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Bacteriophage PMB12 Conversion of the Sporulation Defect in RNA Polymerase Mutants of *Bacillus subtilis*

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The pseudotemperate phage PMB12 was isolated from soil on the basis of its ability to enhance the rate of sporulation of *Bacillus subtilis* 168. PMB12 was subsequently shown to convert the sporulation defect in two genetically distinct classes of sporulation mutants. One class includes those rifampin-resistant mutants that are also spore-negative (mutated at the *rif* locus). The other class includes a strain carrying the sporulation mutation *spoCM-1*. The *spoCM-1* mutation is linked to *cysA14* by PBS1 transduction but is distinct from the *rif* locus. Several other sporulation mutants were not converted by PMB12. PMB12 is related to phage PBS1. However, PBS1 did not convert the above sporulation mutants. The replication of PBS2, a clear-plaquing derivative of PBS1, is rifampin insensitive, apparently due to a phage-induced rifampin-insensitive RNA polymerase. PMB12 replication is also rifampin insensitive.

We recently described a novel biological approach that permits the rapid identification of naturally occurring bacteriophages that convert specific sporulation-deficient mutants of *Bacillus pumilus* to spore positive (2). The method involves screening isolates of pseudotemperate phage for those which, upon infection of wild-type cells, enhance the rate or extent of sporulation as judged by changes in the morphology of cells in the turbid plaque centers (2). The basis for anticipating the existence of spore-converting phages rests on the observation that phage genomes trapped by spores are more resistant to environmental stress than free phage particles (2). Therefore, phages capable of enhancing the probability, or rate, of sporulation of an infected host would appear to have a selective advantage over phages that cannot mediate such conversion. The selective advantage for a spore-converting phage in nature is significantly enhanced if the host normally undergoes sporulation at a very low frequency. For example, many strains of *B. pumilus* are oligosporogenic, forming spores at frequencies of less than 20% (P. S. Lovett, unpublished data), and we estimate that about 10% of the pseudotemperate phages isolated for *B. pumilus* strains are spore-converting phages (K. M. Keggins and P. S. Lovett, manuscript in preparation).

In the present report we describe a modification of our previously described approach that has permitted the isolation of the spore-converting phage PMB12 for *Bacillus subtilis*. Certain of the properties of PMB12 indicate that this phage may be a useful probe for analysis of the genetic control of bacterial sporulation.

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MATERIALS AND METHODS

Bacteria, phages, media, and growth conditions. Strain BR151 (*trpC2 metB10 lys-3*) is a derivative of *B. subtilis* 168. Sensitive strains used were *B. subtilis* 168, W23, and ATCC 7059, 7972, 8188, 15575, 15818, 19659, and 23059; resistant strains used were *B. subtilis* ATCC 4925, 7480, 8472, 15245, 15561, 15562, 15841, *Bacillus licheniformis* 9945A, and *Bacillus amyloliquefaciens* H. (Resistant strains exhibited an efficiency of plating of less than 10^{-8} relative to plating efficiency on BR151.) Phages PBS1 and PMB1 were previously described (2, 10, 20). Incubation was at 37°C unless noted; liquid cultures were grown with rotary shaking. Liquid media used include Spizizen minimal medium (2), Penassay broth (Difco), and SG broth (8). Solid media were tryptose blood agar base (TBAB; Difco), and the above liquid media solidified by adding Noble agar (Difco) to 1.5%. All bacteriophage techniques were performed as previously described (2), except that the semisolid overlays and bottom agar were SG medium containing 0.6% and 1.5% agar, respectively.

Characterization of PMB12 DNA. Radioactively labeled PMB12 particles were prepared by including [^{3}H]adenine (New England Nuclear Corp.) in the semisolid overlays at 0.01 mCi/ml. DNA was phenol extracted from purified phages as described previously (2). Sedimentation of phage DNA through neutral and alkaline 5 to 20% sucrose gradients was as previously described, using T7 DNA as reference

(2). Buoyant density measurements of PMB12 DNA were performed in a model E analytical ultracentrifuge as previously described (19), using PBP1 DNA (ρ = 1.691) or *Escherichia coli* DNA (ρ = 1.710) as reference. Calculations were according to Schidkraut et al. (25).

