

GIL16, a New Gram-Positive Tectiviral Phage Related to the *Bacillus thuringiensis* GIL01 and the *Bacillus cereus* pBClin15 Elements

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One of the most notable characteristics of *Tectiviridae* resides in their double-layer coats: the double-stranded DNA is located within a flexible lipoprotein vesicle covered by a rigid protein capsid. Despite their apparent rarity, tectiviruses have an extremely wide distribution compared to other phage groups. Members of this family have been found to infect gram-negative (PRD1 and relatives) as well as gram-positive (Bam35, GIL01, AP50, and ϕ NS11) hosts. Several reports have shown that tectiviruses infecting gram-negative bacteria are closely related, whereas no information is currently available on the genetic relationship among those infecting gram-positive bacteria. The present study reports the sequence of GIL16, a new isolate originating from *Bacillus thuringiensis*, and a genetic comparison of this isolate with the tectiviral bacteriophages Bam35 and GIL01, which originated from *B. thuringiensis* serovars Alesti and Israelensis, respectively. In contrast to PRD1 and its relatives, these are temperate bacteriophages existing as autonomous linear prophages within the host cell. Mutations in a particular motif in both the GIL01 and GIL16 phages are also shown to correlate with a switch to the lytic cycle. Interestingly, both bacterial viruses displayed narrow, yet slightly different, host spectrums. We also explore the hypothesis that pBClin15, a linear plasmid hosted by the *Bacillus cereus* reference strain ATCC 14579, is also a prophage. Sequencing of its inverted repeats at both extremities and a comparison with GIL01 and GIL16 emphasize its relationship to the *Tectiviridae*.

The bacteriophage GIL01 has a linear double-stranded DNA genome of 14,931 bp delineated by imperfect 73-bp inverted repeats and protected by proteins at its 5' ends. GIL01 is a temperate bacteriophage that was isolated from *Bacillus thuringiensis* serovar Israelensis whose prophage form, designated pGIL01, resides in the host cell as an autonomous linear plasmid without any apparent integration into the host chromosome. It has been suggested that GIL01 belongs to the family *Tectiviridae* (28), whose members are characterized by the presence of an internal lipid membrane. While tectiviruses infecting gram-negative bacteria have been extensively studied at the genetic level, those infecting gram-positive bacteria remain less well characterized. PRD1, for instance, is the family model and infects bacteria harboring conjugative plasmids of the P, N, or W incompatibility groups, such as *Escherichia coli* and *Salmonella enterica*. These plasmids encode the phage receptor but are not otherwise involved in the virus life cycle (22). Five other PRD1-like viruses have been isolated from different parts of the world (PR3 [5], PR4 [26], PR5 [30], PR772 [8], and L17 [4]), and it has been shown that their genomic sequences share approximately 98% identity (21).

In addition to GIL01, three other tectiviruses that infect gram-positive bacteria have been identified so far. The bacteriophages AP50 and ϕ NS11, isolated from *Bacillus anthracis*

(19) and *Bacillus acidocaldarius* (23, 24), respectively, have only been morphologically characterized. Bam35 was isolated from *B. thuringiensis* serovar Alesti in 1978 (1) and was recently sequenced (21), revealing that it differs from GIL01 by 11 bp. A previous distribution analysis of similar prophages among the *B. cereus* group had unveiled the presence of a GIL01-related molecule in *B. thuringiensis* strain B16. PCR and restriction analyses performed with this element, named GIL16, indicated that it differs from GIL01 (28). In order to further analyze this isolate, we sequenced GIL16, and we report its sequence in this study. A genome comparison effectively showed a high degree of conservation with GIL01. We also demonstrate here that both phages have an extremely narrow host range and that GIL16 displays a slightly different spectrum than that of GIL01.

Furthermore, recent genome sequencing of the *Bacillus cereus* reference strain ATCC 14579 revealed the sequence of pBClin15 (11), a 15.1-kb linear plasmid that was previously detected in total DNA preparations from this bacterium (7). Although no experimental evidence is currently available regarding its nature, its genetic organization, similar to that of GIL01 and GIL16, strongly supports the hypothesis that it is a prophage. Moreover, we report the existence of unresolved inverted terminal repeats (ITRs), the common end structure of linear plasmids and bacteriophages.

