Bacteriophage PBS2-Induced Inhibition of Uracil-Containing DNA Degradation

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Degradation of uracil-containing DNA by *Bacillus subtilis* extracts and its inhibition after infection by the uracil-containing DNA phage PBS2 have been investigated to resolve differences between the published reports of Tomita and Takahashi (1975) and Friedberg et al. (1975, 1976). The product of hydrolysis of PBS2 DNA, tritium labeled in its uracil and cytosine residues, is solely uracil and not deoxyuridine. The degrading activity is completely inhibited within 7 min after PBS2 infection, before any other known PBS2-induced protein is detectable. The production of the PBS2 inhibitor (a small, heat-stable protein) continues until 10 to 20 min postinfection.

Bacteriophage PBS1 and its clear-plaque derivative PBS2 synthesize viral DNA containing uracil during infection of *Bacillus subtilis* (9, 13). To make uracil-DNA instead of thymine-DNA, this unique virus induces several proteins that alter the host's deoxyribonucleotide metabolism. These PBS2 proteins include thymidylate phosphohydrolase (10), deoxyuridine triphosphohydrolase (11), deoxyuridyldeninephosphohydrolase inhibitor (8), DNA polymerase (9), and RNA polymerase (1, 12) activities.

Recently, Tomita and Takahashi (15, 16) and Friedberg et al. (3) discovered that *B. subtilis* extracts contained an enzyme(s) that specifically degraded uracil-DNA and was inhibited after PBS1 and PBS2 phage infection. However, there were major differences between these papers (3, 15, 16) in the assay conditions, the nature of the product, and the time course of phage-induced inhibition reported for this activity. Therefore, we designed experiments to resolve the discrepancies between these reports and to compare this new viral function with the other PBS2 proteins we had previously characterized (7-12).

PBS2 phage infection of *B. subtilis* SB19 and harvesting of cells was performed as described previously (9), except that a richer medium was used (see Fig. 1) which typically gives a burst of 100 phage/cell after infection of 2 × 10⁶ cells/ml. Extracts of infected cells were prepared with lysozyme plus gentle sonic treatment and centrifugation (9), using either Takahashi's buffer (50 mM Tris-hydrochloride, pH 7.5, as in reference 15) or Friedberg's buffer (10 mM Tris-hydrochloride, pH 8.0, as in reference 3). Extracts were used immediately or were stored at −20°C, where they were stable for at least a year. The extracts were assayed for uracil-DNA-degrading activity using Takahashi's (15) conditions (375 mM Tris-hydrochloride buffer, pH 8.5, with 0.5 mM CaCl₂ and 5 mM 2-mercaptoethanol) or Friedberg's (3) conditions (10 mM potassium phosphate buffer, pH 7.0, with 1 mM EDTA). Our assays (100 μl total volume) contained 1.5 μg (28,000 cpm) of PBS2 [³H]DNA, prepared as described by Friedberg et al. (3) from phage-infected cultures treated with [6-³H]uracil to label the uracil and cytosine residues (11). After incubation of reaction mixtures at 37°C for 15 min, acid-soluble radioactivity was measured as described (3, 15). The amount of enzyme added was adjusted to ensure less than 40% hydrolysis of the added DNA, so that product formation was proportional to the amount of enzyme and to the time of incubation. Specific activities were calculated from protein concentrations measured as per Lowry et al. (6).

We first investigated the relative specific activities of various enzymes in extracts of *B. subtilis* prepared at different times after PBS2 phage infection (see Fig. 1). The results shown in the lower panel confirm our previous observations (9-11) that PBS2 induces several new activities (deoxothymidylate phosphohydrolase, DNA polymerase, deoxyuridyldenine kinase, and deoxouridyldeninetriphosphohydrolase inhibitor) beginning about 10 min after phage infection. (The extract buffers [3, 15] used here are not optimal for several PBS2 proteins. The PBS2 deoxouridyldeninetriphosphohydrolase inhibitor [11] is unstable here, so the typical 80 to 90% inhibition [8, 11] is not seen in Fig. 1. The PBS2
deoxycytidine triphosphate deaminase is unstable here in the absence of thiol compounds [7], and the PBS2 RNA polymerase activity is undetectably low in these DNA-rich extracts [12]. In contrast, the results in the upper panel indicate that the B. subtilis enzyme(s) degrading uracil-DNA is progressively inhibited from the 3rd to the 7th min after infection. Friedberg et al. (3) had reported that the level of this activity (called an "N-glycosidase" because it produced only uracil) per milliliter of extract declined from the 2nd to the 4th min after infection (although specific activities were not given and protein concentrations varied). Tomita and Takahashi (15, 16) had indicated that the specific activity (which they said was due to a "nuclease" because it produced deoxyuridine) declined from the 10th to the 20th min after infection. In neither case (3, 15) were other PBS2 enzymes measured. Thus, our data (Fig. 1) support the time course of Friedberg et al. (3), who have also shown a similar course (2) when Ca++ is added to [32P]DNA to simulate Takahashi's conditions (15). Our assays, using extracts prepared in Friedberg's or Takahashi's buffer and assayed under both Friedberg's and Takahashi's conditions (see above), indicate that uracil-DNA degrading activity is indeed inhibited early after infection, before the appearance of any other known PBS2-induced protein (3, 9–12, 14, 15). Furthermore, chloramphenicol (see 3 and 15) and actinomycin D prevented this inhibition; rifampin, which blocks host but not phage mRNA synthesis (1, 12), had no effect, indicating that the inhibition of uracil-DNA degrading activity is indeed a phage-controlled event.

