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Lysogeny and Sporulation in *Bacillus* Isolates from the Gulf of Mexico▽†

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Eleven *Bacillus* isolates from the surface and subsurface waters of the Gulf of Mexico were examined for their capacity to sporulate and harbor prophages. Occurrence of sporulation in each isolate was assessed through decoyinine induction, and putative lysogens were identified by prophage induction by mitomycin C treatment. No obvious correlation between ability to sporulate and prophage induction was found. Four strains that contained inducible virus-like particles (VLPs) were shown to sporulate. Four strains did not produce spores upon induction by decoyinine but contained inducible VLPs. Two of the strains did not produce virus-like particles or sporulate significantly upon induction. Isolate B14905 had a high level of virus-like particle production and a high occurrence of sporulation and was further examined by genomic sequencing in an attempt to shed light on the relationship between sporulation and lysogeny. *In silico* analysis of the B14905 genome revealed four prophage-like regions, one of which was independently sequenced from a mitomycin C-induced lysate. Based on PCR and transmission electron microscopy (TEM) analysis of an induced phage lysate, one is a noninducible phage remnant, one may be a defective phage-like bacteriocin, and two were inducible prophages. One of the inducible phages contained four putative transcriptional regulators, one of which was a SinR-like regulator that may be involved in the regulation of host sporulation. Isolates that both possess the capacity to sporulate and contain temperate phage may be well adapted for survival in the oligotrophic ocean.

Lysogeny and sporulation are strategies for phage and host survival, respectively, under adverse conditions. During both processes, the genome of the phage or bacterium is replicated into a form, prophage or endospore, that increases its survival (14). The initiation of both lysogeny and sporulation involves the repression and activation of promoters that are regulated by feedback from their gene products. In coliphage λ, *c*I binds to the lytic promoter P_R during lysogeny while cro binds to P_R during a virulent infection (39). SinR binds to the vegetative promoter P_V in the Sin operon under normal cell conditions, and SpoA activates the sporulation promoter (27). The tertiary structures of the DNA-binding domain of SinR from *Bacillus subtilis* and CI and Cro from the *Escherichia coli* phage 434 are nearly identical (25). Recent work has also demonstrated that CIII and sporulation control protein SpoVM are both inhibited by the FtsH protease (21). These structural and functional similarities indicate a possible evolutionary relationship between prophage induction and sporulation.

Previous work has indicated a possible link between phages and sporulation. Meijer et al. (28) found that lytic development of the virulent *Bacillus* phage ϕ29 was repressed in sporulating cells through inhibition of transcription of early phage genes by SpoA. A phage-encoded sigma factor in the *B. anthracis* virulent phage Fah was negatively controlled by a sporulation anti-sigma factor (29). Lytic development was also found to be suppressed in temperate phages during sporulation, with no virus production occurring during UV induction (35). This phenomenon could be a survival mechanism that allows the phage to be protected in the spore under conditions that are unfavorable to infection, such as low host abundance under nutrient-depleted conditions. Survival would be increased if the prophage contained genes that allow the host to sporulate at a higher frequency than noninfected bacteria.

Phages that can perform this function are known as spore-converting phages, and they have been found in *Bacillus* and *Clostridium* species (7, 47). *Bacillus* phages PMB12 and SP10 are believed to induce sporulation by activating sporulation initiation signals (46). Unfortunately, most of these studies of *Bacillus* phages predate current sequencing technologies. However, the genome of *Clostridium perfringens* temperate phage Φ3226 contained a sporulation-associated sigma factor homolog and a sporulation-dependent transcriptional regulator that was believed to augment host sporulation (53). Although spore-converting phages have been characterized for terrestrial *Bacillus* species, the phages infecting marine *Bacillus* strains have not been studied.

In this study, a collection of 11 *Bacillus* isolates from the Gulf of Mexico were investigated for spore and phage production through traditional induction experiments. Genomic and molecular techniques were used to study the prophages of one of the isolates, B14905, in depth. These studies provide a glimpse into host-prophage relationships of a marine *Bacillus*

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TABLE 1. Collection of *Bacillus* strains from the Gulf of Mexico

Strain	Location	Depth (m)	Environment	Best GenBank match (% similarity)[a]
B14850	Gulf of Mexico	85	Water column	*B. aquimaris* (96)
B14851	Gulf of Mexico	65	Sediment	*B. barbaricus* (95)
B14904	Gulf of Mexico	2	Water column	*B. pumilus* (99)
B14905	Gulf of Mexico	1,500	Water column	*B. fusiformis* (96)
B14906	Gulf of Mexico	85	Water column	*B. cibi* (97)
B14907	Gulf of Mexico	85	Water column	*B. subtilis* (97)
B14908	Gulf of Mexico	10	Water column	*B. pumilus* (97)
B14909	Bimini, Bahamas	1.5	Sediment	*Paenibacillus* sp. (97)
B14910	Tampa Bay	5	Sediment	*B. megaterium* (94)
B14911	Gulf of Mexico	10	Water column	*B. firmus* (96)
B14912	Gulf of Mexico	200	Sediment	*Paenibacillus* sp. (96)

[a] The best GenBank match is based upon partial 16S rRNA gene sequencing previously performed by Siefert et al. (45).

strain and serve as a basis for functional studies to investigate the influence of prophages on host sporulation.

MATERIALS AND METHODS

Isolate collection and phylogeny. Eleven pigmented marine *Bacillus* isolates were collected from different sites and depths during a 1992 research cruise to the Gulf of Mexico, as outlined in Table 1. The collection methods and the 16S rRNA gene sequence for each isolate were previously described by Siefert et al. (45). These strains were maintained in pure culture by monthly plating onto artificial seawater nutrient (ASWJP+PY [38a]) plates and Trypticase soy agar (TSA) plates (Becton Dickinson, Cockeysville, MD).

Induction of sporulation in *Bacillus* isolates. Before the induction experiments, a smear from a colony of each isolate on a month-old plate was observed for the presence or absence of spores using phase-contrast microscopy. This indicated the capacity of each strain to sporulate.

Sporulation was initially induced in the isolates by use of a protocol modified from Mitani et al. (30). Decoyinine is an adenine-ketose antibiotic that inhibits GMP synthetase. Starvation of GMP can initiate sporulation in the presence of ample nutrients, serving as a quick and effective means to study sporulation (30, 49). For each *Bacillus* strain, 1 ml of overnight culture was diluted into 9 ml of fresh ASWJP+PY and incubated at 28°C with shaking. When the optical density at 600 nm reached 0.4, 2-ml aliquots were placed in separate 15-ml tubes, one for a control and one for treatment. Sterile filtered (0.22-μm filter size) decoyinine (0.5 mg ml^{-1}) (Sigma, St. Louis, MO) was added to the treatment tube. After a 24-h incubation, 0.02 μm filtered formalin (1.0%) was added to the control and treatment tubes to stop bacterial growth. Wet mounts of these cultures were prepared in triplicate and immediately viewed. Vegetative cells and spores have different refractive indices that allow differentiation of the two bodies under phase-contrast microscopy. The different cell types were counted on 10 field grids per slide to determine the number of spores (C_s) and vegetative cells (C_v) by use of the equation $C_g/V_g = C_{s,v}$, where C_g is the cell (spore or vegetative-cell) direct count per grid and V_g is the volume (in ml) per grid. V_g was calculated using the equation $(V_s/A_{cs}) \times A_g = V_g$, where V_s is the volume of sample, A_{cs} is the area of coverslip, and A_g is the area of the grid.

The percent population of cells that sporulated (% spore) was calculated by use of the equation $(C_s/C_v) \times 100 = \%$ spore.

Isolates that did not have significant sporulation following decoyinine treatment were subjected to heat and ethanol treatments to induce sporulation. For the heat treatments, 1 ml of log-phase cultures (optical density at 600 nm, 0.4) was incubated at 80°C for 10 min, and 1 ml of the culture left at room temperature served as a control. Serial dilutions from the treatment and control cultures were plated in duplicate on ASWJP+PY plates. Ethanol treatment was performed essentially as described by Koransky et al. (22). One ml of log-phase cultures (optical density at 600 nm, 0.4) was diluted 1:1 with 0.22 μm filtered absolute ethanol for 1 h, while cultures diluted 1:1 with 0.22 μm deionized (DI) water served as controls. Serial dilutions from the treatment and control cultures were plated in duplicate on ASWJP+PY plates. The plates were incubated for 24 to 72 h until colonies appeared.

Prophage induction in *Bacillus* isolates. The presence of prophage in these *Bacillus* isolates was determined by mitomycin C treatment. Five milliliters of overnight culture was diluted into 45 ml of fresh ASWJP+PY medium in separate flasks, one for a control and one for a treatment. When the optical density at 600 nm reached 0.4, mitomycin C (1 μg ml^{-1}) was added to the treatment flask and returned to the shaker. After 24 h, the absorbance measurements were taken and 5 ml was removed from each flask for phage enumeration. Samples were stained with SYBR gold (Molecular Probes, Eugene, OR) as described by Chen et al. (12), with a staining time of 12 min. Virus-like particles (VLPs) were enumerated using blue light excitation with an Olympus BH-2 epifluorescence microscope. At least 200 phage particles in 10 fields per slide were counted. The difference in prophage production between treated and control cultures was calculated. Statistical analysis was done using the pooled Student *t* test for equal variances ($\alpha = 0.05$).

Host range of phage lysates from *Bacillus* isolates. Two different methods were used to address the cross-infectivity of mitomycin C-induced lysates from the 11 *Bacillus* isolates within the collection. Phage lysate was produced for each of the *Bacillus* strains that had significant prophage production as determined by the initial screening described above.

Host sensitivity to the phage lysates was determined using a cross-streaking test (20). Approximately 30 μl of each phage lysate was streaked on an ASWJP+PY plate and allowed to dry before a loopful of an overnight culture of the test *Bacillus* isolate was cross-streaked against the lysate. Plates were incubated at 28°C for 24 to 74 h and then examined for any lysis of bacterial growth.

A spot lysis assay was performed in duplicate by spotting approximately 2×10^7 VLPs of each phage lysate on ASWJP+PY soft agar overlays containing 1 ml of overnight *Bacillus* culture (19). Plates were incubated at 28°C for 24 to 74 h and then examined for any lysis of bacterial growth.

