



Bacterial Endospores as Phage Genome Carriers and Protective Shells

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ABSTRACT Bacterial endospores can serve as phage genome protection shells against various environmental stresses to enhance microbial control applications. The genomes of polyvalent lytic *Bacillus* phages PBSC1 and PBSC2, which infect both *B. subtilis* subsp. *subtilis* and *B. cereus* NRS 248, were incorporated into *B. subtilis* endospores (without integration into the host chromosome). When PBSC1 and PBSC2 were released from germinating endospores, they significantly inhibited the growth of the targeted opportunistic pathogen *B. cereus*. Optimal endospore entrapment was achieved when phages were introduced to the fast-sporulating prespores at a multiplicity of infection of 1. Longer endospore maturation (48 h versus 24 h) increased both spore yield and efficiency of entrapment. Compared with free phages, spore-protected phage genomes showed significantly higher resistance toward high temperatures (60 to 80°C), extreme pH (pH 2 or pH 12), and copper ions (0.1 to 10 mg/liter). Endospore germination is inducible by low concentrations of L-alanine or by a germinant mixture (L-asparagine, D-glucose, D-fructose, and K⁺) to trigger the expression, assembly, and consequent release of phage particles within 60 to 90 min. Overall, the superior resiliency of polyvalent phages protected by endospores might enable nonrefrigerated phage storage and enhance phage applications after exposure to adverse environmental conditions.

IMPORTANCE Bacteriophages are being considered for the control of multidrug-resistant and other problematic bacteria in environmental systems. However, the efficacy of phage-based microbial control is limited by infectivity loss during phage delivery and/or storage. Here, we exploit the pseudolysogenic state of phages, which involves incorporation of their genome into bacterial endospores (without integration into the host chromosome), to enhance survival in unfavorable environments. We isolated polyvalent (broad-host-range) phages that efficiently infect both benign and opportunistically pathogenic *Bacillus* strains and encapsulated the phage genomes in *B. subtilis* endospores to significantly improve resistance to various environmental stressors. Encapsulation by spores also significantly enhanced phage genome viability during storage. We also show that endospore germination can be induced on demand with nutrient germinants that trigger the release of active phages. Overall, we demonstrate that encapsulation of polyvalent phage genomes into benign endospores holds great promise for broadening the scope and efficacy of phage bio-control.

KEYWORDS endospore, bacteriophage, stressors, phage protection, *Bacillus cereus*, microbial control

Received 16 May 2018 Accepted 6 July 2018

Accepted manuscript posted online 13 July 2018

Citation Gabiatti N, Yu P, Mathieu J, Lu GW, Wang X, Zhang H, Soares HM, Alvarez PJJ. 2018. Bacterial endospores as phage genome carriers and protective shells. Appl Environ Microbiol 84:e01186-18. <https://doi.org/10.1128/AEM.01186-18>.

Editor Janet L. Schottel, University of Minnesota

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Bacteriophages are garnering significant interest as self-replicating antibacterial agents in the face of the widespread and accelerating onset of multidrug-resistant bacteria and other bacterial control challenges (e.g., biofouling and microbe-induced corrosion) (1–3). Phages offer numerous advantages over conventional antimicrobial agents, such as self-propagation in the presence of suitable hosts and lower potential environmental impact due to host specificity, resulting in minimal off-target kills (4). The use of phages for microbial control has recently expanded from traditional medical applications to food safety, water and wastewater treatment, and beyond (5–7). However, phage-based biocontrol (i.e., killing of problematic target bacteria) can be hindered by infectivity loss (8). Specifically, adverse environmental conditions (e.g., high temperature [50°C], high copper ion concentration [300 mg/liter], and acidic conditions [pH 2]) during phage delivery could irreversibly damage the phage genome or proteins involved in host recognition within minutes (9, 10). Moreover, phage neutralization by antibodies or other compounds also hinders their infectivity (11, 12). Therefore, micro-encapsulation has been proposed to protect therapeutic phages (13). For example, an alginate/CaCO₃ encapsulation method was used to protect *Salmonella* and *Staphylococcus* phages during transit through the acidic gastrointestinal tract (14, 15). Nevertheless, alginate gel encapsulation can only improve phage resistance to low pH, and the literature is scarce on phage protection during delivery through other adverse conditions (e.g., high temperature) or during long-term storage, which underscores an opportunity for novel strategies that enhance phage survival and application of phages for microbial control.

Bacterial endospores are metabolically dormant and resilient toward various environmental stressors (16), and they can be induced to germinate within minutes upon exposure to germinants, such as L-alanine and L-valine for *Bacillus subtilis* (17). Therefore, endospores could be used as protective shells and delivery vectors for phage genomes that enter into a carrier state, known as pseudolysogeny (18). This is a state of stalled phage development within a host cell, without either phage genome replication or phage genome integration into the host chromosome (19). Pseudolysogeny occurs most frequently under nutrient-deprived conditions, when host DNA replication and protein synthesis stop (20). For example, the genomes of some *Bacillus* phages (e.g., φ 29 and φ e) can be incorporated into developing endospores (21, 22). Accordingly, the endospore-protected phage genomes exhibit dramatically improved resistance to common environmental stressors (23). Upon endospore germination, the phage continues its lytic life cycle, leading to cell lysis and phage release (18). However, no previous studies have considered the use of endospores to enhance phage applications by increasing their tolerance to environmental stressors and their viability after long-term storage.

