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Bacillus subtilis DNA Polymerase III Is Required for the Replication of DNA of Bacteriophages SPP-1 and Ø105

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The replication of the *Bacillus subtilis* bacteriophages SPP-1 and Ø105 is sensitive to 6-(*p*-hydroxyphenylazo)-uracil (HPUra), a selective inhibitor of replicative DNA synthesis of *B. subtilis* which acts specifically at the level of a replication-specific polymerase, DNA polymerase III (pol III). The origin of the HPUra-sensitive polymerase required for phage replication was examined by comparison of the drug sensitivity of phage development in a normosensitive host with that in a host carrying *azp-12*, a *polC* mutation that specifies production of an HPUra-resistant pol III. *azp-12* specified HPUra-resistant phage development, clearly indicating that SPP-1 and Ø105 replication requires the host pol III. The host pol III requirement for SPP-1 replication also was confirmed by the demonstration that phage development was temperature sensitive in a host mutant carrying the *polC* mutation *mut-1* (*ts*). Examination of the pol III activity of crude and purified cell-free preparations derived from phage-infected cells did not indicate any detectable changes in the specific activity, purification behavior, or drug sensitivity of the enzyme.

We have sought to define a relevant in vitro system suitable for the dissection of the structure and function of the replication-specific form(s) of *Bacillus subtilis* DNA polymerase III (pol III; 3, 6, 7, 14). We have considered as a basis for this in vitro system a *B. subtilis*-specific, double-stranded DNA bacteriophage whose replicative DNA synthesis requires the host pol III. We describe below the identification of two bacteriophages, SPP-1 (8, 18) and Ø105 (20), which possess the latter requirements. The characterization exploits: (i) 6-(*p*-hydroxyphenylazo)-uracil (HPUra), a selective, reversible inhibitor of the replicative synthesis of *B. subtilis* (4, 5) which acts specifically at the level of pol III (2, 3, 6, 7, 11, 15, 16); (ii) the observations that the development of phage Ø105 (20) and SPP-1 (8) is sensitive to HPUra; (iii) *azp-12*, a *polC* mutation (13) that specifies a mutant, HPUra-resistant pol III and confers on its host an HPUra-resistant phenotype (6); and (iv) *mut-1* (*ts*), a *polC* mutation (3) that specifies a temperature-sensitive pol III and a corresponding temperature-sensitive phenotype.

MATERIALS AND METHODS

Source and growth of bacteria and bacteriophages. All bacterial strains were derived from *B. subtilis* 168 and were susceptible to lytic infection with the bacteriophages used. 168 *thy ind* was the strain isolated by Farmer and Rothman (9); BD54 (*ile-1, leu-8, metB5*) and BD54 *azp-12*, an HPUra-resistant derivative of BD54, have been described (13). Strain 1443 (*ile-1, metB5, polA1443*) and strain 2355 [*ile-1, metB5, polA1443, mut-1* (*ts*)] were provided by Julian Gross and George Bazill. Ø105C4, a clear plaque mutant of the temperate bacteriophage, Ø105, and SPP-1 were gifts from, respectively, Lars Rutberg and Frank Young.

Growth and phage infection of *B. subtilis* in liquid culture employed M broth, a medium (21) consisting of 1% tryptone (Difco), 0.5% yeast extract (Difco), 1% NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, and 0.5 mM $MnCl_2$. Phages were titered on M-agar (1.5%) plates using a soft M-agar (0.5%) overlay containing BD54 as the indicator bacterium.

Chemicals. Radioactively labeled nucleic acid precursors were obtained from New England Nuclear Corp., and HPUra was a gift of George Wright of this department.

DNA polymerase preparation and assay. Samples of cultures of uninfected and phage-infected (multiplicity, 10) *B. subtilis* were rapidly cooled to 0°C and centrifuged, and each cell pellet was extracted in polymerase buffer (15) by sonication. The soluble portion of the sonic lysate (the portion containing all pol III activity) was freed of cell debris by centrifugation at 4°C at 15,000 × *g* for 30 min. The pol III activity of the supernatant was assayed by measuring, in the absence and the presence of reduced HPUra (300 μM; see reference 6 for method of reduction), its capacity to catalyze incorporation of radioactivity from [^{3}H]TTP into nicked-gapped calf thymus DNA; the standard assay, enzyme units, and protein assay are described in reference 6.