Time series induction of phage particles from isolate B14905. Phage production in B14905 was monitored over a 24-h time series following induction with mitomycin C. Prophage induction was carried out as described above except that absorbance readings were taken every 2 h until 8 h and at 24 h after induction, and 5-ml subsamples were removed from each flask to enumerate bacteria and viruses. Samples were stained with SYBR gold and VLPs were enumerated as described above. The direct-counting method was also used for bacterial direct counts (BDC). Statistical analysis was done using the pooled Student *t* test for equal variances ($\alpha = 0.05$).

Sequencing of the B14905 host and phage genomes. The genome of *Bacillus* sp. NRRL B14905 was sequenced using whole-genome shotgun sequencing by the J. Craig Venter Institute as part of the Gordon and Betty Moore Foundation Marine Microbial Genome Sequencing Project. The genome was partially assembled and annotated by using the subsystems approach in the SEED databank (36).

B14905 phage particles were isolated by use of a large-scale mitomycin C induction procedure, and phage DNA was extracted as described by Mobberley et al. (31). The B14905 phage DNA extract was cloned using linker-amplified shotgun libraries (LASL) as previously described (38). Clones from these libraries were sequenced using an ABI 3100 capillary sequencer (Applied Biosystems, Foster City, CA). The software program Sequencher 3.0 (Genecodes, Ann Arbor, MI) was used to assemble contigs. Gaps between the contigs were resolved using multiplex PCR and by primer walking using phage DNA as the template.

B14905 prophage genome analysis. The gene annotations of B14905 were searched in the SEED database using the term "phage," which generated a list of all open reading frames (ORFs) including the term. When these ORFs were clustered together, the region of the contig was investigated for prophage-related genes, including integrases, repressors/antirepressors, packaging protein genes, and lysis-associated genes. Potential prophage-encoded regions were exported into KODON for further analysis. ORFs were analyzed more in depth by BLASTP analysis of the nonredundant database (2).

Protein analysis by mass spectroscopy. Phage lysate samples were separated on a one-dimensional 12% sodium dodecyl sulfate-polyacrylamide gel run at 4 mA for 16.5 h by use of the Laemmli method (24). Gels were fixed in methanol-acetic acid-water (50:10:40), followed by overnight staining with SYPRO ruby (Bio-Rad, Hercules, CA). Protein-containing bands were numbered and excised from the gel with a scalpel. The excised samples were sent to the Proteomics Core Facility at the Moffitt Cancer Research Center (Tampa, FL) for analysis by matrix-assisted laser desorption ionization–time-of-flight–time-of-flight (MALDI-TOF-TOF) mass spectroscopy as described by Paul et al. (31, 38). The tandem mass analysis produces a collision-induced dissociation (CID) spectrum that can be used for protein identification based on the peptide sequence (52).

PCR with B14905 phage primers. Phage genomic DNA was extracted from a large-scale mitomycin C lysate as described previously (31). B14905 chromosomal DNA was extracted using a Wizard genomic DNA purification kit (Promega, Madison, WI) according to the manufacturer's protocol. The quantity and quality of the DNA preparations were assessed by agarose gel electrophoresis and by use of a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE).

TABLE 2. List of primer sets for B14905 PCR

Primer name	Sequence	Gene	Amplicon size (bp)
1F	GACGACTGGCGAAGTGTTTT	Phage antirepressor (ORF 13)	224
1R	GCAGATGCCCTTGGTTATGT	Phage antirepressor (ORF 13)	224
2F	TCGGAAGAAATCGATGGAAC	Terminase (ORF 18)	538
2R	TAGGCAGCCGCAATAACTCT	Terminase (ORF 18)	538
3F	CGCGAGTCCGATACGATTTA	Hypothetical protein (ORF 34)	206
3R	TTCACCAACAATTTCTTTCA	Hypothetical protein (ORF 34)	206
4F	CACAAAAGAGGCCCAGAAAT	Tail tape measure (ORF 10)	249
4R	GGACGAGTGTCTCGCAACA	Tail tape measure (ORF 10)	249
HostF	ATTTGGCATGGAGCTAGTGC	Noncoding region on contig 126650671	322
HostR	GGAACGAACCGCTTTCTATG	Noncoding region on contig 126650671	322

Unique primer sets were created for each potential prophage genome and for the host, as listed in Table 2. In order to account for the differing amounts of phage DNA present in the preparations, 10 ng of host chromosomal DNA and 1 ng of phage DNA were used in the reactions. GoTaq green mastermix (Promega, Madison, WI) was used as recommended, and the primer sets were added to a final concentration of 0.5 μM per primer (Table 2). All PCRs were performed with a My Cycler thermal cycler (Bio-Rad, Hercules, CA). For phage primer sets 1, 2, and 4 and the host primer set, the following PCR program was used: initial denaturation for 2 min at 95°C; 30 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 2 min; and a final extension at 72°C for 10 min. The PCR program used for phage primer set 3 was as follows: initial annealing for 2 min at 95°C; 30 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C for 2 min; and a final extension at 72°C for 10 min. The amplicons were loaded on a 1% agarose gel with 0.5 μg μl^{-1} of ethidium bromide and run at 89 V for 45 min. The gel was imaged using an AlphaImager 2200 imaging system (Alpha Innotech, San Leandro, CA).

Transmission electron microscopy of B14905 lysate. Transmission electron microscopy (TEM) was used to determine the presence and morphologies of multiple phages from a polyethylene glycol (PEG)-purified phage lysate. Twelve ml of a mitomycin C-induced culture was ultracentrifuged at 38,000 × *g* for 2 h at 4°C. The phage pellet was resuspended in 100 μl of 0.02 μm filtered deionized water, and the VLPs were enumerated with epifluorescence as described above. A Formvar carbon-coated grid (Electron Microscopy Sciences, Hatfield, PA) was floated on a large drop of lysate for 2 h. The grid was air dried and then negatively stained with 2% uranyl acetate for 1 min. The phage particles were visualized with a Hitachi 7100 transmission electron microscope.

Nucleotide sequence accession number. The unfinished genome of *Bacillus* sp. NRRL B14905 was submitted to GenBank under accession number AAXV00000000.

RESULTS

Sporulation induction in *Bacillus* isolates. Month-old colonies of all *Bacillus* strains were observed to contain spores whose morphology was similar to that described by Siefert et al. (45). Sporulation was induced in several of the *Bacillus* isolates after a 24-h treatment with decoyinine (Fig. 1). Isolates B14905, B14906, B14910, B14911, B14850, and B14851 had a significantly higher level of spore production with decoyinine than did the controls. Spore production for decoyinine-treated isolates B14904, B14907, B14908, B14909, and B14912 was not significantly different from that for the controls. Within this group, B14904, B14908, B14910, and B14912 had frequencies of sporulation that were less than 1%, while B14907 had a high frequency of spontaneous sporulation. The difference between spontaneous spore production for the controls and decoyinine-induced spore production for each isolate is displayed in Table 3.

The five *Bacillus* isolates that did not sporulate with decoyinine treatment, as well as B14850, which had high levels of spontaneous sporulation, were subjected to both heat and ethanol treatments to verify that they produced spore spontaneously. There was no significant sporulation in B14904, B14908, B14909, and B14912 with either of the treatments (data not shown). B14907 had a 74% frequency of sporulation with heat treatment, and ethanol treatment resulted in a 7% frequency of sporulation in B14850.

Prophage induction in *Bacillus* isolates. Prophage induction was investigated for 11 *Bacillus* isolates after 24 h of incubation with mitomycin C (Fig. 2). Virus-like particles produced by strains B14851, B14904, and B14911 were not significantly different from those produced by the controls ($P > 0.05$). Isolate B14912 was significantly different from the controls, but there was a high level of spontaneous induction in the control culture. Isolates B14908 and B14910 had significant prophage induction, but it was less than an order of magnitude greater than that for the controls. Phage production in isolates B14905, B14906, B14907, B14909, and B14850 was more than a magnitude greater than that in the controls. Table 3 shows the difference between levels of spontaneous phage production in the control and mitomycin C-induced cultures.

Prophage induction versus spore production in *Bacillus* isolates. The percent difference in prophage production between the mitomycin C-treated and control cultures and the percent difference in spore production between the decoyinine-treated and control cultures for each isolate listed in Table 3 were plotted against each other, as shown in Fig. 3. This shows that while there is no correlation between sporulation and prophage induction, the isolates appear to cluster into 3 groups: those with a high level of prophage production but a low incidence of sporulation, those with a low level of prophage production and a high incidence of sporulation, and those that both sporulated and produced elevated levels of prophage.

Host range of phage lysates from *Bacillus* isolates. Neither cross-streaking nor spot lysis produced visible lysis in isolates from our *Bacillus* collection.

Time series induction of phage particles from isolate B14905. A 24-h time series prophage induction with mitomycin C that was performed on isolate B14905 indicated that isolate B14905 contained inducible phages (data not shown). After 24 h, prophage induction in the mitomycin C-treated culture (3.11×10^{10} VLPs ml^{-1}) was significantly higher than that in the control culture (2.51×10^{8} VLPs ml^{-1}). As expected, bacterial growth monitored by absorbance decreased in the mitomycin C-treated culture, indicative of host lysis.