Mutant isolation. Strain BR151 was mutagenized with nitrosoguanidine (20). The survivors were grown for 5 h in Penassay broth, plated on SG agar, and then incubated for 48 h at 37°C. Colonies that appeared "nonopaque" relative to wild-type colonies were spore negative. Rifampin-resistant mutants of BR151 were selected as spontaneous variants appearing on TBAB containing 5 μg of rifampin per ml (Calbiochem).

Isolation and characterization of spores. About 10^8 BR151 cells were spread on SG agar plates and incubated at 37°C for 3 days. Bacterial growth (mainly spores) was scraped from the plates into 0.1 M sodium phosphate buffer, pH 7.2, and incubated at 37°C for 1 h with 200 μg of lysozyme, 10 μg of DNase, and 50 μg of RNase per ml. The spores were washed repeatedly with buffer and heated at 70°C for 30 min. Spores were then sedimented through 30 to 90% linear Renografin gradients (2). The visible band of spores was collected in a single tube, washed with water, and stored at 4°C. PMB12-induced spores were prepared by spreading about 10^6 PFU with about 10^7 cells on SG agar plates, with subsequent incubation at 37°C for 3 days. Induced spores were purified as above. Spores labeled with [^{3}H]adenine were prepared as previously detailed (2). Dipicolinic acid was measured as previously described (14).

Assays for phage conversion. Plaque assays of PMB12, using the spore-negative mutant CM-1 (or any other convertible, spore-negative mutant) as indicator, gave rise to small turbid plaques that became spore-positive clones during 48 h of incubation at 37°C in the SG medium described above. Spores were detected by phase-contrast microscopy of the contents of the plaques. Bacterial mutants that were temperature sensitive for sporulation (TS1, TS2, TS3, TS4, E2; [30, 31]) were tested for convertibility by PMB12 at the temperature that was nonpermissive for sporulation of the uninfected cells (47°C). When a given spore-negative mutant was found to be converted to spore positive by PMB12 grown on BR151, the phage was subjected to two successive single plaque isolations on the mutant under study. This phage was then assayed for conversion of the mutant in the standard plaque assay.

The following method was used by J. Hoch to screen several sporulation mutants for conversion by PMB12. A 0.1-ml sample of a late log-phase culture of the mutant to be tested (motility confirmed by microscopy) was overlaid onto an SG agar plate in 2.5 ml of SG semisolid. When the top agar had solidified, a drop of PMB12 at 10^9 PFU/ml and a drop at 10^8 PFU/ml were placed in separate areas of the plate. Plates were then incubated for 4 days at 37°C. Sterile Pasteur pipettes were used to remove plugs from the infected and noninfected areas of the plate. Each plug was expelled into 1 ml of sterile distilled water, agitated vigorously, treated with a drop of chloroform, and assayed for viable units on SG agar.

RESULTS

Isolation and properties of the *B. subtilis* spore-converting phage PMB12. Aqueous 20% soil suspensions were heat-shocked (70°C, 30 min) and portions were added to exponentially growing broth cultures of spore-positive auxotrophic derivative of *B. subtilis* 168, strain BR151. After 20 h of incubation at 37°C, the cells were removed by sterile filtration and the filtrate was assayed for PFU using BR151 as indicator. Many turbid-centered plaques were detected. The cells within the turbid centers of a few of the plaques appeared to sporulate more rapidly than the backgound lawn. Phage from each such plaque were transferred to broth, sterilized by filtration, and cross streaked with BR151 on SG agar. By this method we isolated a phage, PMB12, that significantly enhanced the rate of sporulation of BR151.

PMB12 is related to the well-studied *Bacillus* phage PBS1 (10). Antiserum prepared against PBS1 (neutralization constant [K] of 700 against PBS1) neutralized PMB12 with a K value of 660. Electron microscopy of purified PMB12 particles demonstrated that the morphology of PMB12 was similar to that reported for PBS1 (Fig. 1; [7]). PBS1 is a flagella-specific phage (15) and will not infect or adsorb to flagella-negative derivatives of the sensitive host. Similarly PMB12 neither formed plaques on a flagella-negative derivative of *B. subtilis* 168, nor could we detect adsorption of PMB12 to the Fla$^-$ mutant (2 [$\pm$1] $\times$ 10^8 cells per ml adsorbed less than 5% of 10^4 PFU/ml during 30 min of incubation at 37°C in broth). PMB12 propagated on strain BR151 was capable of forming small, turbid plaques on more than half of the *B. subtilis* strains tested.