Considering that all gram-negative tectiviruses identified so far share a very high level of sequence identity, it seemed interesting to investigate whether this close relationship could also be found among members that infect gram-positive bacteria. The present study reports a comparison at the genetic level of three tectiviral bacteriophages, GIL16, GIL01, and Bam35, and of the pBClin15 plasmid.

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MATERIALS AND METHODS

Bacterial strains and phages. The *B. thuringiensis* serovar *Thuringiensis* HER1410 and *Bacillus megaterium* serovar *Tiberius* HER1047 strains were kindly provided by H.-W. Ackermann (Felix d'Herelle Reference Center for Bacterial Viruses, Laval University, Quebec, Canada). Note that molecular and morphological evidence enabled us to classify the *B. megaterium* strain as a member of the *B. cereus* sensu lato group (see Results). The bacteriophages GIL01 and GIL16 were isolated from *B. thuringiensis* serovar *Israelensis* strain AND508, a mutant of the serotype H14 commercial strain NB31 (2), and from *B. thuringiensis* strain B16, originally described as a *B. cereus* strain (S. Kasatiya, Ottawa Public Health Laboratory, Ottawa, Canada), respectively. Phage propagation was performed on the GIL01- and GIL16-receptive *B. thuringiensis* serovar *Israelensis* strain GBJ002 (13). The linear plasmid pBclin15 was extracted from the *B. cereus* reference strain ATCC 14579. The *Bacillus* strains were grown in Luria-Bertani (LB) medium (25) at 30°C with moderate agitation.

Phage propagation. Phage stocks were obtained by centrifuging lysogenic cell cultures at $2,500 \times g$ for 15 min and then filtering the supernatants (0.45- μ m-pore-size filter). Exponentially growing cell cultures (200 μ l) were infected with 200 μ l of a series of diluted (1:10 to 1:100) phage stocks, and the mixtures were incubated at room temperature for 30 min. The phage-bacterium mixtures were plated with molten top agar (0.7%) onto nutrient or Trypticase soy broth plates and incubated at 30°C overnight. Single turbid plaques were used for PCR screening. Clear plaques were picked, and pure lines were obtained by at least three consecutive single plaque purification steps. Clear plaque (cp) variants were used to make lytic stocks by collecting the soft agar (0.3%) from semiconfluent plates. Cell debris was removed by centrifugation ($2,500 \times g$, 15 min). The supernatants were collected for use in phage DNA extractions and PCRs.

DNA manipulation and sequencing strategy. A clear plaque mutant designated GIL16c was isolated from the centers of plaques of *B. thuringiensis* serovar *Israelensis* GBJ002 infected with GIL16. After three runs of single-colony isolations, the phage GIL16c DNA was extracted as previously described for phage GIL01 (28). Two PCR products, of 500 and 2,500 bp, obtained by the use of primer pairs specific for GIL01, were first cloned into the positive selection vector pCR4-TOPO (Invitrogen) and then were sequenced. The remaining sequences of GIL16c were obtained by direct primer walking sequencing of the phage DNA. pBclin15 was isolated by pulsed-field gel electrophoresis (PFGE) of strain ATCC 14579 chromosomal DNA and was extracted by use of a QIAquick gel extraction kit (QIAGEN). Subsequent DNA purification enabled direct sequencing of the extremities by use of the pBclin15-specific primers pBclin-L (5'-CATGCTATGTCATGTTTTCAG) and pBclin-R (5'-CGTTAGAGAAGT TGACAGGTG). DNA purifications were performed with a QIAquick PCR purification kit (QIAGEN).

PFGE of genomic DNA. Chromosomal DNAs were prepared according to the method of Léonard et al. (15), with the exception that the LMP (GIBCO-BRL) agarose-embedded DNA plugs were not digested. PFGE was performed with the CHEF-DR II system (Bio-Rad) at 14°C and 4.5 V cm^{-1} in a 1.0% agarose (Sigma) gel in 0.5 $\times$ TBE (45 mM Tris-borate, 1 mM EDTA). Electrophoresis was performed for 20 h, with the pulse divided into two phases, from 16 to 19 s for 10 h and from 50 to 55 s for 10 h. After being stained with an ethidium bromide solution (1 μ g ml^{-1}), the gel was analyzed with the BioCaptMW, version 99.053, system.

