Restriction and Modification of Bacteriophage SP10 DNA by *Bacillus subtilis* Marburg 168: Stabilization of SP10 DNA in Restricting Hosts Preinfected with a Heterologous Phage, SP18

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SP10 phage cannot propagate in *Bacillus subtilis* Marburg 168 containing the wild-type allele of either gene *nonA* or gene *nonB*. The latter gene codes for the intrinsic cellular restriction activity. SP10 DNA was degraded in *nonB*⁺ derivatives of Marburg 168. The degree of degradation depended upon the previous host in which SP10 was propagated. In the case of SP10 grown in *B. subtilis* W23 (a nonrestricting, nonmodifying bacterium), 90% of the phage DNA was hydrolyzed to acid solubles, and the residual acid-precipitable material was recovered as 0.5- to 1-megadalton fragments. In contrast, if SP10 was propagated in *B. subtilis* PS9W7 (a *nonA nonB* derivative of Marburg 168 that retains modifying activity), 40 to 50% of the input DNA was degraded to acid solubles, and most of the remainder was recovered as 15- to 20-megadalton fragments. In *nonA*⁺ *nonB* cells, SP10 DNA was conserved as unit-length molecules (ca. 80 megadalton). Prior infection of *nonB*⁺ cells with SP18 protected superinfecting SP10 DNA, even when rifampin or chloramphenicol was added before the primary infection. The data are discussed in terms of the following conclusions. (i) The *nonB* gene product of *B. subtilis* Marburg 168 is required for restriction of SP10 DNA. (ii) Some sites on SP10 DNA are sensitive to both the restricting and modifying activities, whereas other sites are nonmodifiable even though they are sensitive to the restriction enzyme. (iii) In some manner, SP18 antagonizes the action of the *nonB* gene product.

SP10 is a pseudodysogenic bacteriophage (2, 10, 33) unable to multiply in *Bacillus subtilis* Marburg 168 (23, 25). Derivatives of Marburg 168 permissive for SP10 development were described recently (25). Permissiveness requires simultaneous mutations in two unlinked genes, designated *nonA* and *nonB* (25). The *nonA* locus is closely linked to the *rif^r* (rifampin resistance) and *strA* (streptomycin resistance) loci (25). The *nonB* locus is identical to the previously described *hsrM* locus that encodes the intrinsic restriction activity of *B. subtilis* Marburg 168 (25, 26); mutations in *nonB*(*hsrM*) abolish restriction activity without affecting modification (25, 26).

For some time now it has been known that prior infection of *B. subtilis* Marburg 168 with phage SP18 permitted limited development of SP10 (9). These latter observations, considered with those related above, suggest that SP18 somehow antagonizes one or both of the gene products involved with nonpermissiveness for SP10 phage.

This communication describes experiments designed to obtain information concerning (i) the general response of SP10 to the restriction-modification system of *B. subtilis* Marburg 168, and (ii) the effect of SP18 on the cellular restriction system. The results indicate that SP10 DNA is rapidly degraded in *nonB*⁺ cells. Phage DNA is evidently modified by passage through a permissive derivative of *B. subtilis* Marburg 168, but the degree of modification is inadequate to protect fully the viral genome from restriction. Finally, it is demonstrated that SP18 can, indeed, antagonize the action of the *nonB* gene product.

MATERIALS AND METHODS

Bacteria and bacteriophages. A clear-plaque variant (19) of SP10 (ATCC 23059B) was used in these experiments. Phages SP18, SP82, SPP1, and ϕ 105c30 were supplied by C. B. Thorne, C. Stewart (via M. Mandel), T. Trautner, and D. Dean, respectively. *B. subtilis* W23 was ATCC 23059. Derivatives of *B. subtilis* Marburg 168 (Table 1) were supplied by H. Saito.

Medium. The medium employed was a modification of MS2 broth (21). It contained 1% tryptone (Difco Laboratories, Detroit, Mich.), 0.8% NaCl, 0.1%

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yeast extract (Difco), 0.03% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.015% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 0.01% $\text{MnSO}_4 \cdot \text{H}_2\text{O}$.