One major hurdle in using spores as phage carriers and protective shells is the narrow host range of most known phages, which precludes infecting both benign spore-forming hosts and target bacteria. However, phage polyvalence (i.e., broad host range) would facilitate their encapsulation by benign bacterial endospores for subsequent targeting of problematic bacteria. Recent studies suggest that polyvalent phages are more widespread than previously recognized (24, 25) and can be preferentially isolated by using sequential hosts (26). Advances in molecular biology and synthetic biology may also be used to extend and tune phage host range (27, 28).

Here, polyvalent phages PBSC1 and PBSC2, which infect both *B. subtilis* and *Bacillus cereus*, were isolated from soil. *B. subtilis* was chosen as the production host because of its safety (29). *B. cereus* was used as a model pathogen for food spoilage and foodborne disease (30). PBSC1 and PBSC2 genomes were incorporated into *B. subtilis* endospores, and sporulation time, multiplicity of infection, and maturation time were optimized to achieve a high efficiency of phage genome entrapment. Phage genome activity was preserved when the endospores were exposed to adverse conditions (e.g., high temperature of 80°C, extreme pHs of 2 and 12, and 10 mg/liter of copper ions) or long-term unrefrigerated storage. We also show on-command phage release from germinated spores (and effective control of target bacteria) when the spores are induced with

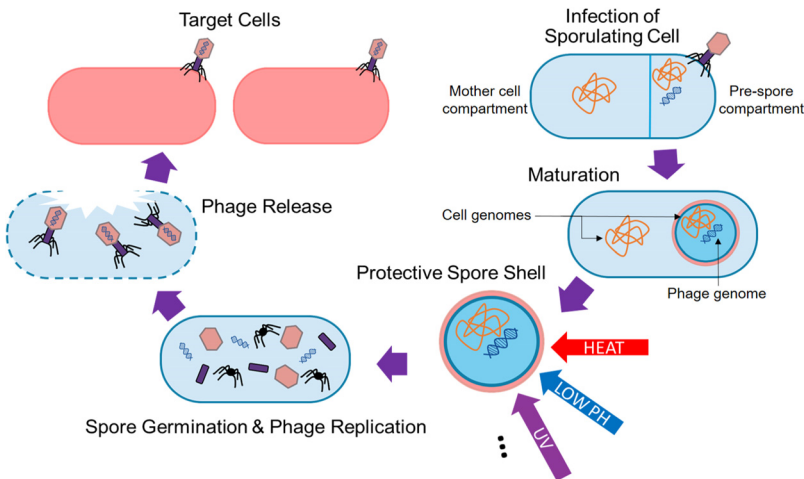


FIG 1 Strategy for phage genome entrapment in spores, protection, and subsequent progeny release. Development of bacterial spores as phage carriers and protective shells by taking advantage of the pseudolysogenic state to enhance both phage resistance against environmental stressors and phage viability after long-term storage. Only phage genomes that enter the prespore component may become successfully entrapped into the mature spores.

common germinants. Accordingly, we propose a novel approach to enhance and broaden phage-based microbial control applications (Fig. 1).

RESULTS AND DISCUSSION

Polyvalent phage isolation and characterization. Soil samples were used for polyvalent *Bacillus* phage isolation because *Bacillus* species commonly inhabit soils and phages usually coexist with their hosts (31). With a sequential multiple-host approach, two polyvalent phages (PBSC1 and PBSC2) were isolated based on their ability to infect both *B. subtilis* subsp. *subtilis* and *B. cereus* NRS 248 cells with a high efficiency of plating (EOP > 0.7; *n* = 6) (Table 1). The electron microscopic images (*n* = 10) showed that PBSC1, with a head of 80 ± 3 nm and a long tail of 70 ± 2 nm, belonged to the family *Myoviridae*, while PBSC2, with a head of 70 ± 1 nm and a barely visible tail, belonged to the family *Podoviridae* (Fig. 2).

Phage PBSC1 displayed greater affinity to *B. subtilis* relative to *B. cereus*, with adsorption rate constants of 1.7 × 10⁻⁹ ml/min for *B. subtilis* and 6.6 × 10⁻¹⁰ ml/min for *B. cereus*. Phage PBSC2 showed lower values of 5.4 × 10⁻¹⁰ and 4.3 × 10⁻¹⁰ ml/min for *B. subtilis* and *B. cereus*, respectively (Table 1 and Fig. S1 in the supplemental material). The latent periods of PBSC1 and PBSC2 in *B. subtilis* were 35 min and 40 min, respectively, and burst sizes were 132 and 99 PFU/cell, respectively (Fig. 2 and Table 1), which indicates that *B. subtilis* can serve as production hosts for both phages. Both PBSC1 and PBSC2 significantly inhibit the growth of *B. cereus* (Fig. 3A2 and 3B2). However, due to the development of phage-resistant mutants (Fig. 3A2 and 3B2), it may be necessary to consider phage cocktails or combining phage biocontrol with other antimicrobial agents to ensure successful application (32, 33).

TABLE 1 EOP^a and life cycle parameters of phages PBSC1 and PBSC2 toward their hosts

Phage	Host	EOP	Adsorption rate constant (10 ⁻¹⁰ ml/min)	Latent time (min)	Burst size (PFU/cell ± SD)
PBSC1	<i>B. subtilis</i> subsp. <i>subtilis</i>	1.00	17.2 ± 0.7	35	132 ± 15
	<i>B. cereus</i> NRS 248	0.74	6.59 ± 0.8	35	101 ± 14
PBSC2	<i>B. subtilis</i> subsp. <i>subtilis</i>	1.00	5.35 ± 0.4	40	99 ± 12
	<i>B. cereus</i> NRS 248	0.76	4.32 ± 0.5	40	86 ± 12

^aEfficiency of plating (EOP) was quantified by calculating the ratio of phage plaque titers obtained with a given host to the phage plaque titers obtained with *B. subtilis*.