Silica gel chromatography with standards (3) demonstrated that [3H]uracil, and no [3H]deoxyuridine or [3H]cytosine, was released from our [3H]-labeled PBS2 DNA under Friedberg's and Takahashi's assay conditions. This confirms Friedberg's data (2, 3), but contradicts Takahashi's report that only deoxyuridine was detectable by chromatography in an isopropanol-HCl solvent (16). However, we find that the 7 M urea present in these authors' nucleoside fraction (16) can cause uracil to migrate like an external deoxyuridine standard, and thus the product may have been misidentified (16). Therefore, it is likely that Friedberg (2, 3) and Takahashi (15, 16) were both assaying an N-glycosidase releasing uracil from PBS2 DNA.

The characteristics of the B. subtilis uracil-DNA-degrading enzyme(s) have been briefly

min after infection, producing \(1.4 \times 10^{10}\) phage/ml by 2 h.
investigated. Tomita and Takahashi (15, 16) and Friedberg et al. (2, 3) found that enzyme preparations from uninfected cells hydrolyzed double-stranded DNA containing uracil, but did not degrade double-stranded DNA containing thymine, 5-bromouracil, or 5-hydroxymethyluracil. We find a similar specificity for native PBS2 DNA in our extracts of infected cells. Furthermore, Tomita and Takahashi (15) had indicated that the specific activity of hydrolysis of single-stranded PBS2 DNA remained constant after infection and that partially purified PBS1 inhibitor did not affect the degradation of denatured DNA. In contrast, we found that 10- to 30-min extracts of infected cells prepared in Takahashi's buffer were 70% reduced relative to the uninfected level of activity under Takahashi's conditions. We also observed that partially purified PBS2 inhibitor could inhibit the hydrolysis of single-stranded PBS2 DNA by about 60% in crude extracts or by 90% in a partially purified enzyme preparation. This is in agreement with the finding that the N-glycosidase from B. subtilis (2, 3) or Escherichia coli (2-5) attacks both single- and double-stranded uracil-DNA, releasing only uracil.

We (Fig. 1) and others (3, 15) had observed that uracil-DNA degrading activity disappeared after phage infection and that extracts of infected cells inhibited the activity of uninfected cell extracts. However, it was not clear whether production of the inhibitor continued in vivo after the inhibition of the uninfected cell's activity was complete. Thus, we quantitated the amount of inhibitor in extracts of cells infected for 10, 20, or 30 min; these infected extracts contained no residual degrading activity (Fig. 1). The results in Fig. 2 show that more inhibitor is present in 20- and 30-min infected extracts than in the 10-min infected extracts. The amount of infected extract protein required to inhibit 50% of the activity of 14 μg of uninfected extract protein was 5 μg for the 10-min and about 1 μg for the 20- and 30-min infected extracts. Even though Takahashi's conditions gave threefold more acid-soluble products in the absence of inhibitor, the amount of inhibitor detected here was similar under Takahashi's or Friedberg's conditions of assay. Thus, the synthesis of the PBS2-induced inhibitor of the B. subtilis uracil-DNA degrading activity continues from 3 min to between 10 and 20 min after infection (Fig. 2), so that the PBS2 inhibitor is present in considerable excess of the amount needed to eliminate the host's activity (Fig. 1). The inhibitor seems to be a small, heat-stable protein (2, 15).

Finally, since uninfected cell extracts contain a potent N-glycosidase activity, it seemed plausible to us that this enzyme would attack injected PBS2 DNA in vivo, releasing part of the uracil before the PBS2 inhibitor was synthesized (Fig. 1). To test this possibility, we prepared CaCl2 gradient-purified PBS2 phage labeled with [2-3H]adenine or [6-3H]uracil (3); we demonstrated by formic acid hydrolysis (11) that all of the tritium was in adenine (over 90%) or in uracil and cytosine (at a 2:1 ratio), respectively. The labeled phage were used to infect cells at a multiplicity of infection of 0.5 (1,700 cpm/ml), 1, 2, 5, or 10 (35,000 cpm/ml); 1-ml samples were removed at 1- to 5-min intervals. At any multiplicity of infection tested, all of the tritium remained acid precipitable throughout the infection. No [3H]uracil was released (less than 5%), even with 100 μg of unlabeled uracil added per ml of medium, whereas 40% of the label was transferred to progeny phage. Therefore, the host's N-glycosidase does not appear to attack parental PBS2 DNA in vivo. Perhaps the phage injects some of its inhibitor with DNA to protect the DNA locally; we previously suggested that PBS2 may inject some of its own rifampin-resistant RNA polymerase (1, 12).

It will be of interest to purify the B. subtilis N-glycosidase and PBS2 inhibitor further (15, 16) to determine the mechanism of inhibition. Host mutants lacking N-glycosidase may indicate whether this enzyme does function to help exclude uracil from cellular DNA, and phage mutants defective in the inhibitor should prove whether this PBS2 protein indeed is necessary

![Fig. 2. Inhibition of uninfected B. subtilis uracil-DNA degrading activity by PBS2-infected extracts. Extracts were prepared in Friedberg's buffer from uninfected or 10-, 20-, and 30-min infected cells. Extracts were diluted in buffer containing bovine serum albumin at 1 mg/ml when necessary. Assays were performed for 15 min under Friedberg's conditions (O) or Takahashi's conditions (□) using 14 μg of uninfected extract protein and the indicated amount of infected extract protein to determine the amount of inhibitor in infected extracts. Similar results were obtained with extracts prepared in Takahashi's buffer.](http://jvi.asm.org/DownloadedFrom/91y41y3wmj165w1671j651671y3wmj16516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516516
during phage infection to allow the synthesis of uracil-DNA.

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LITERATURE CITED