Buffers. Buffer P1 was 50 mM Tris-hydrochloride (pH 7.9)–50 mM NaCl–1 mM MgCl_2 . Buffer P2 was 50 mM Tris-hydrochloride (pH 7.9)–300 mM NaCl–1 mM MgCl_2 –5 μM ZnSO_4 . Buffer L contained 100 mM Tris-hydrochloride (pH 7.9)–20 mM disodium EDTA (pH 7.9). Buffer S contained 100 mM Tris-hydrochloride (pH 7.9)–900 mM NaCl–1 mM disodium EDTA (pH 7.9)–0.5% sodium lauroyl sarcosine.

Conditions of phage infection. Bacteria were grown at 37°C to a density of 2×10^8 per ml. The cells were harvested by centrifugation at $9,000 \times g$ for 2 min, washed once with 1 volume of ice-cold medium, and suspended into 0.95 volume of fresh, warm medium that contained 200 μM 6-(*p*-hydroxyphenylazo)-uracil, an inhibitor of bacterial DNA polymerase III (22) that has no effect on SP10 development (19). Five minutes later, the cells were infected by adding 0.05 volume of medium that contained the phage. If cultures were to be doubly infected, the cells were suspended in 0.9 volume of fresh medium, and each phage was added in 0.05 volume of medium. The input multiplicity was ca. 5 PFU per cell for each phage.

Preparation of SP10 and SP18 phage that contained [^3H]DNA. Cultures (50 ml) were infected as described above, except that 6-(*p*-hydroxyphenylazo)-uracil was excluded. The labeled precursor, [^3H]uracil (5 μCi per ml), was added 40 min postinfection. After lysis, DNase I and RNase A (5 μg per ml each) were added. The lysates were incubated at 37°C for 3 h. Phage were concentrated by the salt-polyethylene glycol method (38) and suspended in 5 ml of buffer P1. Large debris was removed by centrifugation at $2,000 \times g$ for 7 min, and the supernatant was applied to a column (1 by 5 cm) of G-200 Sephadex equilibrated with buffer P1. The column was eluted with buffer P1, and the phage were dialyzed into buffer P2. In the final preparation, $\geq 95\%$ of the radioactivity was precipitable with cold acid and stable in 0.3 N KOH (data not shown), implying that the label was in DNA (17). Moreover, when phage were banded in CsCl (3), 95 to 98% of the input radioactivity cobanded with the PFUs (data not shown). Typically, phage preparations had a specific activity of about 3×10^{-5} cpm per PFU.

Sucrose gradient centrifugation of phage-specific [^3H]DNA isolated from infected cells. Cultures (10 ml) were infected with phage that contained [^3H]DNA. At the desired time, cells were harvested by centrifugation at $9,000 \times g$ for 2 min, washed once with 30 ml of ice-cold buffer L, and suspended in 4 ml of buffer L additionally supplemented with 100 μg of lysozyme per ml and 1% sodium lauroyl sarcosine. The cells were incubated at 37°C for 2 h, by which time lysis was usually complete. Debris was removed by centrifugation at $9,000 \times g$ for 10 min. Pronase (200 $\mu\text{g}/\text{ml}$) was added, and the extracts were incubated at 50°C for 2 h. A 100- μl sample of extract was diluted in 400 μl of buffer S and incubated overnight at room temperature to dissociate aggregates (W. Mego, personal communication). Duplicate 200- μl samples were layered separately onto 4:4-ml linear sucrose gradients (5 to 20% sucrose in buffer S). The gradients were centrifuged at 15,000 rpm (SW60 rotor) for 17 h at 20°C in a Spinco L2-65B Ultracentrifuge. Marker

TABLE 1. Strains of *B. subtilis* Marburg 168

Strain	Genotype ^a		Sensitivity to SP10 phage
	<i>nonA</i>	<i>nonB</i>	
101	+	+	Nonpermissive
HLL3g	–	+	Nonpermissive
PRA2	–	+	Nonpermissive
1019	+	–	Nonpermissive ^b
PS9W7	–	–	Permissive

^a For complete genotype, see reference 25.