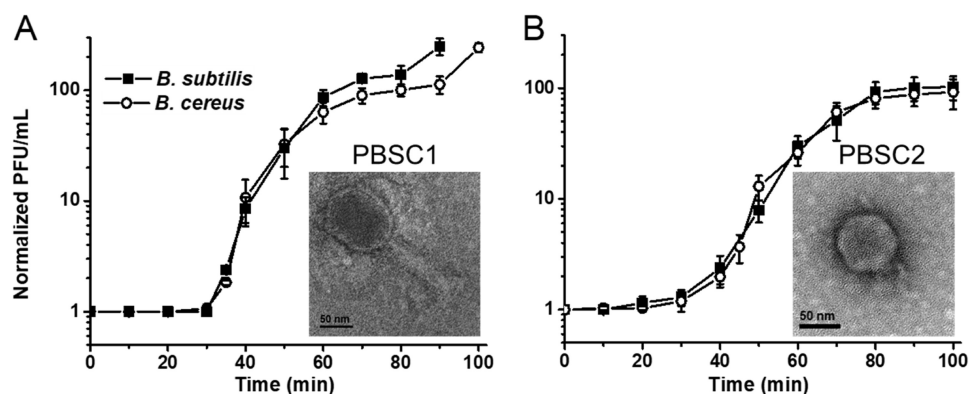


FIG 2 Growth patterns and morphology of polyvalent phages PBSC1 and PBSC2. One-step growth curves of (A) PBSC1 and (B) PBSC2, infecting both *B. subtilis* and *B. cereus*. Error bars in all figures indicate standard deviations from the mean of triplicate independent experiments. The electron microscopic images revealed that phages PBSC1 and PBSC2 belonged to the families of *Myoviridae* and *Podoviridae*, respectively. Bar, 50 nm.

Incorporation of phage genomes into bacterial endospores. To test whether phages PBSC1 and PBSC2 could be incorporated into endospores (in the form of a genome, as shown in Fig. 1) as previously reported for *B. subtilis* phages (e.g., ϕ 29 and ϕ e) (21, 22), we introduced phage PBSC1 and PBSC2 into sporulating *B. subtilis* suspensions. Since thermal treatment (70°C for 30 min) ensured the inactivation of unprotected phages, the production of phage plaques when the endospore suspensions were subjected to double-layer agar assays indicates that the phage genomes were incorporated into and protected by the endospores (see Fig. S2 in the supple-

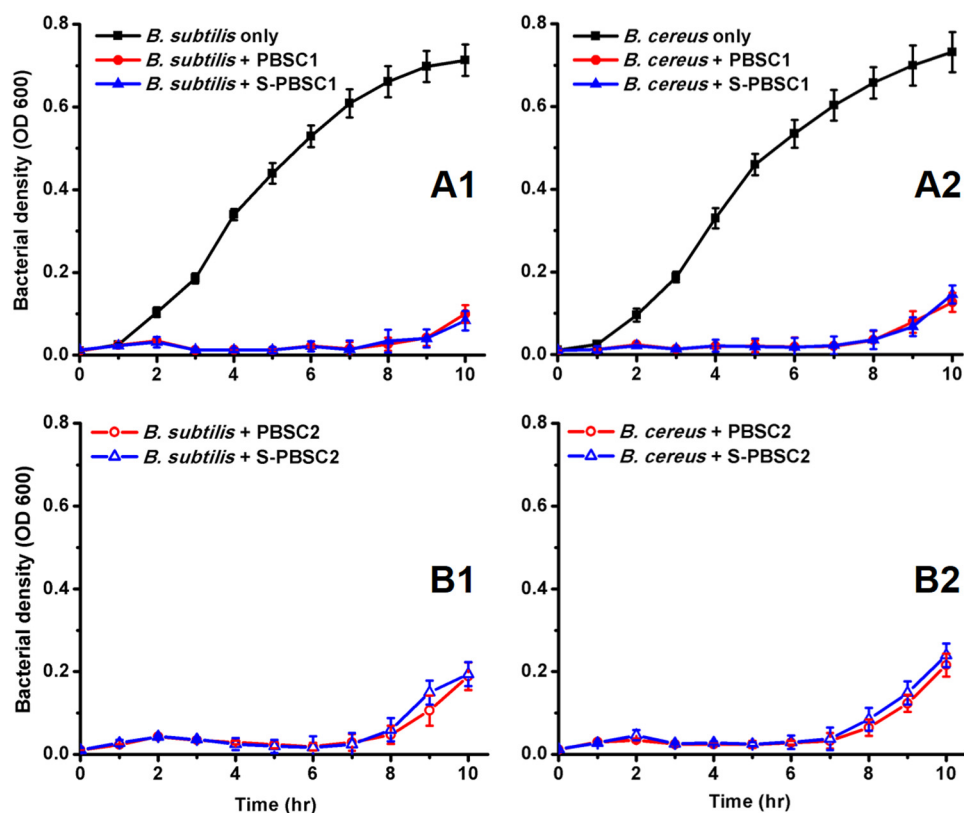


FIG 3 Batch growth experiments showing the inhibitory effects of PBSC1 and PBSC2 on the hosts. The inhibitory effects of free PBSC1 and spore-released PBSC1 (S-PBSC1) on *B. subtilis* (A1) and *B. cereus* (A2). The inhibitory effects of free PBSC2 and spore-released PBSC2 (S-PBSC2) on *B. subtilis* (B1) and *B. cereus* (B2). The initial MOI was 10.