DNA extracted from PMB12 particles had a buoyant density of 1.721 g/cm^3 in CsCl and sedimented at 68 ($\pm$3) S in 5 to 20% neutral sucrose gradients. If it is assumed that PMB12 DNA is a linear duplex molecule, then the molecular weight for the PMB12 genome is approximately 2.2 $\times$ 10^8 (28). Sedimentation of PMB12 DNA through 5 to 20% alkaline sucrose gradients fragmented the DNA into many low-molecular-weight components, suggesting that PMB12 DNA contains numerous single-stranded interruptions or alkaline-labile links. These properties of PMB12 DNA are similar to those reported for PBS1 DNA (12, 32).

Bacteriophage PBS2, a derivative of PBS1, replicates in rifampin-sensitive *B. subtilis* host cells in the presence or absence of the drug

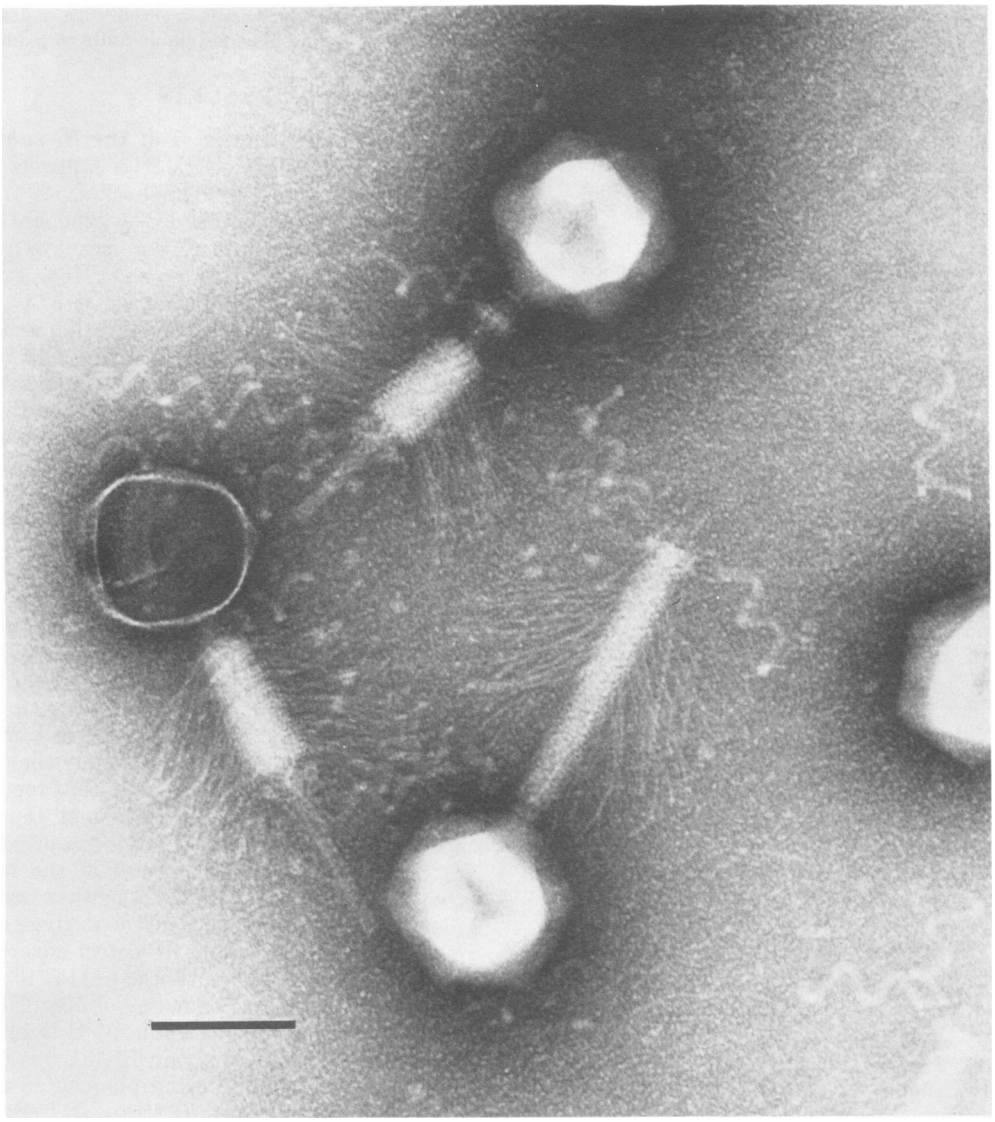

FIG. 1. *Electron micrograph of PMB12. Purified PMB12 particles were negatively stained with 1% potassium phosphotungstate, pH 6.9. Bar represents 0.1 μm.*