^b SP10 does not form plaques on plates seeded with *B. subtilis* 1019. Broth cultures of 1019 lyse 70 to 80 min after infection by SP10, but the burst size is only 1 to 2% of normal (unpublished data). The reduced burst presumably is responsible for the absence of plaques on solid medium.

DNA was spun in a parallel tube. Fractions (100 μl) were collected from the top with an ISCO density gradient fractionator. Each gradient fraction was diluted with 1 ml of a salmon sperm-DNA solution (100 μg per ml in distilled water). After the diluted fractions were chilled in an ice water bath, 1 ml of ice-cold 10% trichloroacetic acid was added. The precipitate was allowed to form for 10 min and was then collected by centrifugation at $12,000 \times g$ for 10 min. The pellet was dissolved in 0.5 ml of 0.3 N KOH, diluted with 1 ml of distilled water, and decanted into a scintillation vial that contained 8 ml of scintillator. The scintillator contained 500 ml of Triton X-100, 1,000 ml of toluene, and 5 g of Solimix I (D. Morrison, personal communication). Radioactivity was measured in a Packard Tri-Carb instrument.

Materials. Most unlabeled materials were obtained from Sigma Chemical Co. (St. Louis, Mo.), Calbiochem-Behring Corp. (San Diego, Calif.), and Fisher Scientific Co. (Itasca, Ill.). G-200 Sephadex was the product of Pharmacia Fine Chemicals (Uppsala, Sweden). Solimix I was obtained from ICN (Irvine, Calif.). Radioactive chemicals were purchased from New England Nuclear Corp. (Boston, Mass.). Antibiotics were purchased from Boehringer Mannheim Corp. (Indianapolis, Ind.).

RESULTS

Solubilization of SP10 DNA in restricting derivatives of *B. subtilis* Marburg 168. In *Escherichia coli*, restriction of phage lambda DNA proceeds in two steps (28). Initially, the resident restriction endonuclease introduces a limited number of double-strand breaks. Thereafter, the primary fragments are hydrolyzed by several nonspecific exonucleases that degrade roughly half the input DNA to acid-soluble material.

Upon infection of restricting hosts by SP10-W23 that contained [^3H]DNA, approximately 90% of the input label was degraded to acid-soluble material over the ensuing 30 to 40 min (Fig. 1A). No further solubilization was evident even upon prolonged incubation (data not

FIG. 1. Stability of SP10 [^3H]DNA in restricting and nonrestricting derivatives of *B. subtilis* Marburg 168. Cultures (12 ml) were infected with either SP10·W23 or SP10·PS9W7 as described in the text. At the times specified in the graph, 1-ml samples were removed, and the amount of label precipitable by cold acid was determined. (A) SP10·W23, 100% = 26,832 cpm/ml. (B) SP10·PS9W7, 100% = 18,146 cpm/ml. Symbols: ○, *B. subtilis* 101; ●, *B. subtilis* HLL3g; □, *B. subtilis* 1019; ■, *B. subtilis* PS9W7.

shown). No solubilization of SP10·W23 DNA was evident in *B. subtilis* 1019, a nonpermissive, nonrestricting host (Fig. 1A).

When restricting hosts were infected with SP10·PS9W7, only 40 to 50% of the input DNA was solubilized (Fig. 1B). These data were interpreted to mean that SP10 DNA can be modified to some extent by passage through the permissive derivative of *B. subtilis* Marburg 168 but that the degree of modification is inadequate to fully protect the SP10 DNA from restriction. Repeated passage of SP10 through *B. subtilis* PS9W7 failed to augment the amount of SP10 DNA that was nondegradable by restricting hosts (data not shown). Inasmuch as $\phi 105$ DNA becomes fully protected by a single passage through PS9W7 (25), the inability to completely protect SP10 DNA is evidently a feature peculiar to that virus. The results presented here are consistent with those of Saito, Shibata, and Ando (25), who reported that $\phi 105$ ·PS9W7 plated with unit efficiency on *B. subtilis* 101 and HLL3g, whereas SP10·PS9W7 failed to form plaques even on HLL3g.