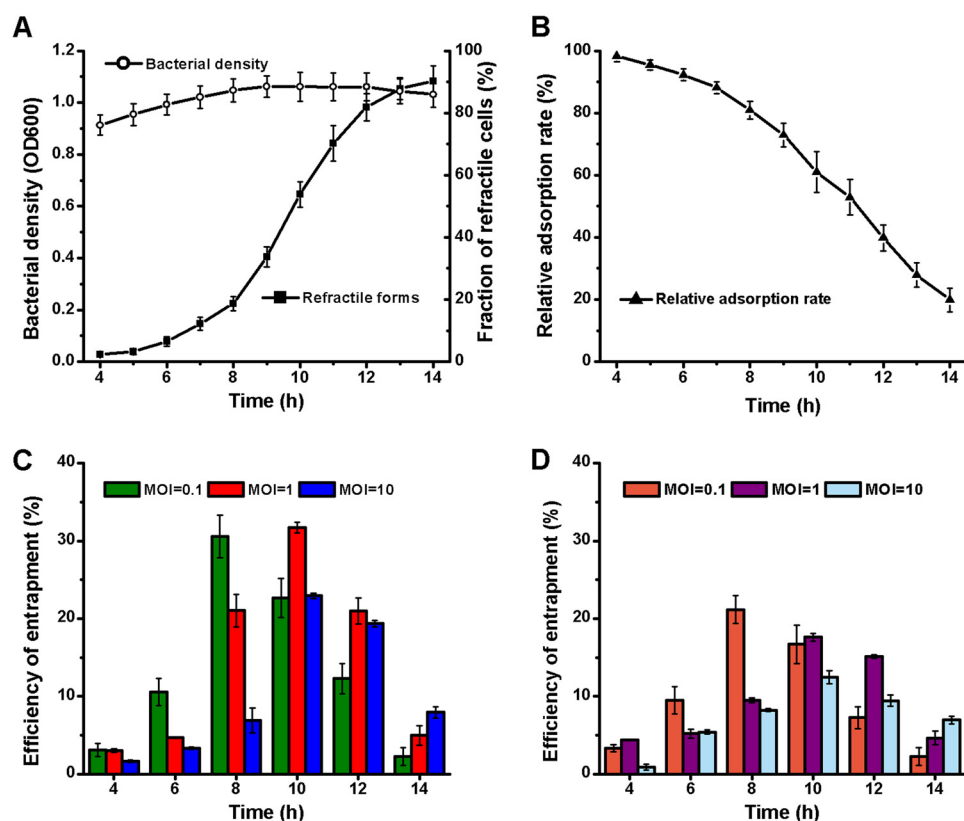


FIG 4 *B. subtilis* sporulation time and efficiency of phage incorporation into endospores. (A) The fraction of *B. subtilis* in refractile forms (e.g., spores) increased with incubation time in sporulation medium. (B) PBSC1 relative adsorption rate during *B. subtilis* sporulation process. Efficiency of entrapment for phages PBSC1 (C) and PBSC2 (D) at different MOIs and incubation times.

mental material). Batch growth experiments showed that phages released from the endospores maintained broad host ranges and exhibited similar infective capability to that of their ancestors (Fig. 3). Electron microscopic images showed no changes in phage morphology due to endospore entrapment, and no endogenous prophages of *B. subtilis* were detected when the spores germinated (see Fig. S3 in the supplemental material).

Sporulation stage and multiplicity of infection (MOI) are crucial factors to achieving high phage genome entrapment. The fraction of refractile forms of *B. subtilis* continuously increased in the sporulation medium while phage adsorption rates decreased (Fig. 4A and B), indicating the sporulation of *B. subtilis*. Although phage PBSC1 had higher entrapment efficiency than PBSC2 under the tested conditions, the two phages showed similar entrapment patterns at the different times tested during the sporulation process (Fig. 4C and D). At MOIs of 1 and 10, the optimal time for phage genome entrapment was approximately 10 h postinoculation, when about 44% of cells were endospores and sporulation was occurring at the highest rates (Fig. 4A). However, at a lower MOI of 0.1, the optimal time was 8 h postinoculation, when about 10% of cells were endospores.

Assuming a Poisson distribution for phage adsorption (34), about 100% of the bacteria would absorb at least one phage at an MOI of 10, compared to 63.2% at an MOI of 1 and 9.5% at an MOI of 0.1. However, higher phage adsorption did not necessarily result in higher phage entrapment. For high MOIs, phage replication may disrupt the sporulation process, mainly due to nutrient release from lysed cells. Late in the sporulation process (stage V, 14 h), we observed a positive correlation between $\ln(\text{MOI})$ and entrapment efficiencies ($R^2 = 0.99$ for both phages).

