Induction of Prophage SPO2 in *Bacillus subtilis* by 6-(*para*)-Hydroxyphenylazouracil

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DNA replication in *Bacillus subtilis* is reversibly inhibited by 6-(*para*)-hydroxyphenylazouracil (HpUra) (1971), whereas replication of temperature phage SPO2 is not affected by the drug. Experiments are presented which show that HpUra will induce bacteria lysogenic for SPO2. Also, prophage ϕ 105, which is sensitive to the drug, is induced by HpUra. Induction of SPO2 lysogenics in the presence of HpUra gives selective synthesis of SPO2 DNA.

DNA replication in *Bacillus subtilis* is reversibly blocked by 6-(*para*)-hydroxyphenylazouracil (HpUra; [5,6]), whereas repair synthesis (e.g., as seen after UV irradiation of the bacteria) is not inhibited by the drug (6). Brown and co-workers (9) have shown that HpUra acts by directly affecting a DNA polymerase. SPO2 is a temperate phage of *Bacillus subtilis*. Replication of SPO2 DNA is not affected by HpUra, presumably because this phage does not employ the HpUra-sensitive host DNA polymerase for replication (12).

A number of different agents which affect DNA metabolism can induce lytic phage development in lysogenic bacteria (4). The mechanism of induction by these agents is not known, but some intermediate formed during DNA repair (which in *Escherichia coli* requires a functional *recA* gene product [7]) is thought to inactivate the phage repressor. In the present paper, we will show that HpUra will induce prophage SPO2 in *Bacillus subtilis*.

MATERIALS AND METHODS

Bacteria and phage. The bacterial strains employed are listed in Table 1. Phage SPO2 was obtained from J. Marmur; *sus* mutants of SPO2 were provided by S. Okubo (16). Phage ϕ 105 was from our collection.

Growth of bacteria and phage. Bacteria were kept on tryptic blood agar base plates (Difco). Liquid cultures were grown in NY broth (10) with aeration or in Spizizen minimal medium (1) supplemented with 5 g of casein hydrolysate (Difco) per liter. Required amino acids were added at 20 mg/liter; thymidine was added at 10 mg/liter. The methods for preparing lysates of SPO2 and ϕ 105 and the methods used for assaying the phages have recently been described in detail (2).

Experimental procedures. Details of experiments are given with the individual experiments. The

methods used for sucrose gradient centrifugation and for sedimentation in CsCl have recently been described (2). Competent cells for transformation and transfection were obtained essentially as described by Anagnostopoulos and Spizizen (1). The procedures for transfection and marker rescue with SPO2 are identical to those previously described for phage ϕ 105 (2).

Chemicals. HpUra was a gift from N. C. Brown. [^{3} H]thymidine (specific activity, 21 Ci/mmol) and [^{14} C]thymidine (specific activity, 62 mCi/mmol) were obtained from the Radiochemical Center, (Amersham, England). Other chemicals employed were of purest quality commercially available.

RESULTS

HpUra has a bacteriostatic effect on 168T⁻ when the cells are incubated for 40 min in the presence of the drug at a concentration of 100 μ M (Fig. 1). However, when 168T⁻ (SPO2) is incubated with 100 μ M HpUra, viability is rapidly lost. 168T⁻ (ϕ 105) gives an intermediate response with about 50% cell survival after 40 min of incubation with 100 μ M HpUra. Lysogenic bacteria were next incubated with HpUra, and at intervals samples were diluted into NY broth. Two hours after dilution into broth the samples were assayed for their content of free phage. About a 100-fold increase in free phage was found for both SPO2 and ϕ 105 lysogenic bacteria incubated with HpUra (Table 2). HpUra thus acts as an inducer of prophage both for SPO2, which can replicate in the presence of the drug, and for ϕ 105, which cannot (13). In the following experiments, we studied further induction of SPO2 by HpUra.