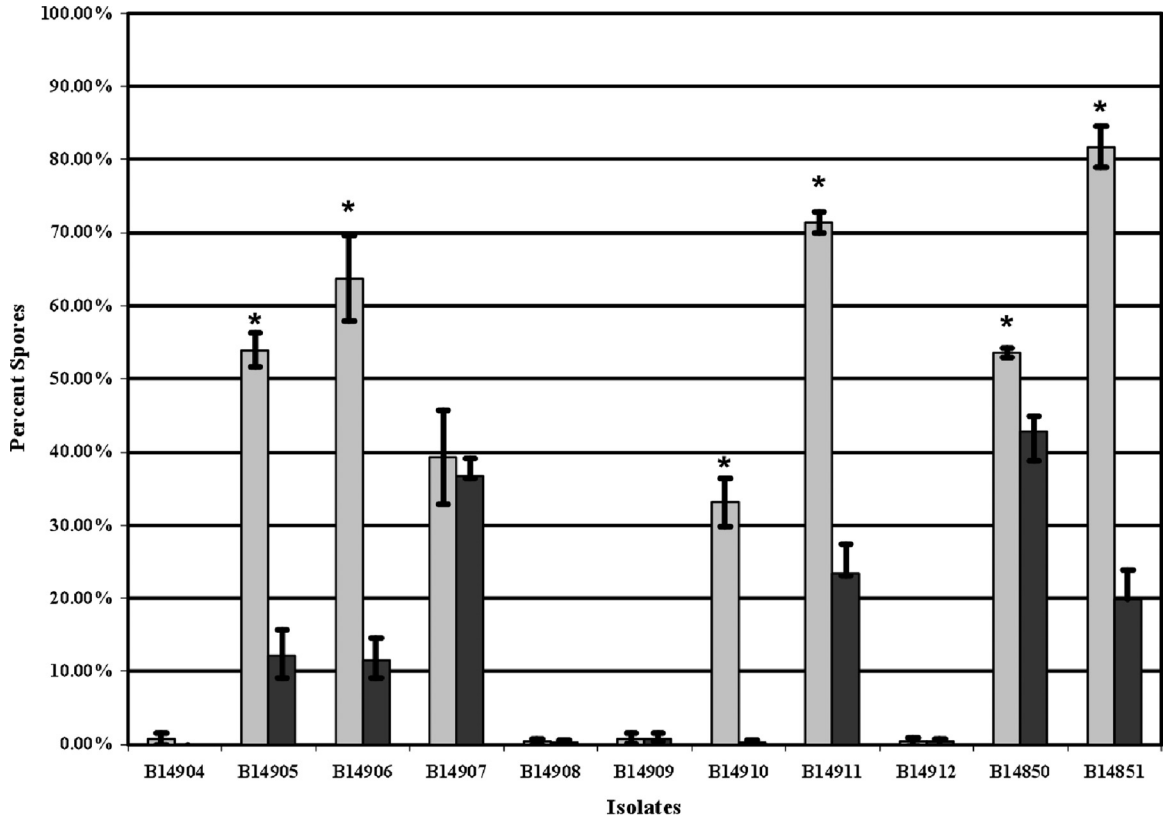

FIG. 1. Frequency of sporulation in *Bacillus* isolates after 24 h of decoyinine incubation. The mean percent sporulation for each isolate was used to chart the frequency of sporulation in decoyinine-treated (light-gray bars) and control (dark-gray bars) cultures. Asterisks denote significantly different frequencies of sporulation between the two treatments ($n = 3$; $P < 0.05$). Standard deviations are represented by error bars.

Analysis of the B14905 bacterial genome. The draft B14905 genome is 4.4 Mbp in length, with a G+C content of 37%. The genome was assembled into 99 contigs and contained 4,626 protein coding regions and 126 RNA genes. Fifty-five percent of the coding regions could be assigned a putative functional assignment.

Analysis of prophage-like regions of the B14905 genome. (i) ΦB05-1. Sequencing of the mitomycin C-induced lysate resulted in a single double-stranded phage genome, ΦB05-1, which is 18,118 bp in length and has a G+C content of 33%. This assembly was verified by comparison to the prophage sequence in the B14905 host genome contained in contig 1000022. Sixteen of the ORFs (61%) shared significant similarity ($e < 10^{-4}$) at the protein level with other sequences in GenBank. Twelve of the ORFs were similar to genes found in other *Bacillus* species and *Bacillus* phages. Significant similarities to other sequences in GenBank and putative functional assignments are listed in Table 4 and Fig. 4. ΦB05-1 is mito-

TABLE 3. Difference in prophage production and spore production[a]

Isolate	Prophage production (VLPs ml^{-1})		% Difference	Spore production (% spores)		% Difference
	Control	Mitomycin C induced		Control	Decoyinine induced	
B14904	7.00E+06	1.50E+07	53	0	0.7	100
B14905	2.50E+08	3.11E+10	99*	13	84.1	85*
B14906	8.30E+07	8.18E+08	90*	11.8	57.2	79*
B14907	1.15E+07	1.35E+09	99*	50.1	66.3	24
B14908	2.44E+09	3.43E+10	93*	0.03	0.4	24
B14909	6.10E+07	3.45E+08	82*	1.0	0.8	−25
B14910	3.12E+07	1.15E+08	73*	0.2	49.6	99.6*
B14911	2.50E+06	2.40E+06	−4	29.9	71.4	58*
B14912	8.25E+08	3.10E+09	73*	0.4	0.7	46
B14850	2.50E+07	3.00E+09	99*	74.8	86.6	14*
B14851	2.60E+06	3.60E+06	0.28	25.6	81.4	69*

[a] Prophage production data are based on ml-1 VLP production in the control and mitomycin C-treated cultures. Spore production data are based on percentages of spores in control and decoyinine-treated cultures. The percent difference between the treatment and the control was calculated for each experiment. An asterisk denotes a significant difference between the two treatments for each experiment.

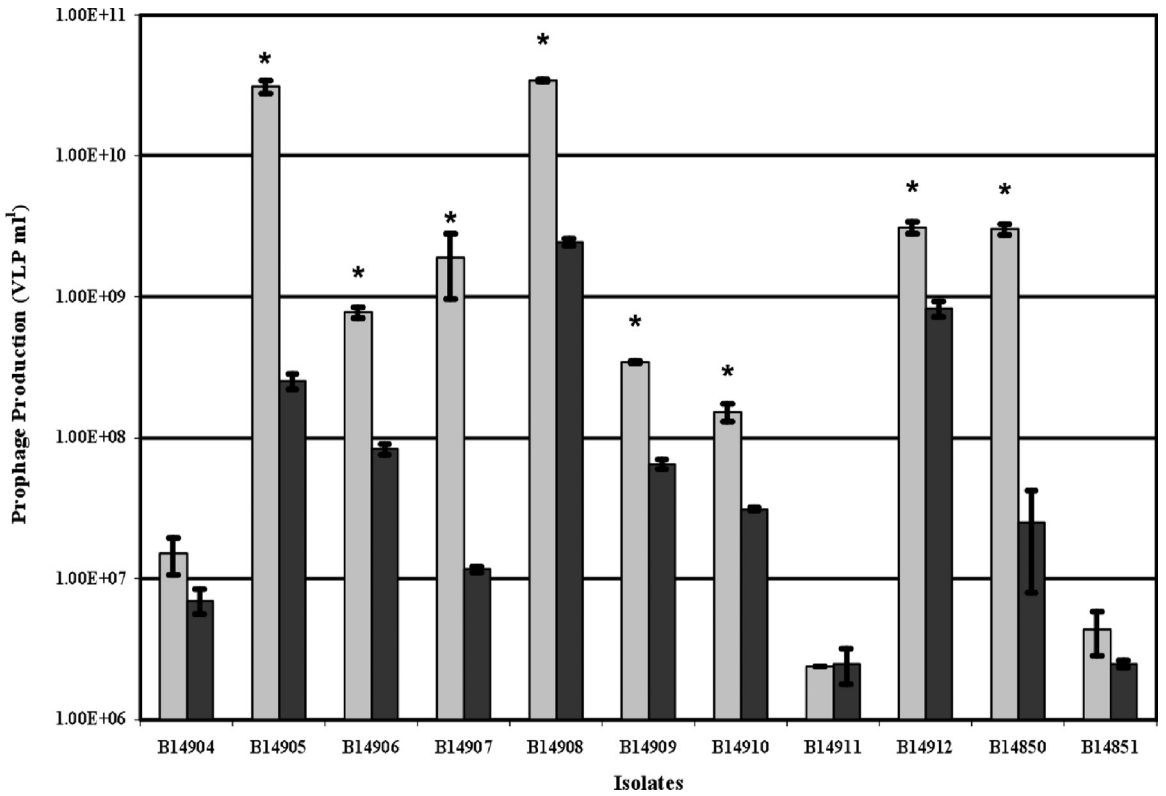

FIG. 2. Prophage production in *Bacillus* isolates after 24 h of incubation with mitomycin C. The mean prophage production for each isolate in control (dark-gray bars) and mitomycin C-treated (light-gray bars) cultures is shown. Asterisks denote significantly different levels of prophage production ($P < 0.05$). Standard deviations are represented by error bars.

mycin C inducible, based on PCR amplification from a purified host-free lysate (Fig. 5, lane 7).

ΦB05-1 contained several genes that were similar to those found in temperate phages. ORF 1 was similar to an integrase and ORF 6 was similar to the replication protein from the *Bacillus cereus* phage BC6A52. ORF 8 shared similarity with ORF 13 of *B. subtilis* phage Φ-105 and ORF 40 of *Clostridium* phage 39-O. ORF 12 of ΦB05-1 was similar to the antirepressor protein of *Streptococcus* phage 10750.4. No phage structural or lytic genes were identified through similarity searching.

The ΦB05-1 genome contained four putative transcriptional regulators, ORFs 2, 3, 17, and 20, based on the presence of helix-turn-helix DNA binding domains. ORF 2 is similar to the DNA binding domains of post-exponential-phase transcriptional regulators from *Bacillus halodurans* C-125 and *Bacillus* sp. NRRL B-14911. Post-exponential-phase transcriptional regulators are involved in expression of genes that allow *Bacillus* to adapt to suboptimal conditions in the transition from active to stationary phase (48). The ORF also shared a weak similarity to SinR-like proteins in *Lysinibacillus sphaericus* C3-41 and *Bacillus cereus* ATCC B4264. SinR is a transition-state regulator that represses stage II sporulation genes and *spo0A* expression during vegetative growth. ORF 3 is a weak hit to a transcriptional regulator found in the genomes of both *Lysinibacillus sphaericus* C3-41 and *Staphylococcus aureus*. It may be that repression of sporulation by phage repressors ensures better prophage survival and that it is more difficult for prophage induction to occur in a spore.

ORF 17 was a very strong hit to a MerR-type transcriptional regulator from *B. cereus* E33L. Transcriptional regulators in this family have been found in bacteria and phages and are known to respond to a variety of stressful stimuli, including metals and antibiotics (8). ORF 18 is part of a group (ORF 17 to 20) that contains 2 ORFs similar to choloylglycine hydrolases from *B. cereus* ATCC 14579, *B. cereus* E33L, and *Clostridium botulinum*. Choloylglycine hydrolases are bacterial products, usually associated with intestinal microflora, that catalyze the degradation of bile salts and result in the production of free amino acids (5). ORF 20 is similar to a transcriptional regulator found in *Streptococcus pyogenes* (3e−8, 32% identity). It also shares similarity to LlaI, part of a phage-encoded restriction modification system in *Lactococcus lactis* (15).