Electron microscopy analysis. Following exposition to UV light (254 nm for 10 s) and incubation at 30°C for 4 h, bacterial debris was cleared by centrifugation. The supernatant, containing viral particles, was filtered through a 0.45- μ m-pore-size filter and then precipitated with a polyethylene glycol 6000 (10%) and NaCl (5 mM) solution. The phage pellet was then resuspended in Tris-HCl (pH 7.5), deposited on carbon-coated Formvar grids, stained with 1% potassium phosphotungstate (pH 7.2), and analyzed under an electron microscope (JEOL) at 80 kV.

Curing experiments. The curing of strain ATCC 14579 was achieved by heat treatment. The strain was initially grown at 30°C in LB medium for a few cycles. This culture was then shifted to 44°C by use of a 1% (vol/vol) inoculum. Growth at this temperature was repeated every 4 h for 2 days with fresh medium, and upon transfer, samples were taken, diluted, plated onto LB medium, and incubated at 30°C for 12 h. Single colonies were isolated and subjected to molecular and plasmid profile analyses by PCR and PFGE, respectively.

PCR amplification. Phage plaques, or bacterial lawns for the negative control, were recovered from titration plates and diluted in 100 μ l of 0.9% NaCl. PCRs were then performed with 1 μ l of these samples by use of the GIL01-specific primers Lex1 (5'-GGATCATGTTGACGCCAAGGG) and R2GIL01 (5'-AAGCTTCAGTCATCCTTCTTCCC) or by use of Lex1 and the GIL16-specific primer R2GIL16 (5'-AAGCTTCTAGTCCTTTTCCGCATTTC). Cell lines



FIG. 1. Electron micrograph of GIL16 particles with a tail-like structure. Bar, 50 nm.

that were cured of pBclin15 were screened by the use of pBclin1 (5'-GTCAA AACATGACATAGCATGC) and pBclin2 (5'-CTACCGCGATTTCGTTTACC). The cereolysin O gene (1,146 bp) was amplified with the primer pair Hem4.1 (5'-ACGTCACCAGTNGATATWTC) and Hem4.2 (5'-TCTCCACCA TTCCCAWGCAAG).

Computational analyses. DNA and protein sequences were analyzed with the EMBOSS package at the Belgian EMBL Node and with the Accelrys DS, version 1.5, gene program. Possible homologies to known proteins were searched with PSI-BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) and ACLAME (<http://aclame.ulb.ac.be>). The Bam35 genomic sequence was obtained with accession number AY257527, and pBclin15 original and recently updated sequences were obtained with the accession numbers NC_004721 and AE016878, respectively.

Nucleotide sequence accession number. The GIL16c nucleotide sequence has been deposited under GenBank accession number AY701338.

RESULTS AND DISCUSSION

GIL16 is morphologically similar to other tectiviral phages.

A dot blot analysis revealed the presence of molecules related to GIL01 in five strains of the *B. cereus* group (B16, B23, DBT012, BGSC4D14, and Bt5), and a subsequent PCR analysis using GIL01-specific primers showed that these elements possess different PCR patterns (28). Further experiments performed with the linear molecule harbored by strain B16 showed that it actually corresponds to the prophage form of a temperate phage named GIL16. Initially reported as a *B. cereus* strain, microscopic visualization indicated that strain B16 is in fact a *B. thuringiensis* strain whose endospores generate the typical bipyrarnidal crystals that characterize this species (data not shown).

GIL16 is able to produce small turbid plaques on *B. thuringiensis* GBJ002, which is also the native host for GIL01 (28). In order to avoid any contamination by other bacteriophages potentially present in strain B16, we isolated *B. thuringiensis* GBJ002 lysogens for GIL16 and confirmed the presence of the pGIL16 prophage by PCR. The release of viral particles by GBJ002 lysogens was then analyzed by electron microscopy analysis. As shown in Fig. 1, GIL16 viral particles displayed a similar morphology to those of other identified tectiviral phages, such as PRD1 (3), AP50 (20), and Bam35 (1, 27). These tectiviruses are characterized by an icosahedral capsid with a diameter of about 50 to 60 nm and are occasionally associated with a tail-like structure serving as a DNA ejection device during infection. The latter could clearly be distinguished on the micrograph (Fig. 1) and had the same size as that previously described for other tectiviruses (1, 10, 20, 21).