B. subtilis strain NB 841 and its HpUra-resistant derivative NB 841 HU/H1 were lysogenized with SPO2; the resistant strain carries a HpUra-resistant DNA polymerase. The two lysogenic strains were incubated with different

TABLE 1. *Bacterial strains*

Strain	Relevant properties	Source
168T ⁻	<i>thyA1, thyB1, trpC2</i>	C. Anagnostopoulos
W 168	Prototrophic	J. Spizizen
BR 95	<i>trpC2, phe-1, ilvA1</i>	J. Spizizen
NB 841	<i>polA⁻, thy⁻, trpC2</i>	N. C. Brown
NB 841 HU/H1	<i>polA⁻, thy⁻, trpC2, pol III</i> , resistant to HpUra	N. C. Brown

FIG. 1. 168T⁻, 168T⁻ (SPO2), and 168T⁻ (φ105) respectively were grown in NY broth at 37 C to about 5×10^7 bacteria per ml. To each tube was added 100 μM HpUra, and viable counts were made at intervals by spreading appropriate dilutions on tryptic blood agar base plates. Symbols: O, T⁻; ●, T⁻ (SPO2); and Δ, T⁻ (φ105).

concentrations of HpUra for 60 min and viable cells were scored. At a concentration of 12.5 μM HpUra, less than 1% of the NB 841 (SPO2) cells survive, whereas NB 841 HU/H1 (SPO2) was essentially resistant to HpUra concentrations up to 50 μM (Table 3). This suggests that induction of SPO2 with HpUra primarily depends on an interaction between the drug and a host DNA polymerase, rather than on a direct interaction, e.g., between the drug and the phage repressor.

DNA synthesis was next measured in 168T⁻ and 168T⁻ (SPO2) in the presence of 100 μM HpUra by following incorporation of [³H]thymidine into acid-insoluble material (Fig. 2). In the lysogenic strain, net DNA synthesis starts to increase 20 to 30 min after addition of the drug.

No significant incorporation of isotope was found during 50 min in the nonlysogenic strain. If the lysogenic cells are starved of thymidine for 30 min after addition of HpUra, DNA synthesis starts a few minutes after addition of thymidine. DNA synthesis is totally blocked in the lysogenic strain when chloramphenicol at 100 μg/ml is added together with HpUra (results not shown). Thus new protein synthesis, some of which is most likely coded for by the phage, is required for DNA synthesis in the lysogenic cells after addition of HpUra.

To test whether the DNA made after HpUra induction is mainly phage DNA, the following experiment was done. 168T⁻ (SPO2) was grown in the presence of [³H]thymidine to about $5 \times$

TABLE 2. *Effect of HpUra on phage production in 168T⁻ (SPO2) and 168T⁻ (φ105)^a*

Min with HpUra	Phage titer $\times 10^{-6}$	
	T ⁻ (φ105)	T ⁻ (SPO2)
0	1.3	6
5	1.0	25
10	11	75
15	60	130
25	100	300
35	140	400

^a 168T⁻ (SPO2) and 168T⁻ (φ105), respectively, were grown in NY broth at 37 C to about 5×10^7 bacteria per ml. HpUra was then added to a final concentration of 50 μM. At intervals, a 100-fold dilution was made into NY broth; 120 min later, chloroform was added to the dilution tube, and the amount of free phage was assayed.

TABLE 3. *Effect of different concentrations of HpUra on survival of B. subtilis NB 841 and NB 841 HU/H1 lysogenic for SPO2^a*

HpUra (μM)	Fraction surviving bacteria after 60 min in the presence of different concentrations of HpUra	
	NB 841 (SPO2)	NB 841 HU/H1 (SPO2)
0	1.0	1.0
12.5	0.002	0.80
25	<0.001	0.72
50	-	0.48
100	-	0.002

^a NB 841 (SPO2) and NB 841 HU/H1 (SPO2), respectively, were grown in NY broth at 37 C to about 5×10^7 bacteria per ml. The bacteria were diluted 100-fold into NY broth containing HpUra at concentrations shown above. Viable counts were made from each tube at the time of dilution and after 60 min in the presence of the drug.

FIG. 2. 168T⁻ and 168T⁻ (SPO2), respectively, were grown in 20 ml each of minimal medium supplemented with 0.5% casein hydrolysate to 5×10^7 bacteria per ml. HpUra was added to a final concentration of 100 μ M, and at the same time 0.1 mCi of [³H]thymidine was added to each culture. At intervals, 0.2-ml samples were taken into 2 ml of 10% cold trichloroacetic acid. The samples were filtered through HA filters (Millipore Corp.) and were washed. The filters were dried in the air and then were counted for radioactivity in a toluene-based liquid scintillation fluid (2). Symbols: ○, T⁻; and ●, T⁻ (SPO2).