RESULTS

Characteristics of HPUra-induced inhibition of Ø105 and SPP-1 development. Esche

(8), studying SPP-1, and Rutberg et al. (20), studying Ø105, noted that addition of HPUra to infected cells inhibited phage development. The results of experiments presented in Fig. 1 and 2 confirm and extend these results and clearly indicate that the action of HPUra on phage DNA synthesis is characteristic of that observed on the synthesis of bacterial DNA (4, 5) and entirely consistent with its established mechanism.

The burst curves depicted in Fig. 1 demonstrate the inhibitory effect of HPUra on phage development and the reversible nature of this effect, a characteristic of drug action on bacterial DNA synthesis. Phage development was completely inhibited by a drug concentration of 50 μM. Exposure of suspensions of infected cells to 50 μM HPUra for a period of 1 h did not reduce the concentration of infected centers or the ability of these centers to generate progeny; removal of HPUra at the end of the 1-h exposure permitted phage production which was kinetically and quantitatively indistinguishable from that of comparable controls.

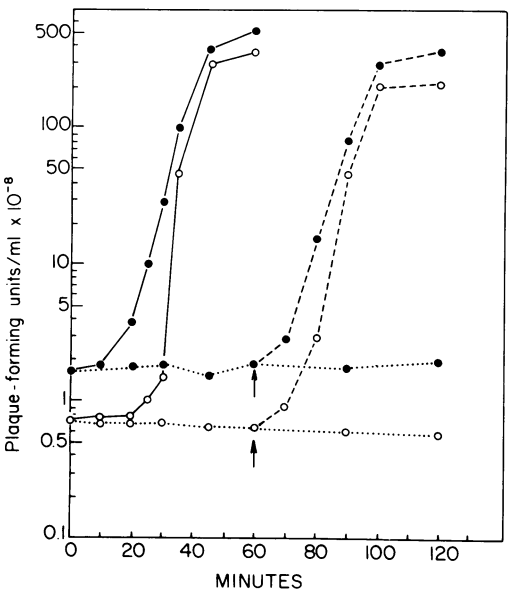

FIG. 1. *Reversible inhibition of phage development by HPUra. Strain BD54 was grown at 37°C to the midexponential phase (cell concentration, approximately 3 × 10⁷ cells/ml). At zero time, portions of the culture received phage (multiplicity, 10) or phage diluent and HPUra at 0 or 50 μM. After 4 min the cells were washed free of unadsorbed phage on sterile membrane filters and resuspended in one-third their original volume in warm (37°C) sterile medium containing the original, respective concentration of HPUra. Incubation was continued, and at interval samples were removed, diluted into cold Spizizen salts (1), and plated to determine the concentration of PFU. At 60 min (indicated by arrows) a portion of each inhibited culture was harvested rapidly by membrane filtration, resuspended in the same volume of sterile, warm medium minus HPUra, and assayed for phage production. Symbols: ○ Ø105; ●, SPP-1; —, no HPUra; · · ·, 50 μM HPUra; - - -, HPUra removed.*

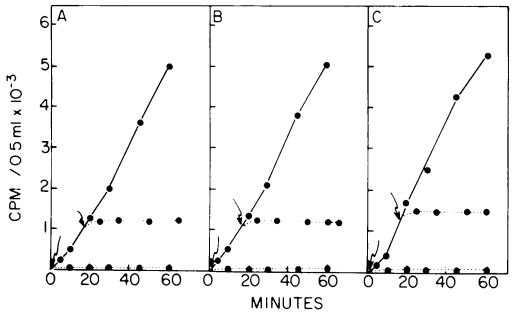

FIG. 2. *Effect of HPUra on DNA synthesis of infected and uninfected cells. Two minutes prior to zero time, an exponentially growing culture of B. subtilis 168 thy ind was divided into three portions; one portion served as a control (A) and each of the remaining two was infected with a multiplicity of 10, one with Ø105 (B) and one with SPP-1 (C). At zero time [¹⁴C]thymidine (specific activity, 1.25 × 10⁷ cpm/μmol; final concentration, 8 μM) was added, and HPUra was added at a concentration of 50 μM to portions of each culture at the times indicated by the arrows. Thymidine incorporation into DNA was followed by determining in 0.5-ml samples of culture, the content of alkali-stable, cold trichloroacetic acid-precipitable radioactivity. Lysis of infected cells was complete 70 min after phage addition. Symbols: —, no HPUra; · · ·, 50 μM HPUra.*