TABLE 1. Nucleotide differences between the GIL01 and Bam35 genomes

GIL01 position ^a	GIL01 nucleotide	Bam35 nucleotide	ORF	Codon modification (amino acid modification) ^b
6-7		G		
194	G	T		
1339	G	A	3	<i>GCC→ACC (Ala→Thr)</i>
5516	G	A	10	GAG→GAA (Glu→Glu)
			11	<i>GCT→ACT (Ala→Thr)</i>
5818	C	T	11	GAC→GAT (Asp→Asp)
6965	G	A	13	<i>GAT→AAT (Asp→Asn)</i>
7606	G	A	16	<i>GGC→GAC (Gly→Asp)</i>
10807	C	T	24	AAC→AAT (Asn→Asn)
11426	G	A	25	<i>GGC→GAC (Gly→Asp)</i>
12554	G	C	28	<i>GTT→CTT (Val→Leu)</i>
14925-14926		C		

^a A range indicates the insertion of one nucleotide between the indicated positions in Bam35.

^b Neutral (no amino acid modification) and minor (similar amino acids) modifications are shown in bold and italics, respectively.

GIL16c, GIL01, and pBclin15 are three closely related elements. Similar to GIL01, GIL16 exists as a prophage within the host cell and occasionally causes cell death with a release of viral particles that are capable of infecting a sensitive host. Among the turbid plaques formed by GIL16 on GBJ002 bacterial lawns, some clear plaque variants could be detected. One of these mutants was isolated, and the corresponding phage was obtained through successive single plaque purification steps. The DNA of this viral particle, designated GIL16c, was prepared and sequenced as described in Materials and Methods. The sequence was then compared to those of tectiviruses that infect the *B. cereus* group, specifically GIL01 and Bam35, and to that of the putative prophage pBclin15. As shown in Table 1, GIL01 isolated from *B. thuringiensis* serovar Israelensis and Bam35 isolated from *B. thuringiensis* serovar Alesti differ by only 11 bp. However, 3 of these 11 differences are present in a noncoding region. In addition, a modification at position 5516 concerns two overlapping open reading frames (ORFs), namely, ORF10 and ORF11, bringing the actual number of codon variations to nine instead of eight. Of these nine differences, three are neutral (encoding the same amino acids), while three others cause minor amino acid substitutions (Ala→Thr or Val→Leu). Considering that all of these modifications do not significantly alter the genetic organization of either bacteriophage, GIL01 may be considered a subspecies of Bam35.

Sequencing of the GIL16c genome resulted in a linear, 14,844-bp double-stranded DNA molecule with imperfect inverted repeats at both extremities. An overall genome com-

parison showed 83.6% identity with GIL01 and a lower level of sequence similarity with pBclin15 (60.8% identity) from *B. cereus*. More precisely, the GIL16 and GIL01 left and right ends share 92% (72 of 78 nucleotides) and 85% (69 of 81 nucleotides) identity, respectively (Fig. 2). Similar to GIL01 and pBclin15, GIL16c displayed short noncoding regions and only a few overlapping ORFs. The new sequence was submitted for a BLAST search, and among 31 predicted ORFs, only 5 with significant similarities to phage-related proteins were identified: these proteins were a B-type DNA polymerase, a LexA-like repressor, a PRD1 DNA-packaging protein homolog, and two endolysins. The same results were obtained for GIL01 and pBclin15, for which these phage-borne predicted functions were also revealed. Although no putative function could be attributed to the other ORFs, most of them are relatively well conserved in GIL01, suggesting that they have similar functions.