10^7 bacteria per ml. The cells were washed and suspended in fresh medium containing 100 μ M HpUra, [¹⁴C]thymidine, and 5-bromodeoxyuridine. The bacteria were incubated at 37 C, and DNA was extracted from parts of the culture at 15, 30, and 50 min after addition of HpUra. The DNA samples were centrifuged to equilibrium in CsCl, fractions were collected, and each fraction was assayed for radioactivity, for bacterial DNA by transformation, and for phage DNA by transfection. The tritium peak served to identify the position of light, unreplicated DNA. The distribution of ³H and ¹⁴C radioactivity in the gradients is shown in Fig. 3. Less than 0.5% of the tritium activity is found outside the major (light) peak in the 15- or 30-min samples. At 50 min, about 2% of the tritium activity is found at a denser (hybrid) position, whereas less than 10% of the ¹⁴C activity is found at a light position at this time. We do not know whether the ¹⁴C activity remaining at a light position at 50 min represents bacterial or phage DNA. On the basis of the distribution of radioactivity, the gradients were divided into three fractions, light, hybrid, and dense, as indicated by the arrows in Fig. 3. The relative amounts of transfecting and transforming

activity in the light, hybrid, and dense regions of each gradient was calculated as the percentage of total respective activity recovered from the whole gradient. This gives a measure of the relative extent of phage and bacterial DNA replication, respectively, during the experiment. The results of these calculations are summarized in Table 4. At 30 min, 40% of the transfecting activity is found in hybrid DNA, whereas no transforming activity was found outside the light DNA. In the 50-min sample, 4% of the transforming activity is found at a position of increased density. We have tested the distribution of a *purB* marker in these fractions and found it to be the same as for *ilvA*, which indicates that the small amount of bacterial DNA found at a hybrid position does not result from selective replication of some part of the bacterial chromosome. From this experiment, we can thus conclude that essentially all biologically active DNA synthesized after induction of 168T⁻ (SPO2) with HpUra is of phage origin.

In the 50-min DNA (Table 4) it is seen that the infectivity of phage DNA is not proportional to the distribution of ¹⁴C radioactivity. This is not merely due to a decreased biological activity of heavily 5-bromodeoxyuridine-substituted phage DNA but may also reflect a lower infectivity of presumably mature SPO2 DNA, as compared with some other form(s) of phage DNA which appears during growth of the phage (Fig. 4). In the experiment shown in Fig. 4 168T⁻ (SPO2) was continuously labeled with [³H]thymidine for 50 min after addition of HpUra. DNA was extracted with phenol and centrifuged in a neutral sucrose gradient. Fractions were collected and each fraction was assayed for radioactivity, infectivity, and marker rescue by superinfecting phage SPO2 ts38 (unpublished data). It is seen that the peak of radioactivity and the peak for marker rescue coincide, whereas the peak for infectivity of phage DNA is associated with material sedimenting slightly faster than the bulk of the radioactivity. We believe that this material represents some intermediate in the replication of SPO2 DNA. The results shown in Fig. 4 are qualitatively similar to what has previously been shown for DNA extracted from ϕ 105-infected cells (14).

DISCUSSION

The present experiments conclusively show that HpUra will induce bacteria lysogenic for phage SPO2. Addition of this drug to the

FIG. 3. 168T⁻ (SPO2) was grown at 37°C in 30 ml of minimal medium supplemented with 0.5% casein hydrolysate (Min-CH), supplemented with thymidine, tryptophan and 40 µg of uracil per ml. To the culture was also added 0.1 mCi [³H]thymidine. When the culture reached about 5×10^7 bacteria per ml it was centrifuged, washed once with Min-CH, and suspended in 30 ml of Min-CH with tryptophan, 40 µg of uracil per ml, 20 µg of 5-bromodeoxyuridine per ml, and 5 µCi of [¹⁴C]thymidine. The bacteria were incubated at 37°C, and samples were removed for DNA extraction at 15, 30 and 50 min after suspension. DNA was extracted with phenol from samples concentrated four times as previously described (2). To 3 ml of DNA solution was added 3.66 g of CsCl, and the mixture was centrifuged in a Spinco SW50 rotor at 30,000 rpm for about 48 h. Fractions were then collected from the bottom of each tube. To each fraction was added 1 ml of 0.2 M NaCl-0.001 M EDTA (pH 8.0). From each diluted fraction, 0.5 ml was added to 2.5 ml of cold 10% trichloroacetic acid which was filtered and counted for radioactivity as shown in Fig. 2. From each fraction, 0.1 ml was then added to

