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Prophage-Mediated Interference Affecting the Development of *Bacillus subtilis* Bacteriophage φe

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Bacteriophage φe shows a reduced efficiency of plating on strains of *Bacillus subtilis* which are lysogenic for the temperate bacteriophage SP02. Although this phenomenon resembles prophage-mediated restriction observed in other bacteria, host-controlled modification of φe was not observed. Mutants of φe which plated with high efficiency on the lysogenic host were isolated.

Although classical DNA restriction and modification systems have not been described in *Bacillus subtilis*, it has been observed that some strains of this bacterium do have mechanisms which can inhibit phage development after the initial stages of infection are completed. Phenomenologically, the infection of the nonpermissive *B. subtilis* strains resembles occurrences observed in bacteria exhibiting phage restriction; the phage adsorb to and even kill the nonpermissive host cells, but few progeny are released because some cellular mechanism prevents completion of the normal lytic cycle (8, 9). The particular *B. subtilis* interference systems described below also resemble the restriction systems of *Escherichia coli* in that a certain number of phage escape the inhibiting mechanism in the host cell, and occasional plaques of the phage appear on lawns of the normally nonpermissive *B. subtilis* strains.

The phenomenon observed in *B. subtilis*, however, does show certain differences from classical phage restriction. There is no evidence that the *B. subtilis* cells contain specific restriction endonucleases similar to those which mediate phage restriction in other bacteria. Moreover, in the studies made by ourselves and others (8), no host modification systems have been found to accompany interference in *B. subtilis*. Because the term phage restriction has become closely associated with DNA restriction and modification, objections can be raised to referring to the observations in *B. subtilis* as phage restriction. To meet this criticism, we have chosen to use another term to describe our observations. *B. subtilis* cells which permit certain phages to adsorb and penetrate, but subsequently prevent development of normal yields of progeny, will be said to exhibit interference. If the interference system is clearly associated with a prophage carried by the host exhibiting interference, we shall use the term prophage-mediated interference. A phage which is sensitive to an interference system will be referred to as an interference-sensitive bacteriophage. It should be noted, however, that there is at least one precedent in the literature in which the term phage restriction has been used to describe a phage-host relationship in *B. subtilis* similar to interference (9).

Most strains of *B. subtilis* are lysogenic for one or more defective prophages (18); since prophages are frequently the source of phage restriction systems in various eubacteria (4, 10), a number of investigators have speculated that the defective prophages of *B. subtilis* might be the source of interference against superinfecting phages (8, 9). Attempts to investigate this hypothesis have been hampered by difficulties in transferring the genome of the defective prophage from one strain of *B. subtilis* to another. Nevertheless, some circumstantial evidence has been obtained which suggests that defective prophages may be involved in interference in this bacterium. Gwinn and Lawton (9) reported that *B. subtilis* 168 cells grown at a high temperature became permissive hosts for normally interference-sensitive phages SP10 and SP20, although the resultant progeny were not modified. Goldberg and Bryan (8) found that pretreatment of *B. subtilis* 168 with ultraviolet light or mitomycin C induced the defective prophage and simultaneously rendered the cells permissive for SP10.

We have recently found that *B. subtilis* cells which are lysogenized with the temperate phage SP02 demonstrate a reduced efficiency of plating for certain virulent phages. In the following

report we examine some of the properties of this interference system and compare it to one of the interference systems found in naturally occurring strains of this bacterium.

MATERIALS AND METHODS

Bacteria and phage strains. *B. subtilis* strain SB11 try⁻, a derivative of *B. subtilis* W23, was obtained from E. W. Nester; *B. subtilis* 168 M and 168 M (SP02) were provided by W. R. Romig; *B. subtilis* 168 (ϕ105) was obtained from S. A. Zahler; and *Bacillus pumilis* was obtained from the stock culture collection of Syracuse Univ. Bacteriophage β22 was supplied by Roy Doi; SP82 was obtained from Charles Stewart; SP02c$_1$ was supplied by W. R. Romig; SP01 and ϕe were obtained from Clifford Yehle; ϕ105c$_1$ was obtained from S. A. Zahler; and ϕ1 was obtained from Frank Young.