Table 2 and Fig. 3 compare the GIL16c predicted gene products with those of GIL01 and the putative prophage pBclin15. An amino acid comparison revealed that among the 31 ORFs identified for GIL16c, 30 ORFs identified for GIL01, and 28 ORFs identified for pBclin15, 24 appeared to be conserved in all three genomes, which corresponds to approximately two-thirds of the genetic information. Similarities between the GIL16c and GIL01 ORFs ranged from 62 to 100%, whereas they varied from 27 to 89% between GIL16c and pBclin15. Besides these conserved predicted ORFs, a few are shared by only two genomes: three ORFs are shared between GIL16c and GIL01 and one ORF is shared between GIL01 and pBclin15. This analysis also indicated that some ORFs are specific to each genome: four, two, and three ORFs are unique to GIL16c, GIL01, and pBclin15, respectively. Interestingly, these variations occur in similar zones in all three genomes (Fig. 3, black arrows), indicating that some parts of the genome may be genetically more flexible than others. The absence of these predicted gene products from related phages may be indicative of the fact that their functions are not essential for the bacteriophage life cycle. However, despite this sequence divergence, the possibility that the predicted products fulfill the same function cannot be ruled out.

From these observations, we concluded that tectiviruses form a family that displays genetic flexibility. While tectiviral phages infecting gram-negative bacteria (PRD1, PR3, PR4, PR5, PR722, and L17) are almost identical, the same extreme relatedness was not observed among tectiviruses infecting gram-positive bacteria. Although Bam35, GIL16, GIL01, and pBclin15 all originated from the *B. cereus* group and clearly share similar genomic architectures (comparable in size, ge-

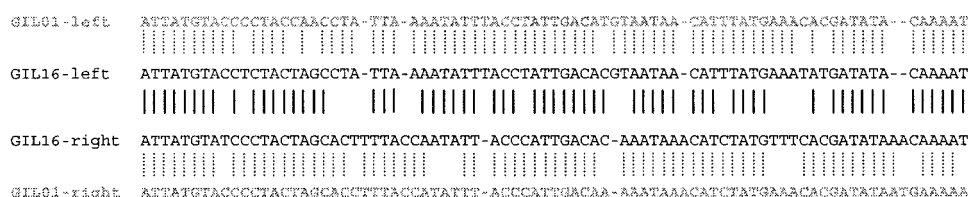


FIG. 2. ITRs at the GIL16 extremities and comparison with those of GIL01. Conserved residues within the GIL16 ITRs are indicated by full vertical lines. Conserved residues between the GIL01 and GIL16 left and right ends are shown by dashed vertical lines.