Effect of SP18 on solubilization of SP10 DNA. Competent *B. subtilis* Marburg 168 are nontransfectable by SP10 DNA unless the cells are first infected with an unrelated phage, SP18 (9). As noted earlier, these observations imply that some aspect of the SP18 infection process overrides the *nonA* or *nonB* gene products or both. Within this communication, attention will

be limited to the effects of SP18 on restriction of SP10 DNA.

The experiments described below involve primary infection of cells with one phage, followed by superinfection with the other. Consequently, it is necessary to show that the primary infection does not affect adsorption or penetration of the superinfecting phage. At the multiplicities used in this study, prior infection of *B. subtilis* with either phage failed to materially affect subsequent adsorption by the other (Table 2). "Blendor experiments" (11) suggested that most superinfecting phage penetrated the cells to which they adsorbed (Table 3).

The initial experiments consisted of infecting parallel cultures of *B. subtilis* PRA2, a restricting host, with SP10·W23 that contained [^3H]DNA and SP18. In some cases, SP10·W23 was added before SP18; in other cases, the order of addition was reversed. Control cultures received a sham addition of SP18. The amount of label converted to acid-soluble material was taken to be a measure of restriction activity.

No protection of [^3H]DNA was evident when SP10·W23 was added before or with SP18 (Fig. 2). Increasing the interval between infection with SP18 and addition of SP10·W23 yielded progressively greater levels of protection. By the criterion applied, the cellular restriction system was completely inoperative 10 min postinfection by SP18. When added 5 min before infection by SP18, chloramphenicol, an inhibitor of protein

TABLE 2. Adsorption of phage to *B. subtilis* PRA2^a

Primary phage (³ H labeled)	Superinfecting Phage (¹⁴ C labeled)	% of total label adsorbed to cells	
		³ H	¹⁴ C
SP18	None	93.6	—
SP18	SP18	97.1	83.2
SP18	SP10	96.7	87.9
None	SP10	—	94.7
SP10	None	96.3	—
SP10	SP10	91.4	84.3
SP10	SP18	97.0	88.8
None	SP18	—	98.6

^a Parallel cultures were infected as described in the text. The final volume of all cultures was 2.0 ml. The primary phage was added at time zero, and the superinfecting phage was added 10 min later. At 20 min postinfection by the primary phage, the cultures were centrifuged at $10,000 \times g$ for 2 min. This latter procedure pelleted the cells and adsorbed phage but not the free phage. The amount of radioactivity in the pellet and supernatant fractions was determined. Labeled phage had the following specific activities: ³H-SP18, 1.3×10^{-5} cpm/PFU; ¹⁴C-SP18, 3.7×10^{-5} cpm/PFU; ³H-SP10, 1.01×10^{-5} cpm/PFU; ¹⁴C-SP10, 2.87×10^{-5} cpm/PFU. The phage were labeled with either L-[methyl-³H]methionine or L-[methyl-¹⁴C]methionine.

synthesis (4), and rifampin, an inhibitor of RNA synthesis (35), failed to prevent inhibition of the cellular restriction system (Fig. 3). Therefore, de novo synthesis of SP18 phage-coded macromolecules is not required for inactivation of the restriction activity of Marburg 168.

Size distribution of restricted and protected SP10 DNA. In principle, the amount of input phage DNA converted to acid-soluble material monitors only the second step in the restriction process. Although generation of acid-soluble material certainly implies that primary fragmentation has occurred, absence of measurable solubilization does not, in itself, mean that primary fragmentation did not take place since most, if not all, primary fragments are probably acid precipitable. Accordingly, neutral sucrose gradient analyses were performed to see whether SP10 [³H]DNA was conserved as unit-size molecules in SP18-infected restricting hosts. The experiments were done in the presence of chloramphenicol to confirm that postinfection protein synthesis is not required to protect superinfecting SP10·W23 DNA.