(ii) ΦB05-2. ΦB05-2 is a prophage-like region 17,159 bp in length with a G+C content of 35.5% that was found on contig 1000019 of the B14905 host genome. This segment is composed of 24 ORFs, 18 (75%) of which shared significant similarity ($e < 10^{-4}$) at the protein level with other sequences in GenBank (Fig. 4). Similarities to other sequences and putative functional assignments are listed in Table 5 and Fig. 4. Replication of ΦB05-2 was not induced by mitomycin C treatment (Fig. 5, lane 8).

The ΦB05-2 segment contained phage-related proteins that are found in other *Bacillus* strains and prophages. ORFs 3 and 4 are similar to proteins from *Streptococcus pyogenes* phage 315.3 and *Pneumococcus* prophage EJ-1, respectively. Two phage replication-associated proteins were found in the ge-

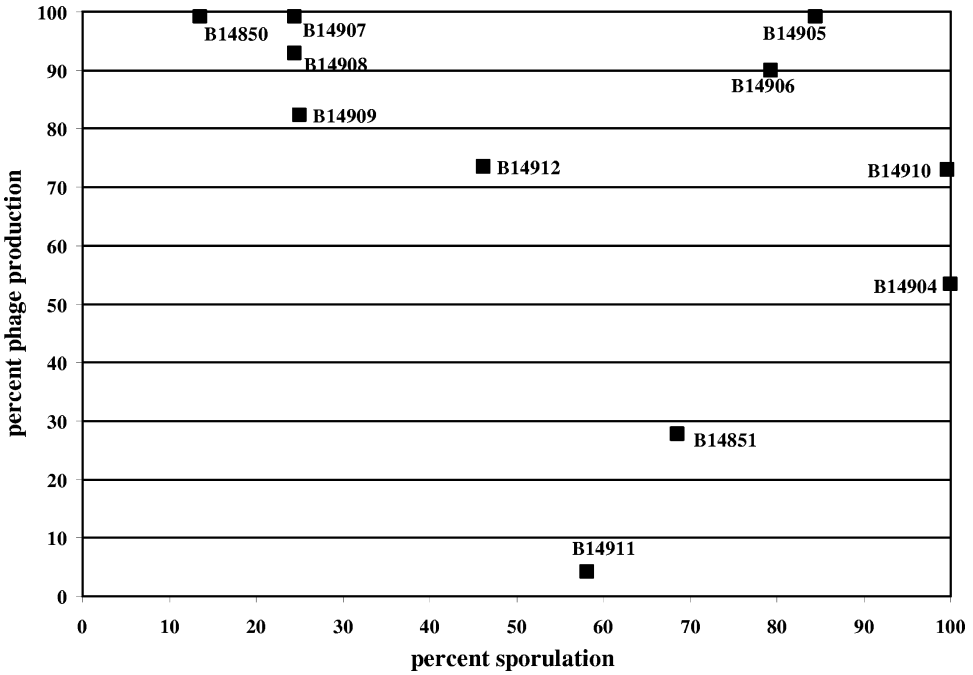

FIG. 3. Prophage production and sporulation in *Bacillus* isolates. For each strain, the percent difference in phage production (mitomycin C treated versus spontaneous) and the percent difference in number of spores, as outlined in Table 3, were graphed.

nome: ORF 5 is similar to a replication protein found in the lytic *Bacillus* phage Fah, and ORF 7 is similar to the single-stranded DNA binding protein of *Staphylococcus aureus* prophage PVL. ORF 18 putatively encodes a terminase small subunit that is similar to the protein in temperate *Bacillus clarkii* phage BCJA1c. The large terminase gene encoded by ORF 19 is similar to those in *Listeria* phage B054, temperate *Haemophilus* phage Aaphi23, and *Lactococcus* phage TP901-1. The final ORF of ΦB05-2 is similar to transposases found in *Bacillus* sp. SG-1 and *Streptococcus pyogenes*. No integrase, lysogeny-related, or structural genes were identified by similarity searching.

(iii) ΦB05-3. The prophage-like region of ΦB05-3 is found on contig 1000024 and is 25,898 bp in length. The segment is composed of 43 ORFs, 27 (62%) of which were similar to other proteins in GenBank (Fig. 4). Table 6 shows the significant similarities to other proteins and putative functional assignments. ΦB05-3 was present in a bacterium-free lysate made from an induced host (Fig. 5, lane 9).

The ΦB05-3 genome contained lysogeny-related and phage replication genes that were similar to those of other temperate phages and prophage regions in bacterial genomes (Table 6). ORF 1 is similar to integrase proteins found in temperate *Listeria* phage A500 and *Mycobacterium* phage Ms6. ORF 3 encodes a phage repressor that is similar to those in *Geobacillus* phage GBSV1 and *B. cereus* phage BC6A51. ORF 7 is similar to the phage antirepressor proteins from *S. aureus* prophage ϕPV83 and temperate coliphage P1. The protein encoded by ORF 10, which has low similarity to a transcriptional regulator from *Geobacillus kaustophilus*, was expressed and detected in a cesium chloride-purified phage lysate (Fig. 6). The estimated size of the protein was similar to the observed molecular size of the protein. ORF 20 and ORF 21 are similar to phage replication genes from several *Bacillus* phages and *Staphylococcus* phage ϕPVL108.

Late phage genes involved in packaging and lysis are found on the right side of the ΦB05-3 genome (Fig. 4). ORF 35 was similar to the terminase small subunits from *Clostridium* prophage ϕC2 and *B. clarkii* temperate phage BCJA1c. ORF 36 encodes the terminase large subunit that shares similarity with genes in *Bacillus* species and *Staphylococcus* phages ϕETA2 and ϕC2. ORF 37 is similar to portal proteins from *Staphylococcus* phages ϕNM4 and ϕETA3. ORFs 41 and 42 are similar to a holin and a lysis protein, respectively, found in temperate *Bacillus* phages. No capsid or tail genes were found based on similarity to known protein sequences.

(iv) ΦB05-4. The ΦB05-4 17,991-bp prophage-like region was found on the host contig 1000003. The genome contained 24 ORFs, 22 (92%) of which shared similarity with other proteins in GenBank (Fig. 4; Table 7). ΦB05-4 shared significant homology (22 ORFs) to a prophage-like region found in *Lysinibacillus sphaericus* C3-41 (18). Twelve of the ORFS were similar to genes from the temperate myoviruses *Clostridium* phage ϕC2 and *Streptococcus* phage EJ-1. Ten ΦB05-4 ORFs also shared similarity to genes of the defective *Bacillus* phage PBSX, a defective prophage-like element known for packaging random portions of host DNA. Based on PCR evidence, ΦB05-4 was not mitomycin C inducible (Fig. 5, lane 10).

ORF 24 was similar to the integrase XkdA from *B. subtilis*, and ORF 23 was similar to a phage repressor protein from *Clostridium* phage ϕCD119. ORFs 7 to 11 and 14 to 21 encode gene products that are similar to tail proteins from the temperate myoviruses ϕC2 and EJ-1 and the defective phage PBSX found on *B. subtilis* (Table 7). Two of these proteins, the tape tail measure (ORF 15) and the tail sheath (ORF 19), were expressed in a PEG-precipitated lysate (Fig. 6). ORF 2, which

TABLE 4. Selected open reading frames of the ΦB05-1 genome and BLASTP hits

ORF no./orientation[a]	Predicted function	Related BLASTP hit(s)[b]
1/−	Integrase	Recombinase XerC-like protein [*Lysinibacillus sphaericus* C3-41] YP_001699767 (1e−133), 58% identity
		Integrase [*Bacillus* phage BC6A52] NP_852561 (4e−35)
2/+	Transcriptional regulator	SinR protein [*Lysinibacillus sphaericus* C3-41] YP_001699766 (2e−21), 34% identity
		Transcriptional regulator of post-exponential-phase responses [*Bacillus halodurans* C-125] (4e−6), 41% identity
		SinR protein [*Bacillus cereus* ATCC B4264] YP_002366064 (2e−4), 40% identity
3/+	Transcriptional regulator	Xre family-like protein [*Lysinibacillus sphaericus* C3-41] YP_001699765 (9e−21), 67% identity
		Putative transcriptional regulator [*Staphylococcus aureus*] CAL23819 (7e−8), 56% identity
4/+		Hypothetical protein [*Lysinibacillus sphaericus* C3-41] YP_001699764 (5e−23), 53% identity
6/+	Replication protein	Phage replication protein [*Bacillus cereus* B4264] YP_002367306 (1e−26), 51% identity
		Replication protein [*Bacillus* phage BC6A52] NP_852568 (3e−26)
		Replication protein [*Lactococcus* phage TP901-1] NP_112676 (3e−15), 37% identity
8/−		Hypothetical protein [*Bacillus licheniformis* DSM 13] AAU42447 (5e−43)
		ORF 13 [bacteriophage ϕ-105] NP_690797 (5e−40)
		gp40 [*Clostridium* phage 39-O] YP_002265457 (1e−23)
10/+		Group-specific protein [*Bacillus cereus* cytotoxin NVH 391-98] ZP_01181082 (2e−18)
		Group-specific protein [*Lysinibacillus sphaericus* C3-41] YP_001699753 (8e−18)
12/+	Phage antirepressor	Bro domain-containing protein [*Desulfotomaculum reducens* MI-1] YP_001113957 (2e−47)
		Phage antirepressor protein [*Streptococcus* phage 10750.4] YP_603395 (4e−37)
16/+		Hypothetical protein [*Bacillus cereus* E33L] AAY60441 (1e−40)
17/+	Transcriptional regulator	MerR family transcriptional regulator [*Bacillus cereus* E33L] AAY60476 (5e−101)
		Transcriptional protein [*Clostridium beijerinckii* NCIMB 8052] ZP_00908099 (2e−27)
18/+	Choloylglycine hydrolase	Conserved hypothetical protein [*Bacillus cereus* E33L] AAY60477 (7e−136)
		Choloylglycine hydrolase family protein [*Clostridium botulinum* A strain ATCC 3502] YP_001254760 (4e−37)
		Hypothetical protein [*Bacillus* sp. NRRL B-14911] ZP_01172832 (2e−16)
19/+	Choloylglycine hydrolase	Choloylglycine hydrolase [*Bacillus cereus*] ZP_04197456
		Choloylglycine hydrolase [*Clostridium botulinum* F strain Langeland] (2e−61)
		Choloylglycine hydrolase family protein [*Clostridium botulinum* A strain ATCC 3502] YP_001254760 (6e−12)
		Hypothetical protein [*Bacillus* sp. NRRL B-14911] ZP_0117376 (7e−11)
20/−	Transcriptional regulator	Hypothetical protein [*Paenibacillus larvae* subsp. *larvae* BRL-230010] ZP_02326137 (6e−13)
		Transcriptional regulator [*Streptococcus pyogenes* MGAS10394] AAT86620 (3e−8)
		LlaIC [*Lactococcus lactis*] AAR19408 (4e−7)
23/+		Hypothetical protein [*Sphingomonas* sp. SKA58] ZP_01305154 (2e−46)
		Appr-1-p processing protein family [*Thiobacillus denitrificans* ATCC 25259] YP_315247 (8e−32)
24/−		Hypothetical protein [*Anaerocellum thermophilum* DSM 6725] YP_002573528 (7e−17)
26/+		Hypothetical protein [*Oceanspirillium* sp. MED92] ZP_01164802 (8e−8)