TABLE 2. Comparison of GIL16c, GIL01, and pBClin15 predicted gene products

GIL16c ORF	No. of residues (positions)	GIL01		pBClin15 ^a		Putative function
		ORF (% identity with GIL16cORF)	No. of residues (positions)	ORFs (% identity with GIL16cORF)	No. of residues (positions)	
1	56 (355–531)	1 (98.3)	58 (356–532)	1	63 (360–551)	
2	166 (547–1047)	2 (85)	167 (548–1051)	2 (86.5)	167 (532–1035)	
3	75 (1119–1343)	3 (95.8)	74 (1123–1347)	3 (46.5)	71 (1110–1325)	
4	245 (1402–2139)	4 (91.4)	245 (1406–2143)	4 (36.1)	233 (1390–2091)	
5	753 (2069–4330)	5 (97.3)	735 (2127–4334)	5 (47.5)	729 (2104–4293)	DNA polymerase
6	66 (4347–4547)	6 (100)	68 (4350–4550)	6 (89.2)	66 (4296–4496)	LexA-like repressor
7	58 (4561–4737)	7 (53.2)	50 (4564–4718)	7 (59.2)	49 (4512–4661)	
		6	48 (4709–4831)			
		9	57 (4950–5123)			
8	84 (4820–5074)					
9	125 (5040–5417)	10 (64.7)	145 (5136–5573)	8 (45.1)	115 (4780–5127)	
10	248 (5243–5989)	11 (79.8)	252 (5387–6145)	9 (38.8)	243 (4938–5669)	
11	80 (5835–6077)	12 (90.1)	80 (5991–6233)	10 (47.6)	81 (5515–5760)	
12	102 (6046–6354)	13 (74.5)	102 (6202–6510)	11 (61)	106 (5729–6049)	
13	212 (6335–6973)	14 (77.6)	212 (6491–7129)	12 (58.4)	216 (6021–6671)	DNA-packaging protein
14	46 (6988–7126)	15 (100)	46 (7144–7284)	13 (78.3)	46 (6684–6824)	
15	45 (7106–7243)					
16	46 (7264–7404)	16 (91.3)	46 (7301–7441)	14 (74.4)	45 (6839–6976)	
17	89 (7406–7675)	17 (65.2)	84 (7443–7697)			
18	356 (7675–8745)	18 (92.1)	356 (7697–8767)	15 (65.1)	355 (6980–8047)	
19	76 (8787–9017)	19 (98.1)	76 (8809–9039)	16 (47.8)	76 (8088–8318)	
20	52 (9021–9179)	20 (27)	68 (9043–9249)	17 (30.8)	79 (8255–8454)	
21	35 (9213–9320)					
22	143 (9253–9684)	21 (97.9)	143 (9329–9760)	18 (63)	140 (8568–8990)	
23	58 (9681–9857)	22 (100)	58 (9757–9933)	19 (69.6)	58 (8991–9167)	
24	48 (9854–10000)	23 (100)	48 (9930–10076)	20 (72.9)	48 (9164–9310)	
25	91 (10012–10287)	24 (96.7)	91 (10088–10363)	21 (37.8)	94 (9316–9600)	
26	204 (10291–10905)	25 (94.6)	204 (10367–10981)	22 (47)	197 (9613–10206)	
27	250 (10909–11661)	26 (95.6)	250 (10981–11733)	23 (74)	243 (10210–10941)	Lytic enzyme
28	170 (11609–12121)	27 (96.4)	170 (11681–12193)	24 (79.2)	173 (10889–11410)	
29	297 (12135–13028)	28 (73.1)	304 (12206–13120)	25 (24.9)	264 (11423–12217)	
30	293 (13032–13913)	29 (93.5)	293 (13124–14005)			
31	288 (13855–14721)					Lytic enzyme
				26	597 (12390–14183)	
				27	95 (14195–14483)	
		30	265 (14021–14818)	28 (67.3)	227 (14495–15179)	Lytic enzyme

^a The pBClin15 coordinates include the newly sequenced ITRs.

netic organization, and the presence of ITRs at both extremities), they display significant variations at the nucleotide level.

GIL16c, GIL01, and pBClin15 phage-borne predicted functions. So far, among the tectiviruses that infect gram-positive bacteria, only the endolysin-degrading activities of GIL01 have been experimentally confirmed by zymogram analysis (29). GIL16c ORF27 shares 95.6% identity with the Mur1 endolysin encoded by GIL01 (ORF26) and 74% identity with the pBClin15 (ORF23) putative endolysin. The high degree of similarity among these three hydrolases suggests that they pos-

sess comparable lytic activities. It is noteworthy that most of the variations observed between the pBClin15 predicted endolysin and those encoded by GIL01 and GIL16 are located at the C-terminal end. Since that domain has been shown to be implicated in cell wall binding (14, 17), the observed differences may reflect the target specificity of these endolysins.

GIL16c ORF5 showed similarity to several B-type DNA polymerases. Its deduced gene product is highly conserved in GIL01 (97.3% identity), whereas only 47.5% identity was observed with its counterpart in pBClin15. All of the conserved

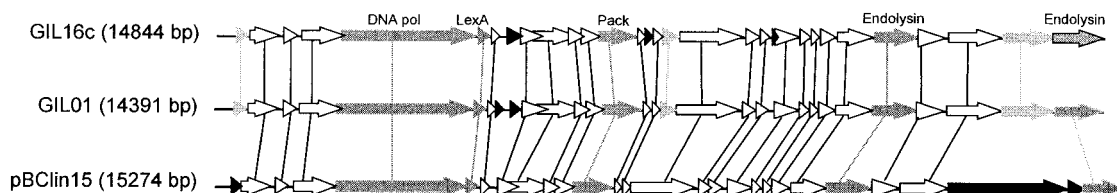


FIG. 3. Genetic map of the GIL16c and GIL01 bacteriophages and of pBClin15. Predicted ORFs are depicted by block arrows and ORFs sharing similarities with known proteins are shown in dark gray. DNA pol, DNA polymerase; LexA, LexA-like repressor; Pack, DNA-packaging protein. Similar ORFs are connected by vertical lines. ORFs shared by two genomes are shown in pale gray, while unique ORFs are shown in black.

