MECHANISMS OF NONPERMISSIVENESS IN ABORTIVE INFECTION OF BACTERIOPHAGE ϕ NR2 IN *BACILLUS SUBTILIS* MARBURG STRAIN

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(Received June 23, 1980)

A mutant strain PS9, permissive to infection of phages SP10 and ϕ NR2, was derived from a nonpermissive Marburg strain of *Bacillus subtilis*. When treated with mitomycin C or ultraviolet irradiation, PS9 cells lysed to produce defective phage PBSX as did the nonpermissive cells. Thus it was suggested that the nonpermissiveness might be independent of the repression system of the defective phage PBSX. As another approach to elucidate the mechanism of nonpermissiveness, DNA and RNA syntheses were analyzed in both permissive and nonpermissive cells infected with phage ϕ NR2. By molecular hybridization with phage DNA, it was found that, in the nonpermissive cells, ϕ NR2 DNA did not replicate but phage-directed RNA was partially synthesized. The RNA synthesized in the nonpermissive *B. subtilis* Marburg appeared to lack late messenger RNA compared to RNA synthesized in the permissive cells.

Cells of *Bacillus subtilis* Marburg strain can adsorb phage SP10 but are nonpermissive to the replication of this phage and thus the Marburg strains can not serve as gene donors in generalized transduction by phage SP10 (1).

GWINN and LAWTON (2) and GOLDBERG and BRYAN (3) reported that the cells of Marburg strain were rendered permissive to infection with phage SP10 by heating or mitomycin C treatment, and suggested that repressors of endogenous defective prophage PBSX may play a role in the nonpermissiveness of the *B. subtilis* Marburg strain.

We have isolated a mutant of *B. subtilis* Marburg strain which is permissive to phage SP10 as well as a newly isolated phage ϕ NR2 (4) and attempted to study mechanisms of the nonpermissiveness in two respects. The first relates to the induction of PBSX in the permissive mutant, and the second is concerned with phage-directed DNA or RNA synthesis in nonpermissive and permissive hosts. In these studies we have used the ϕ NR2 which is similar to phage SP10 with respect to the host specificity concerning the nonpermissiveness (5), since SP10

is inconvenient for these studies because of the difficulty in preparation of high-titer suspension and loss of infectious ability during storage.

MATERIALS AND METHODS

Microorganisms. Strain HLL3g (*purB6 leuA8 thr5 metB5 hisA1 lys21 trpC2*) was derived from *Bacillus subtilis* Marburg by successive transformation with DNA of corresponding auxotrophic strains (6). This strain is nonpermissive to phages SP10 and ϕ NR2. A permissive mutant PS9 was isolated from approximately 5,000 colonies of strain HLL3g treated with nitrosoguanidine. Efficiencies of plating of both phages on PS9 were almost the same as those on the original host strains (4). Auxotrophic genotypes of PS9 was the same as those of HLL3g. In addition to these strains, *B. amyloliquefaciens* 203 (formerly *B. megaterium* 203) was used for growth of phage ϕ NR2. Characteristics of phage ϕ NR2 were described previously (5).

Media and buffers. BTY medium and TCG medium were described previously (5). To certain experiments, Penassay broth (Difco) was used. TCG was usually supplemented with 20 μ g/ml of adenine and 50 μ g/ml of L-tryptophan.

TMN buffer contained 0.01 M Tris-HCl (pH 7.4), 5 mM MgCl_2 , and 10 mM NaN_3 .

Induction of PBSX. Cells were grown in Penassay broth until the cell density reached 0.06 at A_{660} . Mitomycin C (Kyowa Hakko Co., Tokyo) was added to the culture at a concentration of 3 μ g/ml and incubation was continued for 10 min at 37°. The cells were collected by centrifugation, resuspended in the same volume of fresh Penassay broth, and incubated at 37°.

When ultraviolet light was used for induction, a germicidal lamp (20 W, Toshiba Electric Co., Tokyo) was used.

Labelling of DNA. Strains HLL3g and PS9 were cultured in TCG medium supplemented with adenine and tryptophan until approximately 10^8 cells/ml and infected with phage ϕ NR2 at multiplicities of infection of 5 to 10. Then, at intervals, 25 μ Ci/ml of 6- ^3H thymidine (specific activity: 28.0 Ci/mmol) was added and newly synthesized DNA was labelled for 5 min. Then, the culture was mixed with 2×10^{-2} M KCN in the cold, and the cells were harvested for DNA preparation.

DNA was prepared by pH 9-phenol method (7). The radioactivity of this labelled DNA was approximately 1,000 cpm/1 μ g DNA for both strains infected with phage ϕ NR2.