Growth and infection of cells. The medium used in the present experiments for growing cells and preparing phage lysates was a modified M medium as described previously (13). Experiments in liquid medium were carried out at 37 C with cultures grown in 16-mm screw-cap tubes. Optical density (OD) at 600 nm was measured in a Spectronic 20 colorimeter on cells growing in these culture tubes.

Phage were titered on M medium made with 1.5% agar. The phage and the appropriate indicator strain of bacteria were mixed in a 0.7% soft M agar overlay and then poured on the base medium.

Determination of relative burst size. The relative burst size of ϕe and SP01 on different hosts was determined by growing the desired strain of *B. subtilis* in modified M broth as described above. When the cultures reached an OD of 0.3 at 600 nm, ϕe or SP01 was added at the desired multiplicity of infection (MOI), 1 min was allowed for adsorption without shaking, and adsorption was continued with shaking for 5 additional min. The cultures were then centrifuged to remove unadsorbed phage, suspended in M broth and diluted 1:10,000 in the same medium. After lysis, the number of phage particles released was determined by plating on *B. pumilis* or *B. subtilis* 168. *B. pumilis* was used as an indicator in studies involving *B. subtilis* 168 (SP02) because cultures of this lysogenic strain frequently contained considerable numbers of spontaneously induced SP02 which confused results. SP02 did not form plaques on *B. pumilis* while ϕe and SP01 formed plaques at high efficiency on this strain.

Determination of numbers of cells releasing phage. Experiments to determine the number of infected cells which released any phage were conducted in approximately the same manner as those done to determine relative yields of progeny. The desired strain of *B. subtilis* was grown to an OD of 0.3 at 600 nm and infected, 6 min was allowed for adsorption, and the cultures were centrifuged to remove unadsorbed phage. The cells were suspended in M medium and then immediately diluted and plated on *B. subtilis* 168 or *B. pumilis*. Each productively infected cell was expected to produce one plaque regardless of the number of progeny released.

Determination of phage adsorption. To determine the ability of ϕe to adsorb to various *Bacillus* strains, bacteria were grown to an OD of 0.3 at 600 nm and infected at an MOI of 0.2. One minute was allowed for adsorption without shaking. Adsorption was continued with shaking for 5 additional min, the culture was diluted 1:10,000 and treated with chloroform, and unadsorbed phages in the supernatant fraction were titered on *B. pumilis*.

Production of doubly lysogenic strains of B. subtilis. As noted above, strains of *B. subtilis* lysogenic for SP02, or ϕ105 were obtained from other investigators, although several new *B. subtilis* 168 (SP02) strains were made to ascertain that the SP02-mediated interference was not unique to the original strain. A strain of *B. subtilis* doubly lysogenic for ϕ105 and SP02 (*B. subtilis* 168 [SP02][ϕ105]) was made by plating spontaneously induced ϕ105 phage on *B. subtilis* 168 (SP02). Cells from turbid ϕ105 plaques on lawns of this bacterium were streaked on M agar, and cultures were made from isolated colonies. Evidence that the newly prepared strains were doubly lysogenic included the fact that the cultures spontaneously released both ϕ105 and SP02, and the cultures were resistant to both ϕ105c$_1$ (a clear-plaque mutant of ϕ105) and SP02c$_1$ (a clear-plaque mutant of SP02) (see Table 4).

RESULTS

Comparisons of two interference systems in *B. subtilis*. Table 1 compares the relative plaque-forming efficiency of bacteriophages SP82, SP01, and ϕe on *B. subtilis* 168, *B. subtilis* 168 (SP02), (which is a strain of 168 lysogenic for the temperate phage SP02), *B. subtilis* SB11, and *B. pumilis*. The three phages used in the present study are virulent and share in common the fact that their DNA contains the base substitution hydroxymethyluracil (HMU) in place of thymine (7, 12, 16). It will be noted that these phages infected all four *Bacillus* strains, but they formed

TABLE 1. *Relative plaque-forming efficiency[a] of B. subtilis phages on various Bacillus species*

Bacillus species	SP82	SP01	ϕe
B. subtilis 168	1	1	1
B. subtilis 168 (SP02)	1	1	10^{-3}
B. subtilis SB11	10^{-4}	10^{-4}	10^{-4}
B. pumilis	1	1	1

[a] A relative plaque-forming efficiency of 1 was equal to approximately 10^{10} PFU/ml.

plaques 10,000 times less efficiently on *B. subtilis* SB11 than on most other hosts. In addition, ϕe was specifically unable to grow normally on *B. subtilis* 168 (SP02) and formed plaques 1,000-fold less efficiently on this strain than on *B. subtilis* 168.