Addition of HPUra to uninfected, drug-sensitive *B. subtilis* induces the immediate inhibition of replicative DNA synthesis (4, 5); the experiment of Fig. 2, which exploited [³H]thymidine incorporation to measure DNA synthesis, depicts the nature of the effect of drug in SPP-1- and Ø105-infected cultures. Drug addition to cultures simultaneous with infection or at a considerable period after infection inhibited, immediately and completely, all detectable DNA synthesis in a manner indistinguishable from that in uninfected controls.

Effect of host *polC* mutations on phage development. The sensitivity of SPP-1 and Ø105 development to a drug that is an established, reagent inhibitor of a replication-specific DNA polymerase strongly suggested that the drug-sensitive site of phage development is a DNA polymerase, either pol III of the host or another, distinct HPUra-sensitive enzyme encoded by the phage. We attempted to distinguish the origin of the drug-sensitive polymerase by examining: (i) the effect of HPUra on phage development in an HPUra-resistant host, BD54 *azp-12*, which is resistant because it

possesses a drug-resistant pol III (6), and (ii) the effect of nonpermissive temperature on the development of phage in a temperature-sensitive (*ts*) strain carrying *mut-1* (*ts*), a *polC* mutation that specifies a *ts* pol III (3).

The experiment with *azp-12* (Fig. 3) indicated that the host-specific *polC* mutation specified drug-resistant phage development; HPUra at 50 μM, a concentration that completely inhibited phage development in the wild-type host (cf. Fig. 1), had essentially no effect on the development of either SPP-1 or Ø105.

The effect of *mut-1* (*ts*) on phage development is summarized in Table 1; the presence of the mutant, *ts* polymerase clearly conferred temperature sensitivity on the development of SPP-1, reducing phage production at the nonpermissive temperature to approximately one-tenth that observed at the permissive temperature (30°C).

Unfortunately, we could not determine reliably the effect of *mut-1* (*ts*) on the development of Ø105, because Ø105 development in the normal *polC* host background was inhibited too severely by the elevated temperature required for expression of *mut 1* (*ts*).

Pol III activity of phage-infected cells. Our interest in exploiting one of the phages as the basis for an in vitro system with which to characterize a replication-specific form of *B. subtilis* pol III prompted an examination of extracts of cells to determine what effect, if any, phage infection has on the cell content of pol III activity. Of the three polymerases (I, II, and III) detectable in *B. subtilis* (2, 10, 11, 15, 17), pol III is the only enzyme sensitive to inhibition by HPUra (2, 11, 15), and therefore, through exploitation of an active form of HPUra, its activity can be readily detected and specifically measured in crude extracts (6). We determined the specific activity of pol III (see above for procedures of extraction and assay) of crude extracts of uninfected cells and cells harvested at 8, 16, and 24 min after infection (see legend to Fig. 1 for culture conditions; lysis of infected cultures occurred at 45 to 50 min postinfection). The results of these experiments and others involving mixing of extracts strongly suggested that infection with either SPP-1 or Ø105 does not significantly alter the cellular levels of pol III activity; the specific activity of all extracts was 2.6 ± 0.3 units per mg of protein.

We have used our standard procedure (6, 15) to purify further the pol III activity of extracts of cells harvested 30 min after infection with SPP-1 (extracts of Ø105 were not examined as extensively). The purification procedure, which involved DNA removal, ammonium sulfate fractionation, and DEAE-cellulose and DNA-cellulose chromatography, yielded a pol III activity whose behavior was indistinguishable from that of uninfected cells. The activity purified normally, separating from pol I and pol II, and it possessed normal specific activity and normal sensitivity to reduced HPUra.