[a] +, forward orientation; −, reverse orientation. Those ORFs with no significant similarity by BLASTP search are not included.
[b] Accession numbers are from the GenBank database.

is similar to a protein from *Lysinibacillus sphaericus* C3-41, was also found to be expressed in the phage proteome. ORFs 3, 13, and 14 are similar to cell wall hydrolases of ϕC2 and PBSX, and ORF 4 is similar to a holin protein found in ϕC2. No identifiable replication, capsid, or DNA packaging genes were found in ΦB05-4.

Transmission electron microscopy of B14905 lysates. Electron micrographs of negatively stained B14905 lysates showed two different phage morphologies (Fig. 7). A possible myovirus-like particle is seen in panel A, with a capsid diameter of 138 nm and a tail length of 307 nm and width of 23 nm. Panels B and C show smaller particles with icosahedral capsids (103-nm diameter) with thick tails (210-nm length and 35-nm width), consistent with *Myoviridae*. There were also particles that resembled incomplete phage tail components, as seen in panels D, E, and F.

DISCUSSION

Members of the *Bacillus* genus are ubiquitous in nature due to their ability to form spores that are resistant to adverse conditions. The Gulf of Mexico *Bacillus* isolates displayed a range of spore production frequencies when sporulation was induced by artificial means during exponential growth. The isolates that had significant levels of sporulation with decoyinine treatment indicate that this chemical was effective in inhibiting GMP synthesis in these isolates. Strains B14850 and B14907 are interesting because of their high levels of sporulation with both the control and decoyinine treatments after 24 h. This could be due to poorly controlled sporulation initiation caused by a mutation resulting in repression of catabolite sensing (6, 44). Alternatively, these isolates may have salvage pathways that allow them to obtain GMP by other means, so that decoyinine treatment is ineffective in inducing sporulation (40). With the exception of B14907, the five isolates that did not produce spores during chemical or heat treatment had frequencies of sporulation below 1% under both control and treatment conditions. This indicates that these isolates may have tightly regulated initiation of sporulation that prevents any sporulation occurring when nutrients are present. Further work is needed to elucidate the signals that control sporulation in these isolates.

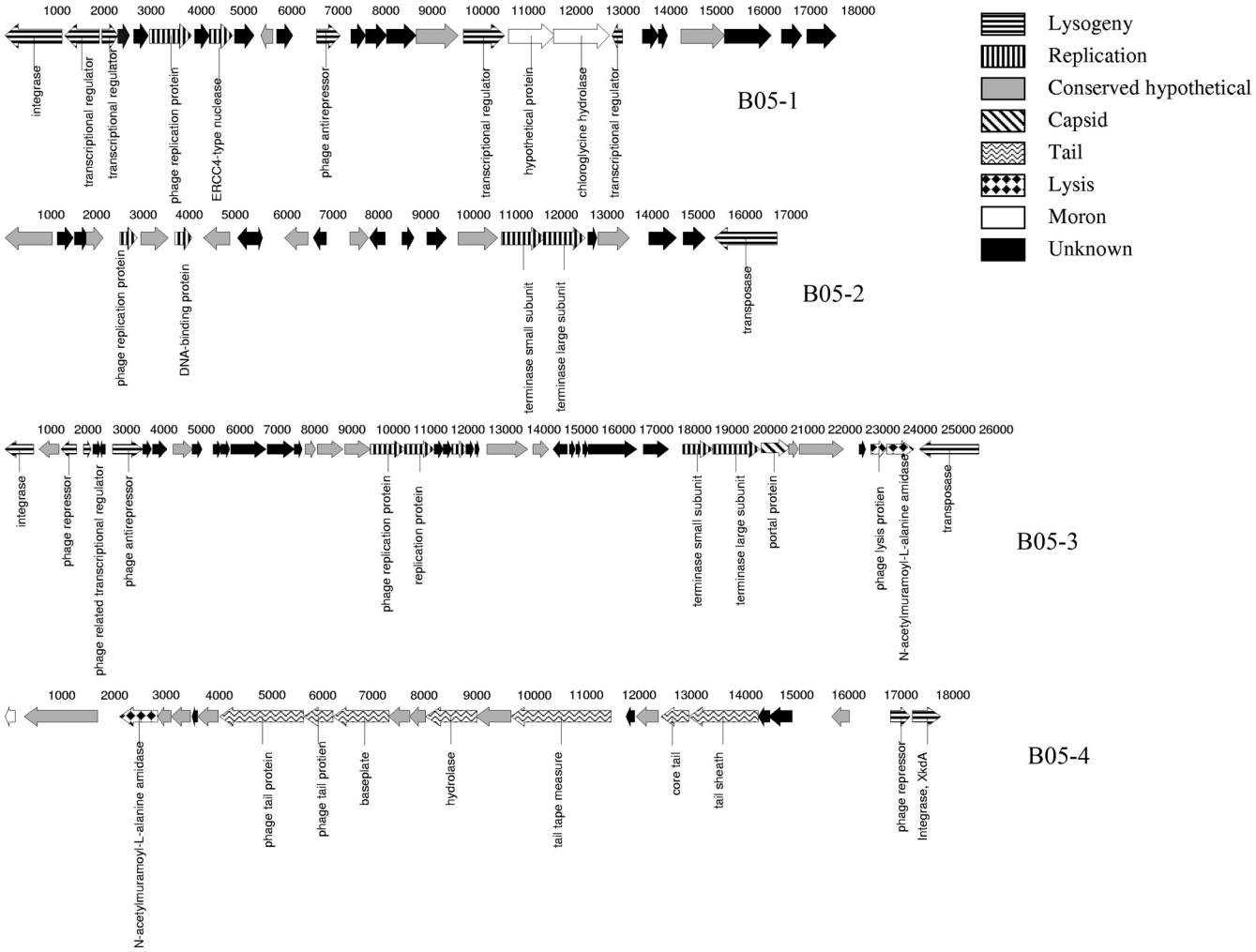

FIG. 4. Genomic map of B14905 prophage-like regions. KODON was used to construct the gene map. Patterns were assigned based on putative ORF function (Table 4).

Eight (66%) of the isolates may have contained temperate phages, since virus-like particles were inducible upon mitomycin C addition. The high incidence of lysogeny in this isolate collection is not unexpected given the prevalence of inducible prophages in the *Bacillus* genus (17). Based on the absence of lytic activity during the cross-infection experiments with induced lysates, the phages from these isolates were either host specific or already lysogenized with a homoimmune prophage.

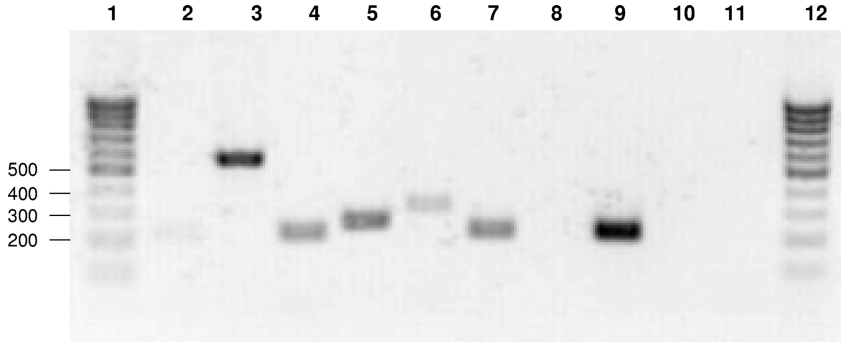

FIG. 5. Agarose gel electrophoresis of B14905 DNA PCR amplicons. Lanes 1 and 12, Fisher 100-bp DNA standard; lanes 2 through 6, host chromosomal DNA (lane 2, ΦB05-1 primer set; lane 3, ΦB05-2 primer set; lane 4, ΦB05-3 primer set; lane 5, ΦB05-4 primer set; lane 6, host primer set); lanes 7 through 11, CsCl phage lysate DNA (lane 7, ΦB05-1 primer set; lane 8, ΦB05-2 primer set; lane 9, ΦB05-3 primer set; lane 10, ΦB05-4 primer set; lane 11, host primer set). Values at left are sizes in bp. Primer sequences are in given in Table 2.