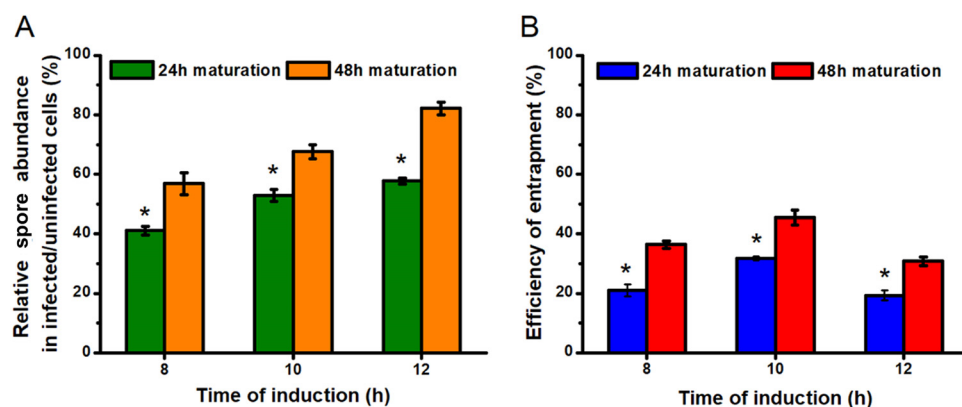


FIG 5 The effect of spore maturation time on the yield of total spores (A) and phage-entrapping spores (B). Bacteria induced for 8, 10, and 12 h were used with a MOI of 1. Panel A shows the percentage of total spores (infected and uninfected spores) formed by phage-infected *B. subtilis* subsp. *subtilis* cells relative to the total of spores formed by uninfected cells. Panel B shows the fraction of the total spores that was infected. The asterisks (*) represent significant difference ($P < 0.05$) between 24 h maturation and 48 h maturation.

The timing of phage introduction is important for efficient phage genome entrapment. Adding phages either too early (4 or 6 h) or late (14 h) in the sporulation process resulted in low entrapment efficiencies (<12%) regardless of MOIs. Bacteria in the early stage of sporulation are still vulnerable to phage lytic cycle progression, while bacteria in the late stage of sporulation can interrupt phage recognition and infection because of changes in cell morphology and surface receptors (35). Specifically, the mother cell can engulf the prespore during stage III of sporulation, which would prevent phage contact with the prespore. After engulfment, slowing down of the prespore metabolism makes it highly unlikely that the phage would still be able to infect the prespore. Even if the phage genome enters the prespore, it might still be recognized and degraded as a foreign nucleic acid (36).

Lengthening spore maturation time from 24 h to 48 h not only significantly increased the abundance of total spores (infected and uninfected spores) formed by phage-infected *B. subtilis* cells (Fig. 4A), but also resulted in a significant increase in phage entrapment efficiency (Fig. 4B). Furthermore, suitable spore crops were obtained within 24 h, but doubling this time achieved higher spore purity (see Fig. S4 in the supplemental material) by promoting vegetative cell lysis (37). The spore maturation time is related to wet heat resistance acquisition by the spore (38), and because purification included thermal treatment (70°C for 30 min), longer maturation resulted in a higher yield of viable spores (Fig. 5A). Similarly, when spores were subjected to thermal treatment, phage genomes were more resistant when encapsulated by more mature (48 h) than less mature (24 h) spores, and they exhibited higher efficiency of spore incorporation (Fig. 5B).

Higher environmental stress resistance for polyvalent phage genomes protected in endospores. Spore-entrapped phage genomes were significantly more resistant than free phages to high temperature, pH extremes, and copper ions. For example, while free PBSC1 and PBSC2 had high inactivation rates after treatment at 50°C (Fig. 6A), entrapped phage genomes retained infectivity even after treatment at 80°C. This is consistent with results of prior studies demonstrating that endospores can resist temperatures over 100°C (39), mainly due to the low water content in the central core and DNA saturation with α/β -type acid-soluble spore proteins (SASP) (40). Entrapped phages also demonstrated resistance to pH extremes, with a maximum decrease in viability of just 25% at pH 2 and pH 12, whereas free phages were dramatically inactivated below pH 4 or above pH 11 (Fig. 6B). Similarly, we observed a significant reduction in toxicity over a wide range of copper concentrations (Fig. 6C), as the low-permeability inner lipid membrane protects the spore core from DNA-damaging chemicals (41). Finally, we observed first-order decay rate coefficient values of 0.20 and

