase assay conditions. Only the host's polymerase III is inhibited by 6-(p-hydroxyphenylhydrazino)uracil. Unlike polymerases II and III, the phage enzyme can use denatured DNA as template. In addition, the subunit size, NaCl optimum for activity, and inhibition caused by araCTP or N-ethylmaleimide for PBS2 DNA polymerase are all quantitatively different from that seen with the 3 host enzymes.

In conclusion, we have purified and characterized the PBS2 DNA polymerase, proving it is a unique enzyme different from the known host DNA polymerases. Although this enzyme probably synthesizes only uracil-containing DNA in vivo, inside a cell containing undegraded thymine-DNA, the phage DNA polymerase does not display any particular specificity for uracil- versus thymine-containing DNA nor for dUTP versus dTTP in vitro. Apparently the triphosphate pools are altered by phage-induced proteins, so that dTTP is excluded, even though dTMP synthesis continues. Then dUTP accumulates in the infected cell, to permit only uracil-DNA synthesis. As yet undiscovered factors must prevent the replication of thymine-containing host DNA after phage infection.

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