lysogenic cells leads to selective synthesis of phage DNA and increased production of mature phage particles. The experiments also show that replication of bacterial DNA cannot be sustained by the phage DNA polymerase (11). Induction of SPO2 prophage by HpUra depends primarily on interaction between the drug and a host DNA polymerase, as evidenced by the finding that SPO2-lysogenic bacteria with a HpUra-resistant polymerase also become resistant to prophage induction by HpUra.

Induction with other agents which interfere with DNA metabolism, such as UV or mitomycin C, depends on the production of a "phage inducer." The production of this inducer in turn depends on the activity of the *recA* gene in *E. coli* (7). A similar requirement for a *rec* gene product for induction of ϕ 105 (15) or SPO2 (unpublished data) with mitomycin C has been found also in *Bacillus subtilis*. Attempts to show a dependence for HpUra induction of

TABLE 4. Distribution of radioactivity and biological activity in DNA from HpUra-induced 168T⁻ (SPO2) density-labeled with 5-bromodeoxyuridine^a

Treatment	Activity (%) of DNA sample		
	Light	Hybrid	Dense
15 Min			
³ H	100	0	
¹⁴ C	100	0	
ϕ	99.4	0.6	
<i>ilv</i> ⁺	100	0	
30 Min			
³ H	100	0	0
¹⁴ C	28	72	0
ϕ	60	40	0
<i>ilv</i> ⁺	100	0	0
50 Min			
³ H	98	2	0
¹⁴ C	9	15	76
ϕ	8	50	42
<i>ilv</i> ⁺	96	4	0

^a Fractions of light, hybrid, and dense DNA from CsCl gradients shown in Fig. 3 were pooled as indicated in Fig. 3. The pooled fractions were assayed for transforming activity and transfecting activity as described in legend to Fig. 3.

competent BR95 cells, and after 40 min for DNA uptake, *ilv*⁺ transformants and infectious centers were scored. The total number of tritium counts recovered from each gradient were 5,647 (15-min sample), 5,896 (30-min sample), 5,127 (50-min sample). More than 1,000 infectious centers and transformants were counted for each gradient.

FIG. 4. 168T⁻ (SPO2) was grown in 20 ml of minimal medium supplemented with 0.5% casein hydrolysate at 37 C to about 5×10^7 bacteria per ml. HpUra was then added to a final concentration of 100 μ M, and at the same time 0.1 mCi of [³H]thymidine was added to the culture. Fifty minutes later, DNA was extracted with phenol. A sample of the DNA was layered on a 5 to 20% linear sucrose gradient and was centrifuged in a Spinco SW50 rotor at 36,000 rpm for 120 min. Fractions were collected from the bottom of the tube and assayed for radioactivity and infectivity as described. The fractions were also assayed for activity of the ts38⁺ marker by superinfection with the ts-mutant phage SPO2 ts 38 (unpublished data). Symbols: $\square$, radioactivity; $\bullet$, infectivity; and $\circ$, marker rescue.

SPO2 on the *B. subtilis* recA gene product have given inconclusive results.

In *E. coli*, a mutant (T44) which will grow at 30 C and 40 C has been described. When lysogenic for phage lambda, however, the bacteria become induced when kept at 40 C. (8). Induction of lambda-lysogenic T44 can be prevented by adding guanosine or cytosine to the medium, or by adding hadacin, a drug known to inhibit the conversion of inosinic acid to guanylic acid. Induction of T44 lysogenic bacteria can be achieved at 30 C by adding adenine. These results suggest that induction of lambda in T44 is due to accumulation of some adenine derivative, and that induction may occur as a consequence of some alteration of the pool of DNA precursors. Measurements of the nucleotide pools of T44 have been inconclusive to show this (11). Alterations in the pool of DNA precursors may well occur also in HpUra-treated *B. subtilis*. The mechanism of induction by HpUra may be basically the same as the mechanism for induction by UV or mitomycin C, although the pathway for inducer formation may be different. HpUra offers certain advantages for studying prophage induction. It selectively and efficiently blocks bacterial DNA synthesis by a well-characterized mechanism without appre-

ciably affecting the synthesis of other macromolecules, but does not affect replication of SPO2 DNA. Phage DNA synthesis, on the other hand, can be controlled by using thymine-requiring bacterial strains and/or DNA-negative mutants of SPO2 (12). Combining these techniques for controlling bacterial and phage DNA synthesis with the techniques of transfection should prove valuable for studying the fate of prophage DNA during the early phases of prophage induction (3).