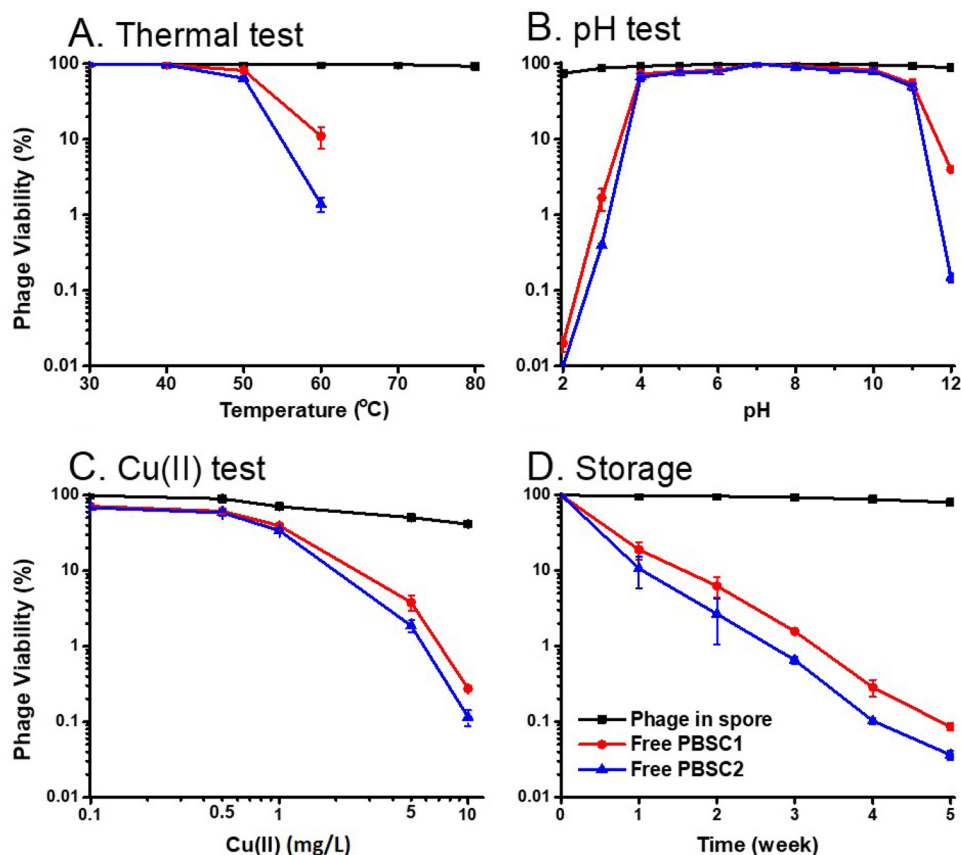


FIG 6 Free PBSC1 and PBSC2 and entrapped phage resistance assays. (A) Phage viability after thermal treatment for 15 min at the stipulated temperatures (no free phages survived 70°C or higher temperature), (B) after 60 min exposure to extreme pH (i.e., pH 2 to pH 12), (C) after exposure to different copper concentrations, and (D) after storage in the dark at room temperature for the stipulated time.

0.22 day⁻¹ for free PBSC1 and PBSC2, respectively, while the air-dried spore-protected phages did not experience a significant loss of viability over 5 weeks (Fig. 6D). We also assessed the relative viability of treated versus untreated spores with and without entrapped phages. No significant differences were observed between normal spores and spores containing phage genomes under the tested conditions (Fig. 7). Overall, these results illustrate the potential for exploiting the pseudolysogenic state of phages and using endospores as phage genome carriers and protective shells during storage (without the need for refrigeration) or delivery through transiently harsh environmental conditions.

Controlled phage release by stimulating spore germination. Spore germination and outgrowth is characterized by high variability in the behavior of individual cells. This process is generally initiated when one or more germinant receptors (GRs) located in the inner membrane sense their cognate germinant and initiate a series of biophysical events (42). To control spore germination and hence phage release, the process may be induced by incubation with germinants, such as L-valine, L-alanine, and an AGFK mixture (L-asparagine, D-glucose, D-fructose, and K⁺) (43). Once the spore GRs recognize the proper nutrients, the spore commits to germination and the process continues, even if the germinant is removed or binding is reversed and inhibited (44). Considering the potential formation of superdormant spores of *Bacillus*, high levels of nutrient germinant mixtures that activate multiple receptors may be required to ensure their timely germination (45).

To facilitate spore germination, we compared the effects of two germinants (L-alanine and AGFK mixture) and their combination, observing the cell behavior (Fig. 8A)

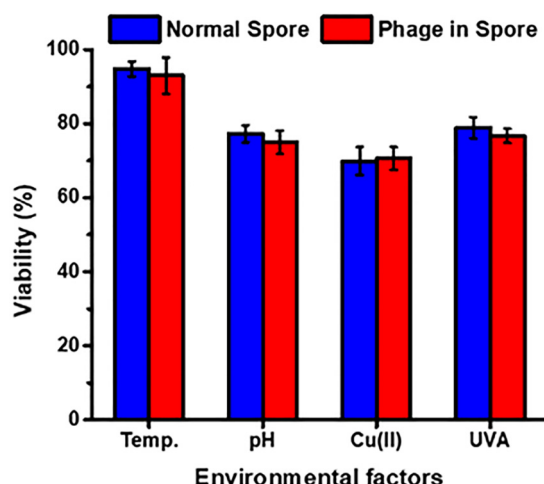


FIG 7 Resistance of normal spores and phage-infected spores. The spore maturation time was 48 h. The viability of normal spores and phage-infected spores was calculated based on relative CFU and COI (centers of infection) before and after treatment, respectively. The normal spores and phage-infected spores had similar environmental resistance toward high temperature (80°C), low pH (2), Cu^{2+} (1 ppm), and UVA (1.2×10^{-9} einstein/cm²/s).

and phage abundance in the medium (Fig. 8B). Based on the observed decrease of optical density at 600 nm (OD_{600}) during spore germination, the germination time was estimated to be 30 to 35 min when induced with 20 mM L-alanine or 20 mM AGFK mixture. Faster germination was induced by the combination of 10 mM L-alanine and 10 mM AGFK mixture. Once phages are incorporated into endospores, the time allowed for phage release should consider not only the endospore germination and outgrowth time but also the phage latent period in vegetative cells. PBSC1 normally has a latent time of 35 min, but, when entrapped, produces only very low titers during the first 150 min of germination. However, when L-alanine or AGFK mixture is added, phage release is observed earlier, after 90 to 120 min. We also observed that latent periods were reduced to around 60 min when cultures were amended simultaneously with both L-alanine and AGFK. In *B. subtilis* spores, the GerA receptor responds to L-alanine, while the AGFK mixture works on the receptors GerB and GerK to trigger germination (17). Consistent with previous work, germinant combinations can be more effective than when applied individually (46, 47). Thus, spores not only integrate but can also amplify