TABLE 5. Selected open reading frames of the ΦB05-2 genome and BLASTP hits

ORF no./orientation[a]	Predicted function	Related BLASTP hit(s)[b]
1/−		Hypothetical protein [*Bacillus weihenstephanensis*] YP_001645941 (4e−111)
		Hypothetical protein [*Lysinibacillus sphaericus* C3-41] YP_001696259 (2e−101)
		Putative YbdG [*Bacillus subtilis* subsp. *subtilis* strain 168] CAB11993 (2e−24)
2/+		Hypothetical protein [*Macrococcus caseolyticus* JCSC5402] YP_002560312 (3e−11)
3/+		Hypothetical protein [*Macrococcus caseolyticus* JCSC5402] YP_002560313 (3e−12)
		Hypothetical protein [*Streptococcus pyogenes* phage 315.3] NP_795502 (3e−8)
4/+		Hypothetical protein [*Macrococcus caseolyticus* JCSC5402] YP_002560313 (2e−32)
		Prophage pi1 protein 12 [*Lactobacillus casei* BL23] YP_001987248 (5e−19)
		Hypothetical protein [bacteriophage EJ-1] NP_945261 (6e−10)
5/+	Phage replication	DnaA analog, DnaD domain protein [*Bacillus cereus* H3081.97] ZP_03235995 (2e−46)
		DNA replication protein [*Bacillus* phage Fah] YP_512343 (2e−41)
		Phage replication protein [*Lysinibacillus sphaericus* C3-41] YP_001697499 (2e−21)
6/+		Phage replication protein [*Lysinibacillus sphaericus* C3-41] YP_00167499 (3e−54)
		Bacteriophage-related protein [*Geobacillus kaustophilus* HTA426] YP_146356 (1e−4)
7/+	ssDNA binding protein[c]	ssDNA binding protein [*Lysinibacillus sphaericus* C3-41] YP_001700342 (4e−33)
		ssDNA binding protein [*Listeria* phage A118] NP_463525 (1e−21)
		ssDNA binding protein [*Staphylococcus aureus* phage PVL] NP_058484 (7e−18)
8/−		Hypothetical protein with collagen triple-helix repeat domain [*Bacillus thuringiensis* serovar *israelensis* ATCC 35646] ZP_00741314 (1e−33)
		Hypothetical protein with collagen triple-helix repeat domain [bacteriophage BC6A52] NP_852575 (2e−8)
9/−		Collagen triple-helix repeat protein [*Bacillus cereus* ATCC 14579] NP_832393 (2e−39)
		Triple-helix repeat-containing collagen [*Bacillus weihenstephanensis* KBAB4] YP_00164372.1 (5e−28)
11/−		Hypothetical protein [*Lysinibacillus sphaericus* C3-41] YP_001698814 (4e−86)
		Hypothetical protein [*Bacillus* sp. NRRL B-14911] ZP_01169056 (5e−60)
		YvdQ [*Bacillus subtilis* subsp. *subtilis* strain 168] CAB15456 (3e−54)
12/−		Hypothetical protein [*Lysinibacillus sphaericus* C3-41] YP_001699285 (4e−12)
13/+		Hypothetical protein [*Lysinibacillus sphaericus* C3-41] YP_001698771 (4e−24)
		YqaQ [*Bacillus subtilis* subsp. *subtilis* strain 168] NP_390499 (2e−9)
16/+		Hypothetical protein [*Lysinibacillus sphaericus* C3-41] YP_001698762 (3e−9)
18/+	Terminase small subunit	Terminase small subunit [*Bacillus clarkii* bacteriophage BCJA1c] YP_164411(1e−51)
		Terminase small subunit [*Bacillus* phage IEBH] YP_002154373 (1e−23)
		Putative terminase small subunit [*Lactobacillus johnsonii* prophage Lj965] NP_958578 (1e−21)
19/+	Terminase large subunit	TerL [*Listeria* phage B054] NP_471068706 (2e−92)
		Terminase large subunit [*Haemophilus* phage Aaphi23] NP_852753 (3e−31)
		TerL [*Lactococcus lactis* bacteriophage TP901-1] NP_112694 (4e−16)
21/+		Hypothetical protein [*Enterococcus faecalis* V583] NP_815182 (4e−55)
		Putative head protein [iodobacteriophage ϕPLPe] YP_002128461 (1e−9)
		Hypothetical protein JK_31 [bacteriophage JK06] YP_277469 (5e−8)
		gp26 [*Burkholderia cenocepacia* phage BcepB1A] YP_024873 (1e−7)
24/−	Transposase	Transposase [*Bacillus* sp. SG-1] ZP_01860268 (1e−180)
		Phage-associated cell wall hydrolase [*Streptococcus pyogenes* MGAS10394] YP_060515 (1e−122)

[a] +, forward orientation; −, reverse orientation. Those ORFs with no significant similarity by BLASTP search are not included.
[b] Accession numbers are from the GenBank database.
[c] ssDNA, single-stranded DNA.

It is also possible (but relatively rare) for phages to lysogenize a host without producing a lytic infection. Isolate B14912 may have a pseudotemperate phage, since the prophage was not inducible with mitomycin C but the strain displayed a high level of spontaneous prophage induction (50). Either the three *Bacillus* isolates that did not produce significant levels of phage do not contain a temperate phage or the prophages are not mitomycin C inducible. The absence of prophage has been confirmed from *in silico* investigation of the draft genome of B14911 (GenBank accession number AAOX00000000). The range of prophage induction in these *Bacillus* isolates indicates that the phages in these *Bacillus* isolates have different relationships with their hosts.

Isolates B14905, B14906, B14910, and B14850 had significant prophage induction and high levels of sporulation (Fig. 3; Table 3). It is possible that these isolates may contain temperate phages that enhance host sporulation, such as temperate phages PMB12, SP10, and Φ3226 (46, 53). Prophage-encoded transcriptional factors may be the mechanism for sporulation enhancement in our *Bacillus* isolates, as was seen for *Clostridium* phage Φ3226 (53).

The lysogen B14905, which produced high levels of spores under decoyinine induction, was selected for sequencing in order to investigate the influence of prophage on sporulation. Sequencing of a mitomycin C-induced lysate resulted in a single phage genome, ΦB05-1. Parallel sequencing of the B14905 chromosomal DNA confirmed the presence of ΦB05-1 as well as three additional prophage-like regions. Genomic analysis of these four regions along with transmission electron microscopy and PCR analysis was performed in order to elucidate which of these regions were inducible prophages.

The genome sizes of the four prophages, ranging from 17,991 bp to 25,898 bp, were much smaller than the sizes of typical tailed prophages (1, 10). Smaller phage genome sizes

TABLE 6. Selected open reading frames of the ΦB05-3 genome and BLASTP hits

ORF no./orientation[a]	Predicted function	Related BLASTP hit(s)[b]
1/−	Integrase	Integrase [*Geobacillus thermodenitrificans* NG80-2] YP_00112487 (5e−27)
		Integrase [*Listeria* phage A500] YP_001468416 (7e−22)
		Integrase [*Mycobacterium* phage Ms6] AAD03774 (9e−13)
2/−		Phage-like element PBSX protein [*Lysinibacillus sphaericus* C3-41] YP_001697496 (1e−24)
		XkdA [*Bacillus subtilis* subsp. *subtilis* strain 168] CAB13107 (2e−21)
3/−	Transcriptional regulator	Immunity repressor protein [*Bacillus cereus* G9842] YP_002447360 (2e−17)
		Immunity repressor protein [*Geobacillus* phage GBSV1] YP_764504 (8e−13)
		Transcriptional regulator [bacteriophage BC6A51] NP_852484 (3e−5)
4/+		Hypothetical protein [*Listeria innocua* Clip11262] NP_471097 (2e−12)
		Prophage DNA-binding protein [*Bacillus anthracis* strain Ames] NP_846364 (5e−9)
7/+	Antirepressor	Antirepressor [*Bifidobacterium bifidum* NCIMB 41171] ZP_03646310 (6e−36)
		Antirepressor [*Staphylococcus aureus* prophage ϕPV83] NP_061599 (3e−26)
		KilA [*Enterobacterium* phage P1] YP_006517 (5e−14)
9/+		Hypothetical protein gp52 [*Geobacillus* phage GBSV1] YP_764508 (1e−9)
10/+		Phage-related protein [*Geobacillus kaustophilus* HTA426] YP_146359 (9e−25)
		Hypothetical protein [*Bacillus licheniformis* ATCC 14580] YP_078625 (1e−11)
14/+		Hypothetical protein [*Macrococcus caseolyticus* JCSC5402] YP_002560312 (1e−104)
		Hypothetical protein [*Paenibacillus larvae* subsp. *larvae* BRL-230010] ZP_02327835 (1e−71)
15/+		Hypothetical protein [*Macrococcus caseolyticus* JCSC5402] YP_002560313 (1e−66)
		ORF 22 [*Streptococcus* phage EJ-1] NP_945261 (3e−31)
16/+		Hypothetical protein [*Geobacillus* sp. Y412MC61] ZP_03558107 (2e−6)
17/+		Hypothetical protein [*Xanthomonas* phage Xp15] YP_239311 (8e−17)
		ORF 205 [*Enterococcus* phage ϕEF24C] YP_001504314 (7e−10)
		ORF 23 [*Pseudomonas aeruginosa* phage F116] YP_164287 (2e−7)
18/+		gp47 [bacteriophage ϕ1026b] NP_945078 (8e−25)
		gp42 [bacteriophage ϕE125] NP_536399 (3e−24)
19/+		gp19 [*Bacillus clarkii* bacteriophage BCJA1c] YP_164397 (1e−5)
20/+	Replication	Replisome protein [*Bacillus* phage gamma] YP_338215 (7e−47)
		Phage replication protein [*Lysinibacillus sphaericus* C3-41] YP_001697499 (3e−29)
		Replication protein [*Geobacillus* virus E2] YP_001285845 (4e−26)
21/+	Replication	Phage replication protein DnaC [*Bacillus anthracis* strain Ames] AAP27845 (8e−69)
		DNA replication protein DnaC [*Bacillus* phage IEBH] YP_002154334 (9e−68)
		DNA replication protein [*Staphylococcus* phage ϕPVL108] BAF41172 (1e−16)
27/+		Hypothetical protein [*Rhodopseudomonas palustris* CGA009] NP_947632 (2e−40)
28/+		Hypothetical protein [*Lysinibacillus sphaericus* C3-41] YP_001698771 (1e−12)
29/+		Hypothetical protein [*Bacillus pumilus* ATCC 7061] ZP_03055888 (2e−8)
34/+		Hypothetical protein [*Clostridium thermocellum* ATCC 27405] YP_001038709 (1e−21)
35/+	Terminase small subunit	Hypothetical protein [*Macrococcus caseolyticus* JCSC5402] YP_002560362 (2e−43)
		Terminase small subunit [*Clostridium* phage ϕC2] YP_001110719 (4e−20)
		Terminase small subunit [*Bacillus clarkii* phage BCJA1c] YP_164411 (3e−11)
36/+	Terminase large subunit	Terminase large subunit [*Bacillus licheniformis* ATCC 14580] YP_078647 (0)
		PBSX terminase [*Bacillus subtilis*] CAA94059 (4e−163)
		Terminase large subunit [*Staphylococcus* phage ϕETA2] YP_001004302 (4e−39)
		Terminase large subunit [*Clostridium* phage ϕC2] YP_001110720 (2e−22)
37/+	Portal	Hypothetical protein [*Lysinibacillus sphaericus* C3-41] YP_001698759 (2e−68)
		Portal protein [*Staphylococcus aureus* phage ϕNM4] ABF73268(9e−44)
		Portal protein [*Staphylococcus* phage ϕETA3] YP_001004371 (4e−43)
39/+		Phage-related protein [*Anoxybacillus flavithermus* WK1] YP_002315042 (3e−98)
		ORF 385 [bacteriophage ϕ LC3] NP_996717 (1e−16)
41/−	Holin	Phage-related lysis protein [*Geobacillus* phage GBSV1] YP_764490 (1e−14)
		Holin [*Bacillus anthracis* phage gamma] YP_338199 (2e−11)
42	Lysis protein	XlyB [*Bacillus cereus* B4264] YP_002368321 (1e−54)
		N-Acetylmuramoyl-L-alanine amidase [*Bacillus* phage gamma] YP_338200 (3e−51)
		ORF 46 [*Bacillus* phage ϕ105] NP_690779 (4e−24)
43	Transposase	Recombinase [*Bacillus halodurans* C-125] NP_241548 (0)
		Transposase [*Staphylococcus* phage ϕPVL108] YP_918946 (3e−163)