The reduced ability of ϕe to form plaques on the two nonpermissive strains of *B. subtilis* is similar to what is observed when certain phage infect bacteria carrying classical restriction systems and invites a comparison of these two interference systems with each other and with true restriction. We first investigated the possibility that ϕe might have a low affinity for the adsorption sites on the two hosts demonstrating interference. However, we found that this interference-sensitive phage adsorbed equally well to *B. subtilis* strains 168, 168 (SP02), and SB11, and was able to kill and lyse both the permissive and nonpermissive hosts.

One difference between the interference systems in *B. subtilis* SB11 and *B. subtilis* 168 (SP02) was observed in studies comparing the number of cells (measured as plaque-forming units [PFUs]) releasing ϕe (Table 2) and the relative yield of progeny (Table 3) at various MOI. At very low MOI, few progeny were produced on either nonpermissive host; in *B. subtilis* SB11 this appeared to reflect a marked drop in the number of cells yielding any progeny, but under the same conditions 30 to 50% (and in some experiments as high as 75%) of the infected *B. subtilis* 168 (SP02) cells produced at least one progeny ϕe. These results suggest that the SP02-mediated interference acted to reduce the yield of progeny rather than to completely prevent productive infection. Additional support for this hypothesis can be seen in the infections at higher MOI. It was possible to overwhelm the SB11 interference at an MOI of 5; under these conditions both the number of SB11 cells producing ϕe and the total yield of progeny was equal to that obtained in the *B. subtilis* 168 controls. However, when *B. subtilis* 168 (SP02) cells were infected at this MOI, the number of plaque-forming units was equal to that of the controls but the total yield of ϕe remained low.

There is other evidence that the SP02-mediated interference may have its most pronounced effect on the number of phage released rather than the ability to produce any progeny. As noted earlier, SP82 and SP01 formed plaques with almost equal efficiency on strains 168 and 168 (SP02). However, we found that the plaques of SP01 on lawns of the former were twice the diameter of those observed on the lysogenic host. Examination of the number of progeny released from SP01-infected *B. subtilis* 168 and 168 (SP02) cells in liquid culture indicated that the burst size on the latter was one half that on the permissive host.

Growth of ϕe on other lysogenic hosts. We next turned our attention to determining whether prophages other than SP02 interfered with the development of ϕe. In these studies, temperate *B. subtilis* phage ϕ105 was used to construct two lysogenic strains of the bacterium. *B. subtilis* 168 (ϕ105) and *B. subtilis* 168 (SP02) (ϕ105) which is lysogenic for both SP02 and ϕ105. Table 4 compares the relative plaque-

TABLE 2. *Relative number of cells releasing ϕe at various MOI*[a]

Host	No. of cells releasing ϕe	
	MOI 0.02	MOI 5
B. subtilis 168	100	100
B. subtilis 168 (SP02)	30	100
B. subtilis SB11	~5	100

[a] A value of 100 in the table ~35×10^5 PFU/ml for MOI of 0.02; and 690×10^5 for an MOI of 5.

TABLE 3. *Relative number of ϕe progeny released from permissive and nonpermissive B. subtilis hosts*[a]

Host	No. of ϕe progeny released		
	MOI 0.02	MOI 5	MOI 10
B. subtilis 168	100	100	100
B. subtilis 168 (SP02)	1	10	14
B. subtilis SB11	10	100	100

[a] A relative burst size of 100 ~25×10^7 PFU/ml for MOI of 0.02; and 200×10^8 PFU/ml for MOI of 5 and 10.

TABLE 4. *Relative plaque-forming efficiency of ϕe, SP02c$_1$ and ϕ105c$_1$ on lysogenic B. subtilis strains*[a]

Host strains	Bacteriophage tested		
	SP02c$_1$	ϕ105c$_1$	ϕe
B. subtilis 168	1	1	1
B. subtilis 168 (SP02)	0	1	10^{-3}
B. subtilis 168 (ϕ105)	1	0	1
B. subtilis 168 (SP02) (ϕ105)	0	0	10^{-3}

[a] A relative plaque-forming efficiency of 1 was ~80×10^8 PFU/ml for SP02c$_1$; 120×10^8 PFU/ml for ϕ105c$_1$; and 75×10^8 PFU/ml for ϕe.

forming efficiencies of SP02c$_1$, φ105c$_1$ (respectively, clear-plaque mutants of SP02 and φ105), and φe on several lysogenic *B. subtilis* strains. It can be seen that the strain which was lysogenic for φ105 alone did not affect the plaque-forming efficiency of φe; cultures doubly lysogenic for φ105 and SP02 did inhibit φe but the interference was no greater than that observed on *B. subtilis* 168 (SP02) itself.