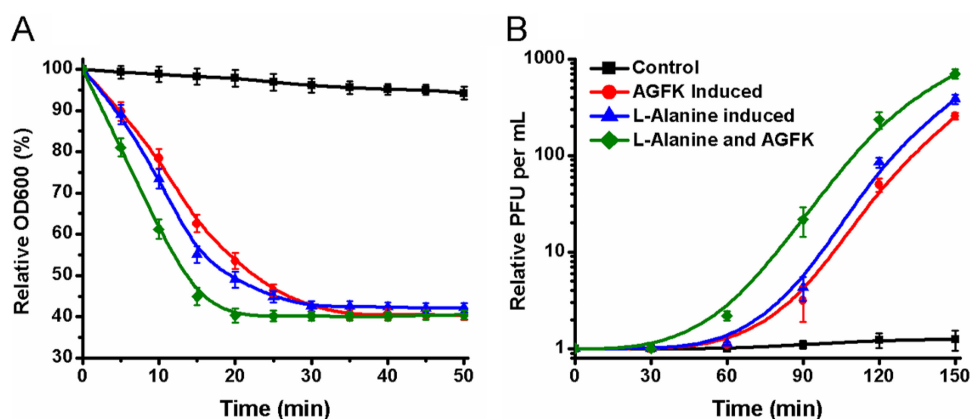


FIG 8 Germination induction of *Bacillus* spores and release of entrapped phage PBSC1. (A) *B. subtilis* germination triggered by different germinants was monitored by changes in optical density (initial $\text{OD}_{600} = 0.8$). (B) Release of entrapped PBSC1 under different inducing conditions. The germinant concentrations were 20 mM for L-alanine, 20 mM (each) L-asparagine, D-glucose, D-fructose, and K^+ (AGFK), or 10 mM L-alanine + 10 mM each AGFK component for the combined treatment.

signals from multiple germinants, and multiple GRs accelerate overall spore germination.

Practical implications and challenges. Phages have demonstrated strong potential to combat antibiotic-resistant bacteria through multiple modes of action (e.g., selective microbial control [48], biofilm dispersal [49], and antibiotic resistance resensitization [50]). The effectiveness of phage treatment depends not only on specific phage-host interactions, but also on the conservation of phage viability during delivery. In the pseudolysogenic state, the genomes of phages infecting sporulating bacteria get entrapped in the mature spores and are consequently protected from harsh environmental stressors. Our results show that spores can be harnessed to serve as cost-effective, resilient storage and delivery vehicles for phages capable of infecting spore-forming bacteria (Fig. 1). Furthermore, polyvalent phages enable the use of a benign surrogate host (e.g., *B. subtilis*) and eliminate the need to separate phages from pathogenic hosts prior to encapsulation (7, 51), which could substantially decrease production cost (52).

This approach could be used to control the growth of spore-forming pathogens, such as *Bacillus anthracis* and *B. cereus* strains causing gastrointestinal diseases (53). For example, polyvalent phage genomes entrapped in *B. subtilis* spores could be seeded in areas containing *B. anthracis* or *B. cereus* spores. Thus, when conditions become suitable for germination, the phages would continue their lytic cycle and subsequently infect these potential pathogens. Spore-entrapped phage genomes could also be considered for gut microbiome manipulations, as they would resist the low pH of the stomach during transit. In such scenarios, spore germination and outgrowth could be triggered by gut nutrients.

While the use of spore-phage system opens up new potential microbial control applications for environmental and public health protection, some challenges should be considered. For example, this approach is dependent on polyvalent phages infecting both endospore formers and targeted bacteria. Thus, the availability of such phages may limit the scope of some applications. Nevertheless, recent studies have shown that polyvalent phages may be more widespread than previously recognized (24, 25), and modification of phage isolation methods could facilitate their enrichment (26).

Conclusions. Overall, this study demonstrates phage applicability for controlling pathogenic endospore formers (e.g., *Clostridium* spp.) and a new method for phage protection during delivery, as well as during long-term, room-temperature storage. Using bacterial endospores as carriers and protection shells for polyvalent phage genomes holds promise for broadening the scope and efficacy of phage biocontrol. Nevertheless, further research with more realistic settings is needed to enhance safe and practical applications, including enhancing the phage genome entrapment efficiency, improving phage release, and optimizing phage dosage and application frequency in microbial control.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study were *Bacillus subtilis* subsp. *subtilis* (ATCC 6051) and *Bacillus cereus* NRS 248 (ATCC 10987). Tryptic soy broth (TSB) medium was used for bacterial culture and phage characterization in terms of growth parameters. *B. subtilis* sporulation and phage incorporation were carried in medium 121B, containing basal medium 121 (0.08 M NaCl, 0.02 M KCl, 0.02 M NH_4Cl , 0.12 M Tris, 1 mM MgCl_2 , 0.2 mM CaCl_2 , 0.002 mM FeCl_3 , 2.5 mM Na_2SO_4 , and 0.002 mM ZnCl_2 ; pH adjusted to 7.5 with HCl) supplemented to final concentrations of 0.2% glucose, 0.2% Casamino Acids, 2.5 mM K_2HPO_4 , 0.8 mM MnCl_2 , 3.4 mM sodium citrate, and 0.15 mM FeCl_3 (21). All bacterial incubations, sporulation, and germination were performed at 37°C.

The double-layer plaque assay used tryptone base-layer agar and tryptone soft agar (26). The numbers of mature spores and phages were enumerated by colony and plaque counting assays, respectively. Bacteriophages were stored at 4°C in SM buffer (50 mM Tris-HCl [pH 7.5], 0.1 mM NaCl, 8 mM MgSO_4 , and 0.01% gelatin). The endospores of *B. subtilis* were kept in ultrapure water at 4°C.