[a] +, forward orientation; −, reverse orientation. Those ORFs with no significant similarity by BLASTP search are not included.
[b] Accession numbers are from the GenBank database.

are usually associated with lytic phages, defective phages, or phage remnants (26). However, a survey of the genomes of 113 marine bacterial isolates indicated that most prophage regions were under 30 kb (37). Gene content, although it can be related to size, is a more important consideration for determining prophages. The genomes of ΦB05-1, ΦB05-3, and ΦB05-4 contained integrases and phage repressor proteins, while that of ΦB05-2 contained only phage replication, terminase, and transposase genes (Fig. 4). Since ΦB05-2 was not amplified by PCR from the mitomycin C-induced phage DNA, we believe this region to be a prophage remnant. Prophage remnants, which can contain functional genes, are common

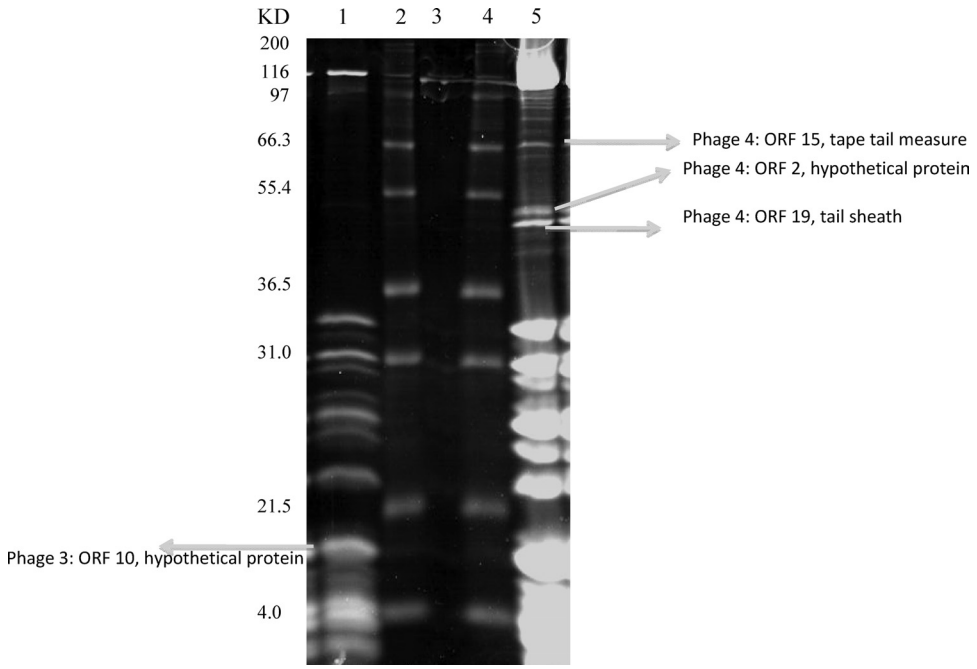

FIG. 6. SDS-PAGE of proteins associated with B14905 lysates. The molecular mass standard is shown in lanes 2 and 4, with sizes in kilodaltons given on the left. Lane 1, CsCl-purified preparation; lane 5, polyethylene glycol-precipitated preparation.

in bacterial genomes and are believed to be a result of decay processes (11).

ΦB05-4 was the only putative prophage of B14905 that contained several identifiable structural genes. These ORFs were similar to tail genes from the temperate myoviruses from Gram-positive bacteria, ϕC2 and EJ-1, and to those of the *B. subtilis* defective phage PBSX. The presence of lysogeny-like genes, along with the absence of any replicative or packaging genes, on ΦB05-4 implies that this segment may encode a defective phage. Defective phages can be mitomycin C inducible, and some are able to form phage particles that have bactericidal activity but are noninfectious (17). In the case of PBSX, random 13-kb portions of the host chromosomal genome are packaged but are not injected into other host cells (3, 51). In this respect, ΦB05-4 may be similar to PBSX since it did not produce a PCR amplicon in the induced phage DNA. A notable difference between the two phages is the lack of identifiable terminase, capsid, and packaging proteins in ΦB05-4. In PBSX, this group of genes, which is about 6,000 bp in length, is located between the *xre* repressor and tail genes (23); this region is not seen in the gene map of ΦB05-4 (Fig. 4). Given the absence of injectable phage DNA and the lack of capsid proteins, ΦB05-4 may be a tail-like bacteriocin. Bacteriocins are usually proteinaceous particles that have bactericidal activity toward closely related strains (13). Some high-molecular-weight bacteriocins resemble phage tails, including the F- and R-type pyocins of *Pseudomonas aeruginosa*, carotovoricin Er from *Erwinia carotovora*, and a bacteriocin from *Bacillus azotofixans* (32, 33, 42). When B14905 mitomycin C-induced lysates were examined by TEM, many myovirus-like tail particles were observed (Fig. 7D to F). The tail tape measure and tail sheath proteins were also expressed in a phage lysate (Fig. 7). Based on the evidence from our experiments, we believe that ΦB05-4 is a tail-like bacteriocin, although additional experiments to identify *in situ* production of tail proteins in an induced lysate are needed to confirm this.

ΦB05-1 and ΦB05-3 are inducible temperate phages of isolate B14905, based on their amplification from induced phage DNA. Given the lack of identifiable structural genes in these two prophages, it was not possible to identify these phages with the tailed phage structures observed by TEM examination of the lysates. Besides the presence of a lysogeny module, the genomic architectures of these two prophages differ substantially. The genome of ΦB05-1 is smaller and does not have the functional modules that are associated with tailed prophages. An interesting feature of the ΦB05-1 phage genome is the presence of four transcriptional regulators. The one encoded by ORF 2 was not similar to phage-encoded gene products but to the Xre-like DNA binding domains in other *Bacillus* transition-state regulators. These transcriptional regulators, which include AbrB and SinR, redirect cellular metabolic activity to use the available source and regulate the expression of sporulation genes during the beginning of the stationary phase (43, 48). SinR specifically inhibits transcription of *spo0A* and stage II sporulation genes until sufficient levels of phosphorylated Spo0A accumulate. Possession of transition-state regulators by prophages may provide an additional level of sporulation control in marine *Bacillus* isolates. The lack of similarity of the N terminus of ORF 2 to any known sequences may indicate that this gene is a novel transcriptional regulator. Further experiments are needed to assess the function of this potential prophage-encoded transcriptional regulator in B14905 sporulation.

ORF 17 is another transcriptional regulator found on the genome of ΦB05-1. This gene is located upstream from two putative choloylglycine hydrolase genes; all three genes are similar to those found in two *B. cereus* genomes (16). Choloyl-