When *B. subtilis* 101 was singly infected with either SP10·W23 or SP10·PS9W7 that contained [³H]DNA, none of the residual, acid-precipitable material was recovered as unit-size molecules (Fig. 4A). Terminal fragments of SP10·W23 DNA had an apparent molecular weight of 0.5×10^6 to 1×10^6 , but this estimate

is tentative because no marker DNA in that weight range was available for this study. A more complicated profile was observed with terminal fragments of SP10·PS9W7 DNA. Here, 30% of the residual acid-precipitable label was recovered in the 0.5- to 1-megadalton range, but the remainder sedimented at a rate consistent with a molecular weight of 15×10^6 to 20×10^6 . When *B. subtilis* 101 was infected with SP18 before the addition of SP10, 80 to 85% of the acid-precipitable label was recovered as unit-size molecules (Fig. 4B), a situation comparable to that observed when *B. subtilis* 1019 was singly infected with either SP10·W23 or SP10·PS9W7 (Fig. 4C). Thus, it seems that prior infection of restricting strains of Marburg 168 with SP18 inhibited primary fragmentation of SP10 DNA.

Specificity of protection. Can other phage protect SP10 DNA from restriction? For these experiments, parallel cultures of *B. subtilis* 101 and HLL3g were preinfected with SP82·101, $\phi 105c30$ ·101, or SPP1·101 for 10 min before infection with SP10·W23 that contained [³H]-DNA. In all these instances, SP10·W23 DNA was degraded to about the same extent as when cells were infected with only SP10·W23 (Table

TABLE 3. Penetration of *B. subtilis* 1019 by primary and superinfecting phage: blender experiment^a

Primary phage (³ H labeled)	Superin- fecting phage (¹⁴ C la- beled)	% of label in pellet			
		Before blend		After blend	
		³ H	¹⁴ C	³ H	¹⁴ C
SP18	None	93.2	—	76.3	—
SP18	SP18	94.1	87.3	82.4	78.4
SP18	SP10	90.9	87.4	81.3	79.6
None	SP10	—	94.9	—	88.5
SP10	None	98.7	—	82.9	—
SP10	SP10	92.4	88.1	78.2	72.6
SP10	SP18	95.3	83.4	80.1	71.5
None	SP18	—	82.5	—	70.2

^a Cells were infected with phage containing labeled DNA. The experimental protocol was basically the same as the one given in Table 2, footnote a. At 10 min after addition of the superinfecting phage, half the culture was pelleted to determine the amount of label adsorbed to cells. The other half was blended (11) and then pelleted to determine what fraction of the label had been injected. In controls performed with phage containing labeled protein, $\geq 85\%$ of the radioactivity was removed from the cells by blending. These experiments were performed in the presence of chloramphenicol (100 μ g/ml) added 1 min before the primary infection to prevent elaboration of phage lytic enzymes; without this precaution, 15 to 25% of the infected cells were broken by blending (our unpublished data).

FIG. 2. Effect of SP18 on solubilization of SP10-W23 [^3H]DNA in *B. subtilis* PRA2. Parallel cultures (2 ml) were doubly infected with SP18 and labeled SP10-W23 as described in the text. A control culture received labeled SP10-W23 but no SP18. The cultures were incubated until 60 min postinfection by SP18, at which point the cultures were treated with cold acid. Percent protection = $(C_x - C/C_0 - C) \times 100$, where C is the residual, acid-precipitable label in the control cultures, C_0 is the input acid-precipitable label, and C_x is the amount of acid-precipitable label remaining in a culture that received SP18. The different symbols represent completely independent repeats of the experiment. In the absence of SP18, 80 to 84% of the input label was solubilized.

4). These data are consistent with the interpretation that protection of superinfecting SP10 DNA required processes carried out, among the phage tested, only by SP18.

Stability of SP18 DNA in *B. subtilis* Marburg 168. SP18 required a full 10 min to inactivate the cellular restriction system (Fig. 2 and 3). During this interval of time, was there any degradation of SP18 DNA? Since all SP18-W23 DNA recovered from *B. subtilis* 101 sedimented through sucrose as unit-size molecules (data not shown), it appears that SP18 DNA is refractile to the restriction activity in question. This problem will be readdressed later.