Polyvalent phage isolation and characterization. A heterogeneous phage stock was obtained using fertile soil samples from the Rice University campus (29.7174° N, 95.4018° W). A sequential multiple-host isolation approach (26) was applied for polyvalent phage isolation, with the host sequence *B. subtilis* followed by *B. cereus* followed by *B. subtilis* again. A single clear phage plaque was harvested from the last plate's bacterial lawn and diluted in SM buffer. The phage was further purified on *B. subtilis* culture three times to remove any contaminant phages according to standard procedures. The isolated

phages were characterized in terms of host range, efficiency of plating (EOP), adsorption rate constant, latent time, and burst size, as previously described (26). In adsorption tests, the fraction of free phages was measured by plaque counts as function of time, and first-order phage adsorption rate constants were determined as the linear regression slope of $\ln(\text{phage concentration})$ versus time. The latent time and burst size were determined by the phage one-step growth curves (54). The morphology of isolated phages was determined via a JEOL 2010 transmission electron microscope (TEM) (JEOL USA, MA) after negative staining with 2% uranyl acetate (55).

Sporulation and phage genome entrapment. After overnight incubation in Trypticase soy broth (TSB), *B. subtilis* culture was synchronized for sporulation in medium 121/121B as previously described (21). Briefly, the *B. subtilis* culture was washed with medium 121, resuspended in medium 121B, and grown in medium 121B until it reached the early stationary phase. After centrifugation, bacteria were harvested and then incubated for sporulation in 121B medium, with shaking at 160 rpm. Phase-contrast images of sporulating *B. subtilis* were obtained by microscopy (DM6000B; Leica). At different times after incubation, sporulating cells were mixed with phage for 15 min at MOIs of 0.1, 1, and 10. The free phages were removed by centrifugation (five times) and the pellet was resuspended in 121B medium (without glucose or Casamino Acids) for spore maturation. The spores were harvested after 24 h or 48 h maturation and then purified as previously reported (56). Briefly, suspensions were treated with 50 mM Tris-HCl (pH 7.2) containing 50 $\mu\text{g}/\text{ml}$ of lysozyme for 1 h at 37°C to inactivate vegetative cells, and then the spores were released by vortexing in 0.05% SDS solution. Heat treatment (70°C for 30 min) was applied to inactivated free cells or vegetative cells. Samples were further washed three times with ultrapure water to remove cell debris and extracellular phages. The morphology and purity of spores were observed by JEOL 6500 scanning electron microscopy (SEM) (JEOL USA, MA) after fixation with glutaraldehyde and dehydration with ethanol (57).

Efficiency of phage genome entrapment. The spore suspensions were spread on nutrient agar for enumeration of uninfected spores and embedded in the soft agar of double-layer plates for entrapped phage quantification. The number of surviving colonies was used to represent the number of uninfected spores, while that of plaques in soft agar represented the number of spores containing phage genomes. The efficiency of phage entrapment was calculated as follows (23):

$$F = 100 \times P / (P + S) \quad (1)$$

where F is the fraction of spores producing infective centers (%), P is the number of infective centers from infected spores (COI/ml), S is the number of surviving colonies from uninfected spores (CFU/ml), and $(P + S)$ is the total number of infected and uninfected spores.

Spore germination and phage release tests. After heat activation in water at 70°C for 30 min and then cooling on ice, spore germination was carried out in 25 mM Tris-HCl buffer (pH 7.4) supplemented with germinants of interest, as follows: (i) 20 mM L-alanine, (ii) 20 mM each L-asparagine, D-glucose, D-fructose, and K^+ (termed AGFK), and (iii) 10 mM L-alanine + 10 mM each AGFK. The initial optical density of spore suspension was 0.8 OD_{600} , and the decrease in OD_{600} in germinating cultures was monitored by plate reader (Molecular Devices, MA). The relative OD_{600} was calculated by division of each reading (A_t) by the initial reading (A_0). To determine the latent time for phage in spores and the pattern of phage release, samples were collected at 30-min intervals and immediately subjected to a double-layer plate assay for phage enumeration.

Resistance tests for free phages and endospore-protected phage genomes. Free phage and endospore-protected phage genome stability were tested with exposure to several environmental stressors, as follows. The initial phage concentration was approximately 10^5 PFU/ml. Wet heat resistance was tested in a water bath for 15 min at temperatures ranging from 30 to 80°C. Samples were cooled to room temperature before submission to double-layer plaque assay. Phage pH resistance was assessed by adding the samples into solutions of various pHs from 2 to 13 for 1 h. The pH was neutralized before phage enumeration. Free phages and protected phages were also subjected to Cu^{2+} resistance assays. Samples were incubated for 1 h in CuSO_4 solutions with Cu^{2+} concentrations of 0.1, 0.5, 1.0, 5.0, and 10 mg/liter. The air-dried endospores containing phage genomes were stored in the dark at room temperature to test the long-term stability of endospore-protected phage genomes.

Statistical analysis. The Poisson distribution was used to describe the distribution of infected cells. The Poisson probability function was used to calculate the fraction of uninfected bacteria (i.e., $P(0) = 1 - e^{-\text{MOI}}$) (58), where MOI is the multiplicity of infection, defined as phages used for infection per number of cells. The Student t test (two-tailed) was used to determine the significance of the differences between treatments. Differences were considered to be significant at the 95% confidence level ($P < 0.05$).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01186-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.6 MB.

ACKNOWLEDGMENTS

This study was supported by an NSF PIRE grant (fund OISE-1545756). N.G. received financial support from the National Council for Scientific and Technological Development—Brazil.

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