Labelling and preparation of mRNA. HLL3g and PS9 cells were labelled at intervals with 20 μ Ci/ml of ^3H -uridine (specific activity: 18.5 Ci/mmol) for 2 min. Cells from 2 ml culture, harvested as described above, were suspended in 0.8 ml TMN buffer supplemented with 500 μ g of lysozyme and 20 μ g of DNase I, and immediately subjected to freezing and thawing repeated 3 times. Then,

lysis was completed by incubation with 0.05 ml of 1 M acetate buffer, pH 5.1 and 0.05 ml of 10% sodium-dodecylsulfate. RNA was extracted by phenol treatment, precipitated with 2.5 volumes of cold ethyl alcohol in the presence of 0.15 M NaCl, and dissolved in $0.1 \times$ SCC containing 0.1% sodium-dodecylsulfate.

Molecular hybridization. DNA-DNA hybridization was carried out as described previously (5), but final washing was done with 3 mM Tris-HCl buffer, pH 9.4, from one side of the filter.

DNA-RNA hybridization was performed by the method of GILLESPIE and SPIEGELMAN (8).

RESULTS

Induction of defective phage, PBSX, in nonpermissive and permissive strains

Cells of HLL3g and PS9 were grown and induced with mitomycin C as described in MATERIALS AND METHODS. Lysis occurred in 60 min, suggesting the induction of defective phage PBSX in both strains HLL3g and PS9 (Fig. 1).

Fig. 1.

Fig. 1. Lysis of cells treated with mitomycin C.

Three μ g/ml of mitomycin C were added as indicated by an arrow (MC) to the culture in Penassay broth of the permissive strain PS9 (●) or the nonpermissive strain HLL3g (○). After 10 min, cells were centrifuged ($\leftrightarrow$) and resuspended in the fresh medium.

Fig. 2.

Fig. 2. Lysis of cells irradiated with ultraviolet light.

Culture in BTY broth of PS9 or HLL3g was irradiated by ultraviolet light (4,000 erg/mm²) at time 0 (●). Unirradiated control was also shown (○).

Similar lysis was seen when the cultures of both strains in BTY broth were irradiated by ultraviolet light at a dose of 4,000 erg/mm² (Fig. 2).

Presence of defective phage PBSX in the lysates was suggested by their killing activity against *Bacillus subtilis* W23 (9). More than 10⁷/ml killing particles were estimated both in the lysates by dilution tests. More direct evidence was obtained by electron microscopy. Phage particles in the lysates were collected by ultracentrifugation, negatively stained, and photographed under a electron microscope. As seen in Fig. 3, sizes and shapes of phage particles were very similar both in the lysates of HLL3g and PS9.

Fig. 3. Electron microphotograph of defective phage PBSX in mitomycin-induced lysate of PS9 (A) or HLL3g (B). The scales represent 0.1 μ m.

Host- and phage-DNA synthesis following infection with phage ϕ NR2

Permissive (PS9) and nonpermissive (HLL3g) cells were infected with phage ϕ NR2 at logarithmic growth phase, and newly synthesized DNA was pulse-labelled with ³H-thymidine at intervals. The labelled DNA was denatured and hybridized with cold, denatured bacterial DNA or phage DNA fixed on Millipore filters (Fig. 4). In the PS9 cells infected with ϕ NR2, the rate of phage-directed DNA synthesis increased markedly after a lag period of about 10 min, whereas the rate of host DNA synthesis decreased. In contrast, in the nonpermissive host, HLL3g, phage DNA synthesis was not observed and no appreciable change in host DNA synthesis was seen after infection with ϕ NR2.

Phage specific mRNA synthesis after infection

HLL3g and PS9 cells were infected with phage ϕ NR2 and pulse-labelled with ³H-uridine. In the permissive host, PS9, the rate of phage-directed mRNA synthesis increased gradually after infection, while the rate of host mRNA synthesis remained constant (Fig. 5A). In the nonpermissive host, HLL3g, the rate of

Fig. 4. DNA synthesis in cells of permissive strain PS9 or nonpermissive strain HLL3g following infection with phage ϕ NR2. DNA was labelled for 5 min with 25 μ Ci/ml of 6- 3 H thymidine, extracted and hybridized, after denaturation, with phage DNA (■) or cellular DNA (□) fixed on a Millipore filter (approximately 5 μ g each DNA had been denatured and fixed on the filter). Radioactivity in hybrid molecules was expressed as percent of input radioactivity (approximately 2,000 cpm).

Fig. 5. RNA synthesis in cells of PS9 or HLL3g following infection with phage ϕ NR2.

RNA was labelled for 2 min with 20 μ Ci/ml of 3 H-uridine, extracted and hybridized with denatured phage DNA (●) or cellular DNA (○). Input radioactivity was about 6,000 cpm. See legend of Fig. 4 for other information.