DISCUSSION

SP10 cannot develop in *B. subtilis* Marburg 168 that contain the wild-type allele of either *nonA* or *nonB* (23, 25). Data presented in this communication are consistent with the interpretation that, in *nonB*⁺ cells, SP10 DNA is degraded to acid-soluble material and acid-precipitable oligonucleotides (Fig. 1 and 4A). The

nonB gene product functions during primary fragmentation as evidenced by the fact that SP10 DNA was conserved as unit-size molecules in *nonB* bacteria (Fig. 4C). No evidence was obtained that the *nonA* gene product played a role in the restriction-modification phenomenon (Fig. 1 and our unpublished data), a conclusion also supported by the available genetic data (25).

SP10-PS9W7 DNA was only partially protected from the restriction system of Marburg 168 (Fig. 1B and 4A). Assuming that only one restriction-modification system exists in Marburg 168 (25), these data would mean that certain restriction sites on SP10 DNA are cleavable but not modifiable. Approximately 20% of the dTMP in SP10 DNA is replaced by a hypermodified nucleotide, designated YdTMP (M. Mandel, personal communication cited in reference 5). If the recognition sequence for the Marburg 168 restriction-modification system contained dTMP, then, in the case of SP10, a fraction of these sites would, inferentially, contain YdTMP. A plausible hypothesis is that YdTMP inhibits the modification reaction but has little or no effect on cleavage by the restriction nuclease. In this context, it is noted that the DNAs of phages ϕ e and PBS2, in which dTMP is completely replaced with 5-hydroxymethyldeoxyuridylylate (HmUMP) and deoxyuridylylate, respectively (14, 32), are poor substrates for *EcoRI*

FIG. 3. Effect of rifampin and chloramphenicol on protection of SP10-W23 [^3H]DNA by SP18. The experimental protocol was basically the same as the one described under Fig. 3, except that either rifampin (10 μg per ml) or chloramphenicol (100 μg per ml) was added 5 min before SP18. Percent protection was computed as described under Fig. 3. Symbols: ○, no antibiotics; ●, rifampin; ▲, chloramphenicol.

FIG. 4. Sucrose gradient centrifugation of restricted and protected SP10-W23 and SP10-PS9W7 [^3H]-DNAs. The hosts were *B. subtilis* 101 and *B. subtilis* 1019. All cultures received chloramphenicol (100 μg per ml) 5 min before infection. In the case of doubly infected cultures, SP18 was added 10 min before infection by SP10. Infections were terminated 30 min after addition of labeled SP10. Sucrose gradient centrifugation is detailed in the text. Arrows mark the position of marker SP10 DNA (molecular weight, 80×10^6) and $\phi 105c30$ DNA (molecular weight, 25×10^6) that were run in a parallel gradient. (A) *B. subtilis* 101 infected with either SP10-W23 (○) or SP10-PS9W7 (●); (B) *B. subtilis* 101 infected with SP18 for 10 min before the addition of either SP10-W23 (○) or SP10-PS9W7 (●); (C) *B. subtilis* 1019 infected with either SP10-W23 (○) or SP10-PS9W7 (●).

TABLE 4. Specificity of protection^a

Primary phage	Acid-precipitable label (% of input)	
	<i>B. subtilis</i> 101	<i>B. subtilis</i> HLL3g
None	18.3	21.7
SP18	98.6	91.3
SPP1-101	12.7	18.6
SP82-101	21.4	23.4
$\phi 105c30$ -101	17.6	19.0

^a Parallel 2-ml cultures were doubly infected as described in the text. The primary phage was added 10 min before SP10-W23 that contained [^3H]DNA. The cultures were acid precipitated 30 min after addition of the labeled SP10.

methylase even though these DNAs are cleaved by the *Eco*RI restriction endonuclease (1). Thus, replacement of dTMP with an unusual analog can affect, to fundamentally different degrees, the activities of a restriction endonuclease and its corresponding modifying enzyme.