Interference-resistant host range mutants of φe. We have examined the phages present in the φe plaques which did form at low efficiency on *B. subtilis* 168 (SP02). Table 5 demonstrates that these phages, which are referred to as φeI^r, were adapted to the nonpermissive host and formed plaques with equal efficiency on *B. subtilis* 168 and the lysogenic strain. More surprising, the adaptation appeared to be a genetic one rather than host-controlled modification. Reculturing of φeI^r on 168 did not phenotypically change the adapted phages so that they again had a reduced plaque-forming efficiency on *B. subtilis* 168 (SP02). Some 20 plaques picked at random were analyzed and all contained phages genetically resistant to the SP02-mediated interference. Table 5 demonstrates, however, that the φeI^r strains were still sensitive to interference in *B. subtilis* SB11.

One troublesome aspect of this finding is that it implies that there was a very high rate of mutation from φeI^s to φeI^r. However, studies in liquid culture discussed above and observations on plates suggest a possible explanation. It appears that the interference of φe in *B. subtilis* 168 (SP02) involved a reduction in burst size rather than a precipitous drop in the number of productive infections. Thus, when φe was plated on *B. subtilis* 168 (SP02), there was probably considerable background growth of the normal phage. Indeed, close examination of the lawns of 168 (SP02) on which φe had been plated usually revealed a large number of very tiny plaques which may have resulted from such background growth of normal φe. Moreover, the plaques of φeI^r which first formed on the nonpermissive host were usually smaller than those seen when the isolates of φeI^r were subcultured on the same strain. Collectively, these observations may mean that the φeI^r mutants were not present when the phage were plated but arose as spontaneous mutants on the plates. Under these conditions the real mutation rate could be much lower than the apparent rate of mutation.

TABLE 5. *Relative plaque-forming efficiency of φe and φeI^r on Bacillus strains*[a]

Host	Bacteriophage	
	φe	φeI^r
B. subtilis 168	1	1
B. subtilis 168 (SP02)	10^{-3}	1
B. subtilis SB11	10^{-4}	10^{-4}

[a] A relative plaque-forming efficiency of 1 was ~10^{10} PFU/ml.

Phage were also isolated from the occasional plaques of φe observed on lawns of *B. subtilis* SB11 to determine whether these phage represented a second class of φe which was adapted to grow on this nonpermissive host. However, the phage obtained from these plaques did not demonstrate an increased plaque-forming efficiency on *B. subtilis* SB11 and thus were apparently neither host range mutants nor phenotypically modified to grow more efficiently on *B. subtilis* SB11. It is possible that they originated from occasional productive infections of cells in the lawn of the nonpermissive host, with the resulting burst of phage multiply infecting neighboring cells to produce a plaque. It should be noted, however, that host range mutants of HMU-containing phage have been isolated by other methods. Brodetsky and Romig (5) have reported the selection of mutants of several such phages which form plaques on *B. subtilis* SB19 at a higher efficiency than do the wild-types. It is not unlikely that mutants of φe which have an extended host range do occur, but these are difficult to distinguish among the normal background plaques of escape infections.

Sensitivity of other bacteriophages to SP02-mediated interference. In addition to the bacteriophages noted in Table 1, a number of other phages have been studied to determine if they were sensitive to the SP02-mediated interference. Virulent phages φ29 and β22 were not affected. However, recently Ronald Yasbin and Frank Young (personal communication) found that bacteriophage φ1 was sensitive to the interference system in *B. subtilis* 168 (SP02). Our examination of φ1 revealed that this phage was completely inhibited in *B. subtilis* 168 (SP02) and no progeny were produced even at very high MOI. No φ1 plaques have been observed on lawns of the lysogenic host, and our attempts to isolate interference-resistant mutants of φ1 have failed. Like φe, φ1 was found to be specifically affected by SP02 prophage and formed plaques with high efficiency on strains lysogenic for φ105. Aside from sharing a common sensitivity to SP02-mediated interference, however, φ1 is not related to φe. Its DNA does not contain HMU. Moreover, φ1 was not sensitive to the *B. subtilis* SB11 interference and formed plaques with equal efficiency on *B. subtilis* strains 168

and SB11. This latter observation further indicates that the two interference systems were not related.