rifampin (24). The rifampin insensitivity of PBS2 replication has been correlated with the appearance of a new rifampin-resistant species of RNA polymerase detected only in PBS2-infected cells (4). PMB12 was, therefore, tested for its ability to replicate in BR151 (which is inhibited from growing by 2 μg of rifampin per ml) in the absence and presence of rifampin. In the absence of rifampin, the latent period and burst size for PMB12 were 30 (±3) min and 100, respectively. Addition of rifampin (20 μg/ml) to BR151 cells 3 min before infection with PMB12 had no detectable effect on the latent period (32 min) or the burst size (105).

Isolation and properties of a spore-negative mutant of BR151 that is converted to spore positive by PMB12. Only one of more than 50 uncharacterized, independently isolated nitrosoguanidine-induced spore-negative mutants of BR151 was converted to spore positive by PMB12. This mutant, designated CM-1, exhibited a sporulation frequency below 10^{-7}. PMB12 propagated on CM-1 then purified by differential and equilibrium gradient centrifugation was plated in a typical plaque assay, using CM-1 as indicator. Each turbid plaque detected after 24 h of incubation became a white, opaque spore-positive clone within 48 h

of incubation. One plate that initially contained about 300 PMB12 plaques was photographed after 5 days of incubation (Fig. 2). Due to the small size of PMB12 plaques, the spore-positive clones appear as small (1 to 2 mm in diameter) white spots (Fig. 2).

Phage conversion of CM-1 to spore positive in SG broth could be demonstrated but generally occurred at very low frequency (Table 1). The yield of phage-induced spores generated by infection of CM-1 in SG broth rarely exceeded 10^6 spores per ml. A similar problem of low frequency induction of sporulation by a *B. pumilus* spore-converting phage in liquid medium has been reported (2).

PMB12-induced CM-1 spores were purified, diluted, and plated on TBAB. After overnight incubation, 583 of the resulting colonies were tested for the presence of PMB12 infectious activity by transferring portions of each colony to plates inoculated with BR151. Each colony tested harbored infectious activity. However, PMB12 is not stably maintained by vegetatively growing cells. In a typical experiment,

TABLE 1. *Induction of sporulation of CM-1 in liquid SG medium by PMB12 infection*[a]

Culture	Viable units/ml	
	Total	Heat resistant
CM-1	1.1×10^8	<20
CM-1 + PMB12	4×10^7	2×10^6

[a] CM-1 was grown in SG broth to mid-log phase. The culture was split, and half was infected with PMB12 at a multiplicity of infection of about 20. Incubation was continued for 48 h, and samples were then assayed for colony forming activity on TBAB. Heat-resistant viable units were assayed after heat shocking at 70°C for 30 min.