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LITERATURE CITED

1. Anagnostopoulos, C., and J. Spizizen. 1962. Requirements for transformation in *Bacillus subtilis*. *J. Bacteriol.* **81**:741-746.
2. Armentrout, R. W., and L. Rutberg. 1971. Heat induction of prophage ϕ 105 in *Bacillus subtilis*: replication of the bacterial and phage genomes. *J. Virol.* **8**:455-468.
3. Arwert, F., and L. Rutberg. Induction of prophage SPO2 in *Bacillus subtilis*: prophage excision in the absence of bacterial or bacteriophage DNA synthesis. *J. Virol.* **14**:1476-1481.
4. Borek, E., and A. Ryan. 1973. Lysogenic induction, p. 249-300. In J. N. Davidson and W. E. Cohn (ed.), *Progress in nucleic acid research and molecular biology*. Academic Press Inc., New York and London.
5. Brown, N. C. 1970. 6-(p-hydroxyphenylazo)-uracil: a selective inhibitor of host DNA replication in phage infected *Bacillus subtilis*. *Proc. Nat. Acad. Sci. U.S.A.* **67**:1454-1461.
6. Brown, N. C. 1971. Inhibition of bacterial DNA replication by 6-(p-hydroxyphenylazo)-uracil: differential effect on repair and semiconservative synthesis in *Bacillus subtilis*. *J. Mol. Biol.* **59**:1-16.
7. Hertman, I., and S. E. Luria. 1967. Transduction studies on the role of a rec⁺ gene in the ultraviolet induction of prophage lambda. *J. Mol. Biol.* **23**:117-133.
8. Kirby, E. P., F. Jacob, and D. A. Goldthwait. 1967. Prophage induction and filament formation in a mutant strain of *Escherichia coli*. *Proc. Nat. Acad. Sci. U.S.A.* **58**:1903-1910.
9. Mackenzie, J. M., M. N. Neville, G. E. Wright, and N. C. Brown. 1973. Hydroxyphenylazopyrimidines: characterization of the active forms and their inhibitory action on a DNA polymerase from *Bacillus subtilis*. *Proc. Nat. Acad. Sci. U.S.A.* **70**:512-516.
10. Okubo, S., and W. R. Romig. 1966. Impaired transformability of *Bacillus subtilis* mutants sensitive to Mitomycin C and ultraviolet irradiation. *J. Mol. Biol.* **15**:440-454.
11. Ruff, W., E. P. Kirby, and D. A. Goldthwait. 1971. Cell division and prophage induction in *Escherichia coli*: studies of nucleotide levels. *J. Bacteriol.* **106**:994-1004.
12. Rutberg, L., and R. W. Armentrout. 1972. Deoxyribonucleic acid polymerase activity in a deoxyribonucleic acid polymerase I-deficient mutant of *Bacillus subtilis* infected with temperate bacteriophage SPO2. *J. Virol.* **10**:658-660.

13. Rutberg, L., R. W. Armentrout, and J. Jonasson. 1972. Unrelatedness of temperate *Bacillus subtilis* bacteriophages SPO2 and ϕ 105. *J. Virol.* **9**:732-737.
14. Rutberg, L., and B. Rutberg. 1970. Characterization of infectious deoxyribonucleic acid from temperate *Bacillus subtilis* bacteriophage ϕ 105. *J. Virol.* **5**:604-608.
15. Rutberg, L., and B. Rutberg. 1971. Growth of bacteriophage ϕ 105 and its DNA in radiation-sensitive mutants of *Bacillus subtilis*. *J. Virol.* **8**:919-921.
16. Yasunaka, K., H. Tsukamoto, S. Okubo, and T. Horiuchi. 1970. Isolation and properties of suppressor-sensitive mutants of *Bacillus subtilis* bacteriophage SPO2. *J. Virol.* **5**:819-821.