Evidence that *Bacillus subtilis* Bacteriophage SPO2 is Temperate and Heteroimmune to Bacteriophage ϕ 105

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Some characteristics of *Bacillus subtilis* phage SPO2 which show that it is a temperate phage are presented. Wild-type SPO2 forms turbid plaques, similar to those of other temperate phages. SPO2 lysogenic strains which are resistant to SPO2 can be isolated; these strains remain stable lysogens despite the fact that they can no longer adsorb SPO2. SPO2 lysogenic strains can be grown for many generations in SPO2 antiserum and remain lysogenic. Phage SPO2 plates on ϕ 105 lysogens and phage ϕ 105 plates on SPO2 lysogens; this indicates that SPO2 and ϕ 105 are heteroimmune. Phage ϕ 105 plates on an SPO2-resistant strain; this indicates that SPO2 and ϕ 105 adsorb to different receptor sites on the bacterial surface.

There has been considerable interest in the recent discovery of temperate bacteriophages in *Bacillus subtilis* (5, 6, 9, 11, 12, 17; Bernard E. Reilly, *personal communication*). The phages fall into two classes: those which are able to lysogenize and replicate in suitable hosts (6, 9, 11, 12; Bernard E. Reilly, *personal communication*), and those which are unable to inject phage deoxyribonucleic acid (DNA) into sensitive hosts (5). In studying *B. subtilis* phages of the former category, we have observed that several of them have many properties in common, as do the lambdoid phages of *Escherichia coli* (2). Although many isolates of these phages must ultimately be examined to establish the validity of the hypothesis that *B. subtilis* has a family of closely related inducible temperate phages, current evidence rests mainly on a comparison of SPO2 and ϕ 105. The close morphological and serological similarity of these two phages raises the question of whether they are, in fact, different isolates of the same phage. This paper provides compelling evidence that they are not.

Several phages of *B. subtilis* which are semi-temperate have been reported (3, 10, 13, 14, 16; Jun Takagi, *personal communication*). Phage SPO2 was described as a temperate phage capable of forming lysogens (6). This paper presents additional evidence to establish that SPO2 is maintained in its host cell as a prophage, rather than by persistent infection.

MATERIALS AND METHODS

Strains and media. The lysogenic strains were 168M (SPO2) from Okubo (6) and 168 (ϕ 105) from Reilly (11). The indicator strains were 168B (4) and 168M

from Okubo (6). Phages ϕ 105 and SPO2 were obtained by inducing 168 (ϕ 105) and 168M (SPO2), respectively, with mitomycin C. Phage SPO2 c_1 (6) was obtained from Okubo and propagated on 168B. Lysates of SPO2 were induced and assayed, by using indicator strain 168B, on NY medium (7). Lysates of ϕ 105 were induced and assayed, by using indicator strain 168M, on TY medium (8).

Induction of 168M (SPO2) and 168 (ϕ 105) by mitomycin C. Liquid cultures of the lysogens were incubated to a turbidity of 0.5 optical density units by use of a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.) at 600 nm. Mitomycin C (Nutritional Biochemical Corp., Cleveland, Ohio) was then added to a final concentration of 0.2 μ g/ml, and incubation of the cultures was continued until lysis occurred. This usually took place in 2 to 4 hr, and lysates with phage titers of 10^9 to 5×10^9 phage/ml were obtained.

Preparation of SPO2-resistant bacterial strains. Strains of *B. subtilis* which do not adsorb SPO2 were isolated after adding phage SPO2 c_1 at a multiplicity of 100 to cells in liquid culture. At 1 hr after adding the phage, the culture was plated on NY medium. Most colonies of the surviving bacteria were unable to adsorb SPO2, as estimated by standard methods (1).

Assay of individual cells lysogenic for SPO2. The lysogenic cells were detected by their ability to form plaques on strain 168B after induction by ultraviolet irradiation. A vegetative culture of the lysogenic cells was first treated with rabbit antiserum against SPO2 to remove free phage. Plates of NY medium containing 200 of these treated cells and 10^7 spores of 168B in a soft-agar layer were irradiated for 30 sec at a distance of 50 cm from a General Electric 15-w germicidal lamp. The spores were prepared according to the method of P. C. Huang, H. Eberle, L. B. Boice, and W. R. Romig (Genetics, *in press*) and were used instead of vegetative cells because of their low sensitivity

to ultraviolet irradiation. After incubation of the plates for 24 hr, plaques were counted. The number of plaques formed was the same as the number of lysogenic cells added to the plates.

Preparation of SPO2 antiserum. The method of Boice et al. (2) was used, except that Freund's adjuvant was omitted and the neutralizing capacity of the serum was not measured before the immunization of the rabbit was begun.

RESULTS

Morphology of SPO2 plaques. Phage SPO2 forms turbid plaques. Several clear-plaque mutants have been isolated by Shunzo Okubo (*personal communication*). One of these, c_1 , has been described (6). The contrast between SPO2 turbid plaques and the clear plaques of SPO2 c_1 is similar to that between turbid plaques of *E. coli* phage λ and clear plaques of mutants (18). The plaques of SPO2 c_1 , like the plaques of $\lambda C1$, contain no growth of bacterial colonies.

Strains resistant to SPO2. As previously described, several strains of *B. subtilis*, lysogenic for SPO2, but unable to adsorb extracellular phage, were isolated. An example of these strains is 168M (SPO2)/SPO2. That this strain is unable to adsorb SPO2, and yet approximately 100% of the cells continue to liberate SPO2, indicates that re-infection by an extracellular phage is not responsible for phage production; presumably it is due to a prophage.