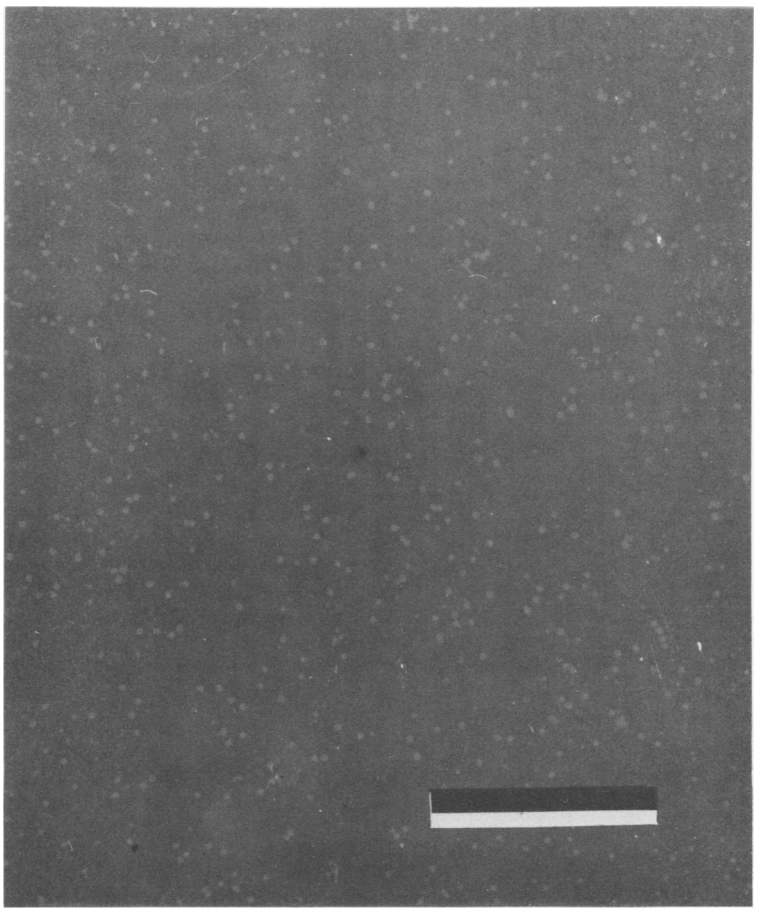

FIG. 2. *Plaque assay conversion of CM-1 to spore positive by PMB12. Plaque assays of PMB12 were performed, using CM-1 cells as indicator. A plate that contained about 300 plaques after 24 h of incubation was incubated for 5 days at 37°C, and a portion of the plate was photographed. Each plaque detected on day 1 yielded a spore-positive clone within 48 h of incubation. Magnification ×3; bar represents 1 cm.*

PMB12-induced CM-1 spores were germinated and grown overnight on TBAB. Individual colonies were then restreaked, and after an additional 20 h of incubation, the daughter colonies were tested for their ability to form spores (on SG agar) and for maintenance of PMB12 infectivity (by transferring colonies to lawns of BR151). More than 95% of the daughter colonies examined (463) were spore negative and did not retain PMB12 infectious activity. The remaining colonies were spore positive and harbored PMB12 infectious activity.

The dipicolinic acid content of PMB12-induced CM-1 spores and uninfected spores of BR151 was 83 (±7) μg/mg of dry weight. In addition, we could detect no difference between phage-induced spores of CM-1 and uninfected BR151 spores with respect to the rate of inactivation at 85°C.

PBS1 is related to PMB12. However, PBS1 propagated on BR151 did not convert CM-1 to spore positive in plaque assays, nor did PBS1 appear to enhance the rate of sporulation of BR151.

Genetic mapping of the sporulation mutation in CM-1, designated *spoCM-1* by PBS1 transduction, demonstrated linkage to the *cysA14* mutation (J. Hoch, personal communication). The recombination between *spoCM-1* and *spoOJ93* (12) was subsequently found to be 9.6% by transformation (Hoch, personal communication). It, therefore, appears that *spoCM-1* is located in the same or an adjacent cistron to that marked by *spoOJ93*. These data also indicate that *spoCM-1* is not a mutation in the *rif* locus. More extensive mapping of *spoCM-1* is in progress.

PMB12 conversion of rifampin-resistant, spore-negative mutants to spore positive. The spore-negative mutation in CM-1 mapped to a site near, but distinct from, the location of rifampin-resistant (Rif^r) mutations in *B. subtilis* (9). The *rif* locus presumably marks the cistron coding for the β subunit of RNA polymerase (18). It is well established that many Rif^r mutants are also spore negative or produce structurally altered spores (6, 16, 26, 27). Moreover, reported evidence suggests a probable key role for RNA polymerase in the regulation of sporulation (5, 17, 22, 29, 30). Based on our view of the general mode of action of spore-converting phages, it seemed possible that the spore-negative mutation in CM-1 might represent a genetic lesion that altered RNA polymerase structure and/or function (see below). Although this hypothesis remains untested, it prompted us to test Rif^r spore-negative mutants for their convertibility to spore positive by PMB12. Each of 18 independently isolated Rif^r spore-negative mutants of BR151 was converted to spore positive by PMB12. Each PFU initiated the conversion of a spore-positive clone. Spontaneous spore-positive revertants of each of five of the Rif^r mutants tested simultaneously acquired sensitivity rifampin, suggesting that the spore-negative phenotype and rifampin resistance were the consequence of a single genetic lesion. Five spore-negative Rif^r mutants of *B. subtilis* blocked at known stages of sporulation (LS3 [stage 0]; TS1 [stage O → I]; TS2 [stage II]; TS3 [stage III]; TS4 [stage III → IV]) (26, 30) were each converted to spore positive by PMB12 infection. The ability of PMB12 to convert LS3, TS1, TS2, TS3, and TS4 to spore positive was unaffected by including rifampin (5 μg/ml) in the agar overlays. Accordingly, PMB12 conversion of the sporulation defect was insensitive to rifampin.