Fig. 6.

Fig. 7.

Fig. 6. Competition to hybridization between ϕ NR2 DNA and RNA labelled for 2 min at 30 min after infection of permissive PS9 cells.

Cold RNAs for competition were prepared from PS9 at 12 min ($\circ$) or 30 min ($\bullet$) after infection and also from nonpermissive HLL3g at 30 min ($\triangle$) or 42 min ($\blacktriangle$) after infection. Data were shown as percent radioactivity of non-competed hybrid (approximately 500 cpm).

Fig. 7. Competition to hybridization between ϕ NR2 DNA and RNA labelled for 2 min at 15 min (A) or 30 min (B) after infection of PS9 cells.

Infection was carried out in the presence of 100 μ g/ml chloramphenicol. Cold RNAs for competition were prepared from PS9 at 30 min after infection ($\circ$) and from HLL3g at 10 min ($\blacktriangle$) or 30 min ($\triangle$) after infection. See legend to Fig. 6.

phage-directed mRNA synthesis was decreasing with the lapse of time, although appreciable synthesis continued for longer than 50 min after infection (Fig. 5B).

The phage specific mRNA synthesized in the nonpermissive host HLL3g was characterized by competition experiments. First, 3 H-uridine labelled phage specific mRNA, synthesized during 30 to 32 min after infection in permissive host PS9, was hybridized with denatured phage DNA fixed on a membrane filter in the presence of various amounts of cold RNAs. Cold RNA was prepared from PS9 at 12 min or 30 min after infection with phage ϕ NR2, and cold RNA from HLL3g was prepared at 30 min or 42 min after infection. As shown in Fig. 6, almost complete competition was observed with excess amounts of RNA from PS9 at 30 min after infection, while other RNAs competed only 40% of the hybridization. These results suggest that phage-directed RNA synthesized in nonpermissive cells of HLL3g is somehow deficient, possibly lacking late mRNA.

In order to confirm the deficiency described above, labelled mRNA was prepared from cells of PS9 infected in the presence of 100 μ g/ml of chloramphenicol (CM), and hybridization between the CM-RNA and phage DNA was competed

with cold RNAs (Fig. 7A). Obviously, excess amounts of cold RNA from infected HLL3g cells, either at 10 min or 30 min after infection, competed the hybridization almost completely, while excess of RNA from PS9 at 30 min after infection left 50% of bound radioactivity uncompleted. When the labelled CM-RNA was prepared at 30 min instead of 15 min after infection, the competition patterns were almost the same (Fig. 7B).

Reciprocal experiments were conducted with labelled RNA from cells of HLL3g at 10 min after infection and cold CM-RNA from cells of PS9 at 10 min after infection. In these combinations competition remained at only 50%, suggesting that the CM-RNA consisted of fewer molecular species (possibly only immediate early mRNA) than RNA from cells of HLL3g (data not shown).

DISCUSSION

It has been proposed that repressors for the defective prophage PBSX might play a role in the abortive infection by phage SP10 of *Bacillus subtilis* Marburg (2,3). SIEGEL and MARMUR reported that, in a temperature-sensitive mutant of *B. subtilis*, induction of PBSX and a temperate phage SP02 occurred at 48° (10). Later THURM and GARRO found that another temperate phage ϕ 105 was also induced in this temperature-sensitive mutant by temperature shift up (11). These findings give support for a common repression system effective on various *B. subtilis* phages including the defective phage PBSX. Results of the present studies, however, have reduced the possibility that the growth of phages SP10 and ϕ NR2 are inhibited by repressors of the defective phage PBSX. We have obtained a mutant, PS9, permissive to both the phages SP10 and ϕ NR2. Upon treatment with mitomycin C or ultraviolet irradiation, the mutant PS9 lysed and produced defective phage particles as did the parental nonpermissive strain. This result suggests that the nonpermissiveness to phages SP10 and ϕ NR2 in *B. subtilis* Marburg is determined by a host function independent of the PBSX-repression system.

We have studied DNA and RNA syntheses in both permissive and nonpermissive hosts after infection with phage ϕ NR2. It was found that the nonpermissive host did not allow the replication of phage DNA but permitted partially the phage-directed RNA synthesis. The RNA synthesized in the nonpermissive host seemed to comprise both immediate early and early mRNAs. Although the study is still preliminary, we propose that the nonpermissiveness to phages SP10 and ϕ NR2 might be related to a matter of the suppression in transcription.

Genetic studies on the determinants of the nonpermissiveness have been reported previously (4). One mutation associated with the permissiveness located near *rflm* and the other located near *purB*.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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