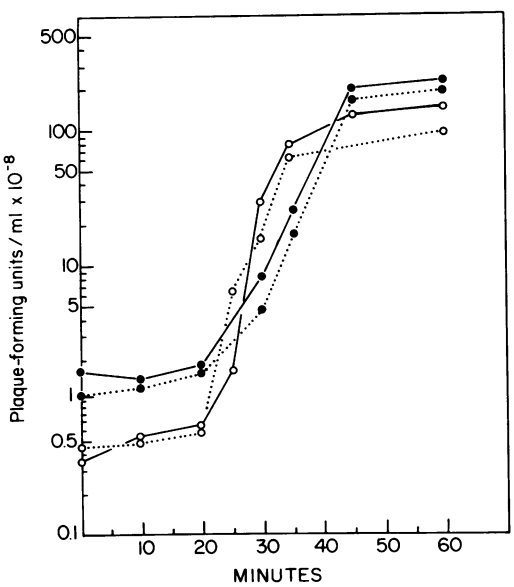

FIG. 3. *HPUra resistance of phage development in a host carrying the polC mutation azp-12. Samples of a culture of BD54 azp-12 were infected with a multiplicity of 10 in the presence or absence of 50 μM HPUra and were freed of phage, resuspended, and analyzed for phage development according to the protocol described for BD54 in Fig. 1. Symbols: ○, Ø105; ●, SPP-1; —, no HPUra; · · ·, 50 μM HPUra.*

TABLE 1. *Temperature sensitivity of SPP-1 development in a ts polC mutant host*

Host	Burst size[a]	
	33°C	47°C
1443 (normal *polC*)	400	580
2355 [*mut-1* (*ts*)]	420	41

[a] Midexponential cultures (density, 5 × 10^7 cells/ml) growing at 33°C were shifted to 47°C [the nonpermissive temperature for *mut-1* (*ts*)] and, after 5 min, infected with SPP-1 at a multiplicity of 10. After a 5-min adsorption period, bacteria were freed of unadsorbed phage by membrane filtration and resuspended at 47°C in fresh medium at the original volume. After removal of samples for assay of infected centers, the culture was divided; one portion remained at 47°C, and the other was shifted to 33°C. After 75 min of incubation, samples of each culture were treated with chloroform and assayed to determine the average burst size.

DISCUSSION

The sensitivity of Ø105 and SPP-1 development to HPUra, a property of these phages

noted elsewhere (8, 20) and dissected in detail in the experiments of Fig. 1 and 2, suggested that the host pol III, the established target of drug action, is required for the replication of phage DNA. The experiment of Fig. 3, which exploited the host *polC* mutation *azp-12*, verified the latter suggestion, excluding the possibility that the drug-sensitive target was a phage-specific polymerase. We were able to confirm, in the specific case of SPP-1 development (see Table 1), the role of the host pol III by exploiting the temperature sensitivity of the enzyme specified by the *polC* mutation *mut-1* (*ts*).

SPP-1 and Ø105 are not the only *B. subtilis*-specific phages that require the host pol III for their replication. A third phage, Øe, also displays this requirement, albeit in a manner that is not identical to that demonstrated by SPP-1 and Ø105 (12). Øe development, similar to that of SPP-1 (and putatively Ø105), is temperature sensitive in a *mut-1* (*ts*) host; however, it is not sensitive to inhibition by HPUra. Lavi et al. (12) have explained this apparent paradox by postulating that Øe infection causes modification of the host pol III, creating a functional, but drug-resistant, enzyme. The nature of the Øe-specific modification, if it occurs, is not known.

Infection of *B. subtilis* by SPP-1 and Ø105 does not significantly alter the specific activity of the host pol III in crude cell extracts; infection also does not alter significantly, at least in the case of SPP-1, the only case studied in detail, the sensitivity of pol III to HPUra or its behavior during purification. Whether phage infection results in a specific alteration of the host pol III, i.e., by addition of "co-proteins," by proteolytic modification, or by modification of specific amino acid residues (19), has not been determined. Preliminary experiments (P. McIntosh, S. D. Rowley, and N. C. Brown, unpublished data), which have examined the amount and structural nature of the pol III of the DNA-cellulose fraction of SPP-1-infected cells by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, have not yet detected any change in the molecular weight or the amount of the 167,000-dalton pol III core.

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