TABLE 7. Selected open reading frames of the ΦB05-4 genome and BLASTP hits

ORF no./orientation[a]	Predicted function	Related BLASTP hit(s)[b]
1/−		Hypothetical protein [*Lysinibacillus sphaericus* C3-41] YP_001697519 (5e−28)
		Hypothetical protein [*Bacillus* sp. NRRL B-14911] ZP_01171046 (3e−6)
2/−		Hypothetical protein [*Lysinibacillus sphaericus* C3-41] YP_001697518 (0)
3/−	Hydrolase	Cell wall hydrolase [*Lysinibacillus sphaericus* C3-41] YP_001697517 (2e−136)
		Lysin [*Lactobacillus* phage A2] NP_680500 (1e−25)
		N-Acetylmuramoyl-L-alanine amidase [*Listeria* phage A511] YP_001468459 (2e−39)
		N-Acetylmuramoyl-L-alanine amidase [*Streptococcus* phage EJ-1] NP_945312 (5e−20)
4/−	Holin	Hypothetical protein [*Lysinibacillus sphaericus* C3-41] YP_001697516 (6e−40)
		Holin [*Clostridium* phage φC2] YP_001110752 (3e−12)
		Holin [*Clostridium* phage φCD27] YP_002290909 (1e−11)
5/−		Hypothetical protein [*Lysinibacillus sphaericus* C3-41] YP_001697515 (1e−38)
		Hypothetical protein [*Bacillus anthracis* phage lambda Ba01] AAP24508 (2e−6)
7/−		Hypothetical protein [*Lysinibacillus sphaericus* C3-41] YP_00169514 (6e−73)
8/−	Tail fiber	Hypothetical protein [*Lysinibacillus sphaericus* C3-41] YP_00169513 (0)
		Phage variable tail fiber protein [*Clostridium botulinum*] YP_001921094 (3e−11)
9/−		Hypothetical protein [*Lysinibacillus sphaericus* C3-41] YP_001697512 (4e−96)
		ORF 26 [*Clostridium* phage φC2] YP_001110743 (7e−17)
		ORF 62 [*Streptococcus* phage EJ-1] NP_945301 (2e−9)
		XkdU [*Bacillus subtilis*] CAB13131 (1e−5)
10/−	Baseplate	Hypothetical protein [*Lysinibacillus sphaericus* C3-41] YP_001697511 (0)
		Baseplate J-like protein [*Clostridium phytofermentans* ISDg] ZP_01354786 (1e−88)
		ORF 59 [*Clostridium* phage φC2] YP_001110742 (6e−83)
		Baseplate tail protein [*Streptococcus* phage EJ-1] NP_945300 (1e−42)
		XkdT [*Bacillus subtilis*] CAA94041 (1e−33)
11/−		Hypothetical protein [*Lysinibacillus sphaericus* C3-41] YP_001697510 (5e−70)
		ORF 60 [*Streptococcus* phage EJ-1] NP_945299 (3e−13)
		XkdS [*Bacillus subtilis*] CAA94040 (6e−9)
12/−		PBSX-related protein [*Lysinibacillus sphaericus* C3-41] YP_001697509 (1e−44)
13/−	Hydrolase	Hypothetical protein [*Lysinibacillus sphaericus* C3-41] YP_001697508 (1e−174)
		Putative hydrolase [*Clostridium* phage φC2] YP_001110740 (7e−74)
		ORF 58 [bacteriophage EJ-1] NP_945297 (7e−30)
		XkdQ [*Bacillus subtilis*] CAA94050 (2e−22)
14/−	Lysin	Hypothetical protein [*Lysinibacillus sphaericus* C3-41] YP_001697507 (9e−108)
		Putative LysM [*Clostridium* phage φC2] YP_001110739 (6e−41)
		XkdP [*Bacillus subtilis*] CAA94038 (3e−15)
15/−	Tail tape measure	Putative tail tape measure protein [*Lysinibacillus sphaericus* C3-41] YP_001697506 (0)
		Putative tail tape measure protein [*Clostridium* phage φC2] YP_001110738 (2e−44)
		Tail length tape measure protein [*Streptococcus* phage EJ-1] NP_94529 (2e−23)
17/−		XkdN1 [*Lysinibacillus sphaericus* C3-41] YP_001697505 (2e−78)
		ORF 16 [*Clostridium* phage φC2] YP_001110734 (1e−28)
		XkdN [*Bacillus subtilis*] CAA94036 (2e−9)
18/−	Tail core	Hypothetical protein [*Lysinibacillus sphaericus* C3-41] YP_001697504 (1e−196)
		Putative core tail [*Clostridium* phage φC2] YP_001110732 (4e−50)
		Core tail protein [*Streptococcus* phage EJ-1] NP_945293 (1e−24)
		XkdM [*Bacillus subtilis*] CAA94035 (5e−23)
19/−	Tail sheath	PBSX-like protein [*Lysinibacillus sphaericus* C3-41] YP_001697503 (0)
		Putative sheath tail protein [*Clostridium* phage φC2] YP_001110731 (5e−131)
		Sheath tail protein [*Streptococcus* phage EJ-1] NP_945292 (7e−51)
		XkdK [*Bacillus subtilis*] CAA94066 (7e−22)
20/−		Hypothetical protein [*Lysinibacillus sphaericus* C3-41] YP_001697502 (1e−17)
21/−		Hypothetical protein [*Lysinibacillus sphaericus* C3-41] YP_001697501 (1e−73)
		ORF 12 [*Clostridium* phage φC2] YP_001110730 (7e−8)
22/−		Hypothetical protein [*Lysinibacillus sphaericus* C3-41] YP_001697500 (1e−51)
23/+	Phage repressor	Phage-related transcriptional regulator [*Lysinibacillus sphaericus* C3-41] YP_001697497 (7e−63)
		Xre [*Bacillus subtilis* subsp. *subtilis* strain 168] NP_389133 (6e−10)
		Xre-like repressor [*Clostridium* phage φCD119] YP_529596 (2e−8)
24/+	Integrase	PBSX-like protein [*Lysinibacillus sphaericus* C3-41] YP_001697496 (3e−87)
		XkdA [*Bacillus subtilis*] CAA94051 (1e−17)

[a] +, forward orientation; −, reverse orientation. Those ORFs with no significant similarity by BLASTP search are not included.
[b] Accession numbers are from the GenBank database.

glycine hydrolases are bacterial proteins that degrade bile salts in the mammalian intestine (5). Since *B. cereus* is an opportunistic intestinal pathogen, these genes might serve as a survival mechanism for this bacterium. The presence of the genes in an inducible prophage of a marine *Bacillus* isolate might be indicative of a horizontal gene transfer event mediated by transduction.

The genome of ΦB05-1 also contained two other ORFs that

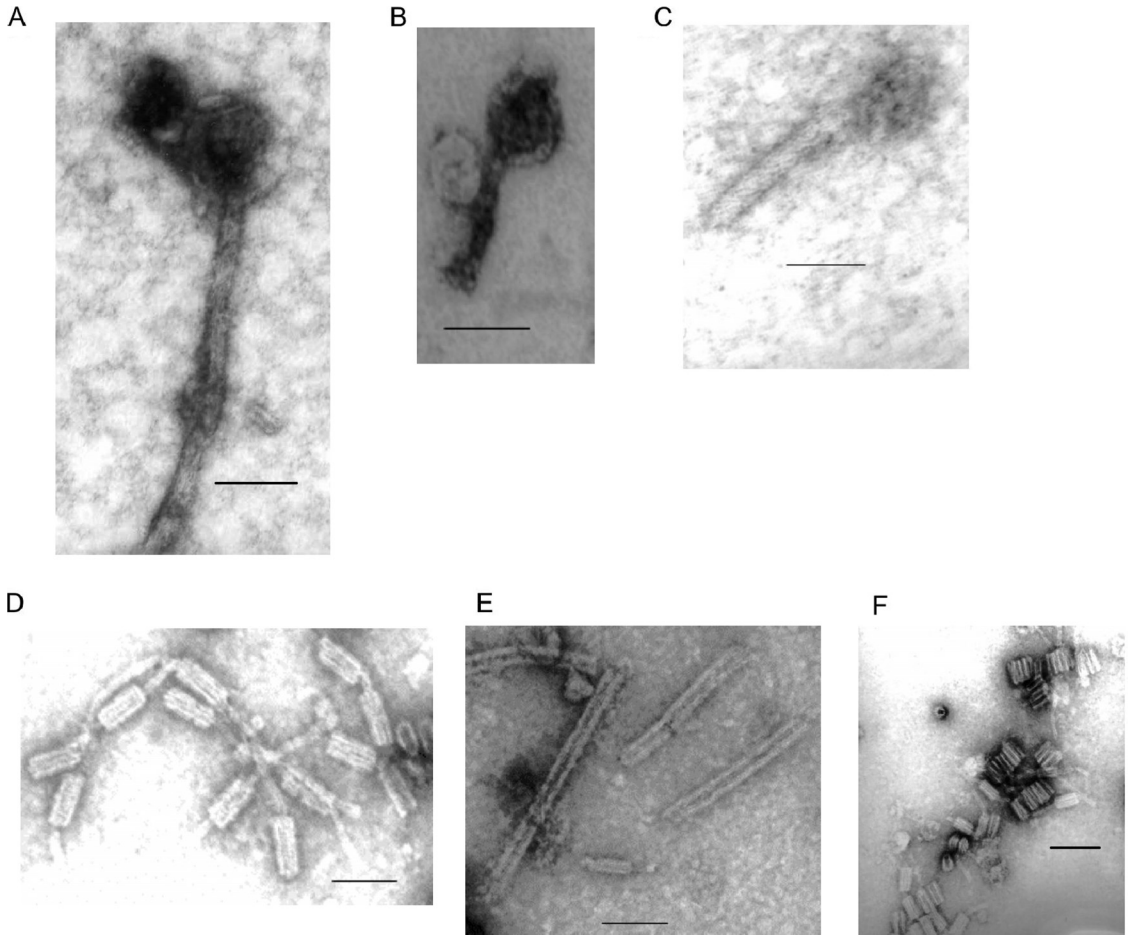

FIG. 7. TEM micrographs of B14905 induced phage lysates. (A, B, E, and F) Magnification, ×100,000. Bar, 100 nm. (C and D) Magnification, ×150,000. Bar, 50 nm.

had weak hits to transcriptional regulators found in bacterial genomes. It is possible that ORF 3 is involved in lytic gene expression, given that its location and orientation are similar to those of cro repressors in other temperate phages (9). Given its similarity to experimentally characterized protein LlaI, ORF 20 may be involved in phage-encoded resistance. The location of this ORF in the genome is similar to those of methyltransferase proteins found in lytic DNA modification modules encoded by other temperate phages, such as VHML and N15 (34, 41). The variety of transcriptional regulators in ΦB05-1 may be indicative of the role of these proteins in regulating host and phage functions. For example, Chen et al. (12) clearly showed that the *c*I repressor of coliphage λ not only bound to the operator regions of the lytic λ genes but also could bind to the operators of host metabolic genes. There were multiple *c*I binding sites in the operator of the host gene *pckA* (phosphoenol carboxykinase), the first gene involved in gluconeogenesis. Downregulating metabolically expensive or wasteful pathways could provide lysogens an advantage during starvation survival, as occurs in most of the oligotrophic oceans (37).

The genome of ΦB05-3 is the largest of the prophage-like regions of B14905 and is the closest in genome content to that of a classic tailed phage (11). Genes involved in phage replication, phage particle assembly, and lysis are similar to those found on temperate phages. Even though the genomic and PCR evidence suggests that ΦB05-3 is an inducible prophage, the genome of this phage was not recovered by sequencing of an induced phage lysate as ΦB05-1 was. It is possible that this phage was induced at a low copy number relative to that of ΦB05-1, so that it was at too low of a concentration to be sequenced. Further functional studies are needed to determine the relationship this phage has with the host strain.

The combination of *in silico* analysis of prophage in the B14905 genome and *in vivo* molecular studies of the induced phage lysate provided information about host-phage interactions that would not be possible using either approach alone. The results from this study indicate that polylysogeny occurs in marine *Bacillus* strains, as has been observed for terrestrial strains (17, 23). The diversity of the two inducible prophage and two prophage-like elements found on this single bacterial genome supports metagenomic studies that have determined that the diversity of phages in the ocean is vast (4). This work provides further suggestive evidence that prophages and hosts have coevolved advantageous adaptations to survive under adverse conditions.

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