DISCUSSION

A number of situations have been described in which a bacteriophage is able to infect a potential host cell but is unable to complete the lytic cycle and release progeny. The first cataloging of these phenomena was made by M. H. Adams (1, 2) who coined the term "abortive infections" to describe these conditions in which there was an irreversible loss of the plaque-forming potentiality of a phage-infected cell. The best understood abortive infections are those in which DNA restriction endonucleases selectively degrade the phage genome into several pieces (4, 15). *E. coli* cells which contain F^+ or substituted F' sex factors demonstrate a second type of inhibitory mechanism which causes abortive infections of T7 and several other phages. In this instance the episome apparently directs the synthesis of some product which interferes with synthesis of several classes of phage proteins (11).

The two interference systems discussed in the present paper should probably be considered examples of abortive infections. This is certainly true of $\phi 1$ infection of *B. subtilis* 168 (SP02) because no progeny were released from the infected cells. However, these same lysogenic cells infected with ϕe or SP01 released a reduced yield rather than no progeny at all, and thus did not precisely meet the definition of abortive infection as given above. The naturally occurring interference observed in *B. subtilis* SB11 appeared to be directed specifically against certain HMU-containing phages, and blocked phage development only at low MOI. The latter interference system would meet the definition of abortive infection but only at these low multiplicities of infection.

We do not yet understand the molecular basis of either of the *B. subtilis* interference systems, but several preliminary studies should be considered. It is important to note that our observations and those of others (8) indicate that the interference in *B. subtilis* was not accompanied by modification as is the case in phage restriction systems which are mediated by DNA restriction endonucleases. The association of DNA modification with DNA restriction is thought to reflect a need to protect the host's DNA from the same endonucleases which breakdown the heterologous phage DNA (4). The lack of modification in the *B. subtilis* interference systems could then be interpreted to mean that endonucleases were not involved. The *B. subtilis* SB11 interference system could include a nuclease without a modification enzyme, however, if the nuclease specifically inactivated phage DNA containing HMU as in SP82, ϕe, and SP01. In such a hypothetical situation, the cell's DNA would be protected by virtue of the fact that it contained thymine rather than hydroxymethyluracil. On the other hand, the SP02-mediated interference affected unrelated phages, only one group of which contained HMU. The fact that some progeny were released when the superinfecting phage was ϕe or SP01 would suggest that the DNA of these phages was not destroyed. Indeed preliminary studies of DNA synthesis in *B. subtilis* 168 (SP02) cells infected with ϕe not only indicate that ϕe DNA remains intact in the nonpermissive host but that new phage DNA is synthesized.

Two abortive infection systems in *Enterobacteriaceae* show similarities to SP02-mediated interference in that they are related to lysogeny and neither is accompanied by modification. T2 and T4 infections of *E. coli* W which is lysogenic for phage ω resemble restriction in that the entering phage DNA is degraded, but several lines of analysis indicate that the DNA breakdown may be secondary to the actual mechanism of interference. It has been hypothesized that abortive infections in this bacterium may be related to changes in the *E. coli* membrane resulting from lysogeny (14, 17). The T-even and T5 coliphages give abortive infections in *Shigella dysenteriae* lysogenic for phage P2. Here again the failure to support productive infection is thought to reside in changes in the membrane related to prophage conversion. It has been suggested that P2 prophage may prevent resealing of the cell envelope after penetration of superinfecting phage (6). Interference in *B. subtilis* could be related to differences in the cell membrane, but one would not expect that such a mechanism would be overwhelmed at high MOI as was the case in *B. subtilis* SB11 interference. Indeed, one might predict that in such a system the number of infectious centers would decrease even further at high MOI. This was not observed in either *B. subtilis* SB11 or *B. subtilis* 168 (SP02). We are continuing our study of possible mechanisms to explain interference in *B. subtilis*.

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