Response of additional sporulation mutants of *B. subtilis* to PMB12. PMB12 was tested for ability to convert the sporulation defect in several strains of *B. subtilis* that harbor known sporulation mutations (23). Strains carrying the following sporulation mutations were not detectably converted to spore positive in spot tests with PMB12: *spoOJ87*, *spoOH81*, *spoOH12*, *spoOH17*, *spoOH75*, *spoOA12*, *spoOB136*, *spoOF221*, *spoOA9V*, *spoIVF88*, *spoIIIB2*, *spoIVB165*, *spoIIA26*, and *spoIIIA59*. The sporulation of strains carrying the mutations *spoOJ93* and *spoOE11* appeared slightly increased in the area where phage was spotted relative to the background lawn. However, both mutations are "leaky", and it is, therefore, difficult at this time to interpret the apparent increases in sporulation. We also tested PMB12 for ability to convert the temperature-sensitive sporulation defect in an erythromycin-resistant mutant (strain E2) of *B. subtilis* (31). By both plaque assay and spotting techniques (see above), E2 was not detectably converted by PMB12.

DISCUSSION

Sporulation-converting phages of *B. pumilus* and *B. subtilis* were initially identified on the basis of their ability to enhance the rate or extent of sporulation of essentially wild-type (i.e., nonmutant) cells (2; Keggins and Lovett, manuscript in preparation). Subsequent to identification of the phages, it has been possible to identify specific spore-negative bacterial mutants that are totally dependent on the phage for sporulation. That both PMB1 (2) and PMB12 enhance wild-type cells to form spores

suggests that these phages probably regulate key "switch" functions that increase the rate or probability of transition of cells to sporulation. It would not seem to be of major selective advantage for a phage genome to code for a spore-specific protein such as, for example, spore coat protein. Although this protein is required for sporulation, it is not obvious that the presence of additional copies of such a gene would enhance sporulation of wild-type cells. By contrast, if a phage genome provided an RNA polymerase (or modified the host enzyme) such that key spore gene promoter sites were recognized and appropriate transcription occurred, this would be one type of control that could enhance the sporulation of wild-type cells.

Losick and Sonenshein (22) suggested that a change in template specificity of host RNA polymerase was a key control mechanism in the transition of vegetative cells to sporulation. Although the precise biochemical basis responsible for the change in template specificity is not clear, the evidence accumulated in recent years supports their general proposal (5, 17, 18, 30). The information available for PMB12 indicates that this phage mediates conversion at the level of RNA polymerase. PMB12 converts sporulation-deficient mutants in which the genetic lesion is in the cistron coding for the β subunit of RNA polymerase (Rif^r mutants). In contrast, the sporulation-converting phage for *B. pumilus*, PMB1, was not capable of converting Rif^r spore-negative mutants of *B. pumilus* (K. M. Keggins and P. S. Lovett, unpublished data).

PMB12 conversion of Rif^r spore-negative mutants was insensitive to the presence of rifampin. Thus, the postulated RNA polymerase that mediates conversion must also be rifampin resistant. The core enzyme of the Rif^r mutants should be rifampin insensitive, and modification of its transcribing specificity could be accomplished by addition of a phage-determined subunit. Alternatively, PMB12 might induce or activate a host-specified, normally rifampin-insensitive RNA polymerase, possibly similar to the activity detected in *E. coli* (1). Lastly, the genomes of certain phages code for RNA polymerase (3), and it is possible that PMB12 complementation of sporulation mutations results from induction of a phage-specified RNA polymerase that can, at least partially, replace the sporulation-specific functions of the host RNA polymerase. In this regard, it is significant that PMB12 replication is rifampin insensitive, as is the related phage PBS2 (24). PBS2-infected cells contain a species of RNA polymerase that is rifampin insensitive and physically distinct from the host enzyme (4, 24). Moreover, we have detected a rifampin-insensitive RNA-synthesizing activity in PMB12-infected cells that was not present in uninfected cells (R. Taylor and P. S. Lovett, unpublished data). If PMB12 mediates conversion only at the level of RNA polymerase, then it would, therefore, seem likely that all sporulation mutations complemented by the phage mark cistrons that determine the function of the sporulation form(s) of RNA polymerase (21).

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