GIL16-ORF12	1--MQAIPDQ	HVRIAGKIGS	GKSLAEVYL	AGYD--HVIM	LDTKGOSLER
GIL01-ORF13	1--MNGIPTDE	HVFIAGKIGS	GKSLAEVYL	AGYE--HVIM	LDTKGOCLEER
pBclin15-ORF12	1MSLTITIGDE	HVFLSQGTGT	GKSLAEVYL	SGVTDDVVK	LDTKGKVHER
PRD1-P9	1MTIRMPNDRO	RILVLGKTGT	GKTCAAVWHU	SQKD-----	FKRKAWIVLN
GIL16-ORF12	RKKGENLWYG	LKGGKDFVLV	ETLEEVAEAR	TKKVIYCPIF	EBODEEYIDA
GIL01-ORF13	RKKGKELWYG	LKGGKDFVLV	ETLEEVAEAR	TKKIIYCPIF	EBODEEYIDA
pBclin15-ORF12	RKKKEPIWYG	LKGGKDFVTV	ERLADIDDOE	TRKIIYAPIF	EBQEGEYVKK
PRD1-P9	HKGDD--LID	SIEGANTVDL	N---PRPKK	PGLYIYHPIF	-DVEDAEVTVQ
GIL16-ORF12	LMKWYPERHN	TILWIDELMQ	VCPSPSKYYP	HMKHLYQRGR	FVDSVWVACT
GIL01-ORF13	LMKWYPEREN	TILWIDELMQ	VAFSPFKYPP	HLRALMTGRG	SKEATVWACT
pBclin15-ORF12	LMKWYMEREN	TILWIDELME	VCPSHFYKYP	YLKGLMTRGR	SKEATVWACT
PRD1-P9	ELWDIHAMGD	IGVYVDEGVM	IP---NRDP	AFQALLTQGR	SKKIPMITLS
GIL16-ORF12	QRPAITSDI	MSNSTHFFIF	DLNKVADRKR	VADDLSGDMF	MDKPGYRNFV
GIL01-ORF13	QRPATIRIDV	FNDSHFFIF	DLNIPADRDR	IVKATGCPPE	YEKPGKYNFV
pBclin15-ORF12	QRPAITPAIV	MGNATHFFIF	DMNLPODREK	IAKTTGAMFE	LEKPGYKFW
PRD1-P9	QRPVNLTRFA	ISESDFFOIF	QLGDORDROT	VQGFVVDLE	KLMQAPVNTV
GIL16-ORF12	YMRSDSDEPV	RATLKL----	-----	-----	---212/212
GIL01-ORF13	YMRSDSDEPV	RATLKL----	-----	-----	---212/212
pBclin15-ORF12	YFRSGWDEPV	LATLKL----	-----	-----	---216/216
PRD1-P9	PALKKFHSIY	YDVGANNCVI	MTPVPTADAV	LARFDLGKKR	KQTL227/227

FIG. 4. Amino acid comparison between the putative DNA-packaging proteins of GIL16 (ORF12), GIL01 (ORF13), and pBclin15 (ORF12) and the P9 protein of PRD1. The ATP-binding motif (GXXGXGKXXXXXXXXL) is framed, and conserved residues are indicated by asterisks. All residues of GIL16 that are present in at least two other ORFs are indicated by gray boxes.

motifs of this protein family are found in the three proteins. The B-type family includes several DNA polymerases encoded by linear plasmids carrying genes for terminal proteins at their extremities. Linear molecules, such as ϕ 29 and PRD1, are replicated via a protein-priming mechanism in which the terminal proteins are used as primers to initiate replication. The presence of genes at the 5' extremities of the GIL01 genome was experimentally confirmed (28), supporting the idea that the two related linear molecules GIL16 and pBclin15 may also possess a similar end structure.