Both dTMP and YdTMP in mature SP10 DNA evidently arise by postreplicational modification of HMdUMP in nascent DNA (5, 20, 37). Presently, it is not known whether the conversion of HMdUMP to dTMP and YdTMP occurs randomly or in a specific pattern. An analysis of the 15- to 20-megadalton fragments of SP10-PS9W7 DNA (Fig. 4A) could shed some

light on this problem, provided the inability of Marburg 168 to modify certain restriction sites on SP10 DNA is due to the presence of YdTMP. If YdTMP occurs in a specific pattern, it follows that the same sites on all SP10-PS9W7 DNAs are unprotected which, in turn, means that the solubilization reaction (Fig. 1B) will remove the same sequences from all SP10-PS9W7 DNAs; therefore, the 15- to 20-megadalton fragments should not contain all sequences originally present in parental DNA. Conversely, if the replacement of dTMP with YdTMP is random, on a population-wide basis, all possible sites on SP10-PS9W7 DNA will be protected, and the 15- to 20-megadalton fragments should contain global sequences. The relevant experiments are currently in progress.

Prior infection of restricting derivatives Marburg 168 with SP18 protected superinfecting SP10 DNA (Fig. 2 and 3). Since SP10 adsorbed to and penetrated SP18-infected cells (Tables 2 and 3), protection was mediated intracellularly. Most of the protected SP10 DNA was recovered as unit-size molecules (Fig. 4B), suggesting that SP18 antagonized the primary fragmentation step of the restriction process, i.e., the step carried out by the *nonB* gene product (Fig. 1 and 4C). It does not necessarily follow that this antagonism entails direct inactivation of the restriction nuclease of Marburg 168.

Interestingly, inactivation of the Marburg 168 restriction system by SP18 did not require post-infection synthesis of phage-coded RNA and protein (Fig. 3 and 4B). Such data are consistent with the interpretation that the inactivating element was injected with the viral DNA, although other interpretations are equally valid in light of the existing data. If the cellular restriction system was overridden by elements injected with SP18 DNA, it is difficult to see why inactivation required a full 10 min to go to completion (Fig. 3). In the case of coliphage T4, processes carried out by injected proteins, e.g., alteration of the alpha subunit of host RNA polymerase (12), typically are complete within a few minutes of infection (39). We explored the possibilities that SP18 is a slow adsorber or, as is the case with coliphage T5 (16), that there is a long interval between adsorption and full penetration, but neither of these alternatives seems to be an adequate explanation of the problem at hand (data not shown).

Considering the long period of time required by SP18 to inactivate the restriction system of Marburg 168 (Fig. 2 and 3), it is necessary to ask why SP18 DNA was not cleaved during the interval when the restriction system was presumably still operative. This is an especially compelling point considering the rapidity with which SP10 DNA was evidently attacked (Fig. 1). Perhaps SP18 DNA simply lacks the sequence recognized by the Marburg 168 restriction enzyme. Alternatively, SP18 may possess some other active mechanism to protect its DNA during that interval when the cellular restriction system is still operational; the nature of such a mechanism is purely speculative but, if it exists, it presumably cannot protect a heterologous DNA that shares a common cytoplasm with SP18 (Fig. 2 and 3). Resolution of this problem will require isolation of the Marburg 168 restriction enzyme and characterization of SP18 mutants that are sensitive to the Marburg 168 restriction-modification system.

Several phage are able to directly antagonize cellular restriction systems. T3 and T7 each elaborate a protein that specifically inactivates the Type I restriction enzymes in *E. coli* strains B and K (7, 15, 29-31), whereas phage ϕ NR24rH, a mutant of ϕ NR2, inactivates restriction endonuclease BamN_x found in *Bacillus amyloliquefaciens* N (18). Data presented here suggest that SP18 can somehow override the restriction system of *B. subtilis* Marburg 168 (Fig. 2, 3, and 4B). Our preliminary data (not shown) indicate that SP18 grown on nonrestricting, nonmodifying hosts can plate with unit efficiency on the following restricting strains: *B. subtilis* IAM1247

(13, 27), *B. subtilis* R (34), *B. amyloliquefaciens* N (26), *B. amyloliquefaciens* H (36), and *Bacillus brevis* ATCC 9999 (8, 24); indeed, the only host tested so far that restricts SP18 is *B. subtilis* subsp. *globigii* (6). It will be of interest to determine the basis for the resistance of SP18 to so many restriction systems.

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