Growth of an SPO2 lysogen in medium containing SPO2 antiserum. A culture of 168M (SPO2) was diluted to 1.4×10^3 vegetative cells/ml in fresh NY medium containing SPO2 antiserum at an inactivation constant of approximately 126. After incubating the culture for 24 hr, viable cells, lysogenic cells, and free phage were assayed. A portion of the culture was centrifuged and filtered through a membrane filter (Millipore Corp., Bedford, Mass.) with 0.45- μ m pores, and the antiserum activity of the filtrate was tested. The inactivation constant was never significantly different from its original value, indicating that the antibody was stable under these conditions.

Another portion of the culture was transferred to fresh NY medium containing SPO2 antiserum; after incubation for 24 hr, the culture was assayed in the same manner as before. Subculture into fresh medium containing SPO2 antiserum was repeated a third time, and the same assays were again performed. The results of the assays of viable and lysogenic cells at the end of each transfer are shown in Table 1.

Isolated colonies, obtained after 24-hr growth of three successive serial transfers in broth with SPO2 antiserum in a repeat experiment, were

TABLE 1. *Results of assays of viable cells and lysogenic cells*

Transfer no.	No. of cells ^a	
	Phage-yielding	Colony-forming
1	4.0×10^8	1.2×10^9
2	3.6×10^8	1.1×10^9
3	2.4×10^8	6.6×10^8

^a The amount of free phage found in the culture fluid was negligible. The ratios of phage-yielding to colony-forming cells for transfers 1, 2, and 3 were 0.33, 0.33, and 0.36, respectively.

tested for their ability to yield phage. Of 10 colonies tested from each of the three serial transfers, 10 yielded phage.

The bacteria doubled about 20 times during each transfer. Since the proportion of bacteria which yield phage remained constant (100%) throughout some 60 generations of bacterial growth, it is apparent that SPO2 antiserum does not cure SPO2 lysogens.

The most probable reason that the ratio of phage-yielding cells to colony-forming cells is not 1.0 at the end of each serial transfer shown in Table 1 is that the medium allows sporulation to occur, and the method of assaying individual lysogenic bacteria does not efficiently detect lysogenic cells which have sporulated. The ratio for one serial transfer in the repeat experiment was measured 7.5 hr after dilution into fresh medium and found to be 1.1; at this time the bacteria were still in the vegetative phase of growth and sporulation was negligible.

Plating phage SPO2 on 168 ($\phi 105$) and phage $\phi 105$ on 168M (SPO2). Phage SPO2 forms plaques with equal efficiency on 168B and 168 ($\phi 105$). Phage $\phi 105$ forms plaques with equal efficiency on 168M and 168M (SPO2). Thus, SPO2 is insensitive to the immunity produced by the $\phi 105$ prophage, and, correspondingly, $\phi 105$ is insensitive to the immunity produced by the SPO2 prophage. Accordingly, phages SPO2 and $\phi 105$ are heteroimmune, as defined by Thomas and Bertani (15).

Plating phage $\phi 105$ on an SPO2-resistant strain. An SPO2-resistant strain, 168M (SPO2)/SPO2, was tested for sensitivity to $\phi 105$. Phage $\phi 105$ was able to form plaques on it, and this observation indicates that in all probability $\phi 105$ has a different adsorption site on the bacterial host than does SPO2.

DISCUSSION

Two lines of evidence have been presented here that SPO2 is a temperate phage. First, stable

phage-resistant SPO2 lysogens which cannot adsorb SPO2 were isolated. Second, an SPO2 lysogen was grown in broth with SPO2 antiserum without being cured of SPO2; the experiment was continued for 60 bacterial generations, and at the end of each transfer approximately 100% of the viable cells still yielded phage. These results contrast with a similar experiment of Bott and Strauss (3) which demonstrated that cultures derived from spores carrying SP10 can be cured of SP10. They found that cultures containing SP10 decreased from 72 to 93% to 0.3 to 0.0% phage-yielding colonies in one serial transfer in broth containing SP10 antiserum.

SPO2 appears to form a prophage which is integrated into the bacterial chromosome, rather than existing in its host cell as a prophage that replicates as an extrachromosomal unit like prophage P1 of *E. coli* (H. Ikeda and J. Tomizawa, Cold Spring Harbor Symp. Quant. Biol., *in press*). Evidence for this notion derives from a comparison of the sedimentation properties of SPO2 "prophage DNA" and mature phage DNA in sucrose gradients (9). "Prophage DNA" was found to sediment at nearly the same rate as do fragments of bacterial DNA, and at a considerably faster rate than mature phage DNA (9).

The evidence set forth in this report suggests that SPO2 and $\phi 105$ are not isolates of the same bacteriophage. Thus, not only are they hetero-immune but they also appear to have different sites of adsorption on the surface of their bacterial host. This evidence is extended by recent observations that their prophages also occupy different places on the bacterial chromosome. The chromosomal site for the SPO2 prophage has been located close to the erythromycin (*ery-1*) locus (J. W. Inselburg, T. Eremenko-Volpe, L. Greenwald, W. L. Meadow, and J. Marmur, *unpublished data*). The site for the $\phi 105$ prophage is between the *phe-1* and *ilvA1* bacterial markers (11; Bernard E. Reilly, *personal communication*). Accordingly, their morphological and immunological similarity, as previously described (2), must be attributed to a close relationship rather than to identity. Therefore, the idea that they are members of a cluster of related temperate phages remains valid.

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