One interesting BLAST result was the DNA-packaging protein P9 of PRD1, the prototype member of the *Tectiviridae*. GIL16c ORF13, GIL01 ORF14, and pBclin15 ORF12 had 26, 21, and 27% identities, respectively, with this protein. Protein P9 is thought to be involved in PRD1 genome encapsidation by providing the required energy (9, 18). The hypothesis that these predicted proteins correspond to DNA-packaging proteins was reinforced by the presence of an ATP-binding consensus motif (28) in all three ORFs (Fig. 4).

GIL16 phage versus pGIL16 prophage. GIL01 and GIL16 were shown to be temperate bacteriophages that are capable of remaining in the host bacteria as prophages with an occasional induction of host cell lysis. It is notable that the phages described in this study are all able to establish a plasmid state, whereas tectiviruses infecting gram-negative hosts are only known to have a lytic cycle. This observation can be explained by the existence of a transcription regulator in GIL16 and GIL01 that represses the expression of lytic genes during lysogeny. Similar to other temperate bacteriophages, their lytic enzymes might be controlled by a viral repressor and at least one operator (6, 12, 16). One ORF that shares homology with LexA-type transcription regulators of several bacterial species and phages was indeed found in both GIL01 (28) and GIL16 (ORF6). Its putative gene product possesses a DNA-binding domain that characterizes LexA-type repressors similar to

those encoded by *B. cereus* ATCC 14579 and *Bacillus subtilis*. PRD1 and its relatives apparently lack any type of repressor, thus explaining the absence of a prophage state. However, we cannot rule out the possibility that these tectiviruses may at some point have possessed a transcription regulator. It is noteworthy that pBclin15 also harbors this putative repressor (Table 2). Interestingly, this region is highly conserved in all three genomes. ORF6 of GIL16c is identical to that in GIL01 and shares 89.2% identity with that of pBclin15.

Occasionally, clear plaques can be observed among the turbid plaques formed by GIL01 and GIL16 on GBJ002 lawns. Clear plaques are produced by lysogeny-defective phages, designated clear plaque (cp) mutants. These phages are characterized by mutations either in the repressor or in the operator sequence recognized by the repressor, leading to permanent expression of the lytic genes and the production of clear plaques. The GIL16 sequence reported here corresponds to that of a cp mutant (GIL16c). The region that is supposed to code for the putative repressor as well as the downstream predicted gene (ORF7) was sequenced from a wild-type (wt) particle and several cp mutants in order to identify putative variations. No mutation was observed in ORF6, which encodes the putative repressor, but two types of mutations were identified in a specific region of ORF7. As shown in Fig. 5, wt GIL16 harbors an 11-bp motif that is repeated twice with just one mismatch. In the cp1 mutant, corresponding to GIL16c, one of these 11-bp motifs was deleted, while in the cp2 mutant, the mutation was a repetition of a 28-bp motif that is present once in the wt. Interestingly, both mutations occurred at nearly the same position in ORF7. The corresponding regions in wt GIL01 and in two cp mutants have also been investigated, giving similar results. The GIL01 cp1 and cp2 mutants harbored a repetition or a deletion of the 11-bp motif identical to that implicated in the GIL16 mutation (Fig. 5). It is intriguing that all of the isolated GIL16 and GIL01 cp clones had an insertion or deletion of a particular motif in ORF7, while no mutation was ever observed in ORF6. These motifs may be part of repressor binding sites controlling the lytic genes. Since it is common within phage genomes to find genes encoding DNA-binding proteins situated close to the site of action of the gene product (12), the possibility of ORF6 and/or ORF7 being involved in the regulation of the lysogenic cycle should be explored. Future research will consist of establishing the precise role of these ORFs as well as identifying potential repressor binding sites across the phage genomes.

GIL01 and GIL16 have different host spectrums. In an effort to assess the host ranges of both GIL01 and GIL16, we tested the strains used by Ackermann et al. to propagate the phage Bam35. A PFGE analysis of genomic DNAs confirmed the initial absence of linear molecules in both the HER1410 and HER1047 strains (Fig. 6, lanes 7 and 8), and these results were sustained by the absence of amplification with GIL01- and GIL16-specific primers. Interestingly, only one of these strains was sensitive to GIL01, while both were infected by GIL16 (Table 3). wt GIL16 and the cp mutants produced turbid and clear plaques, respectively, on the lawns tested. GIL01, however, lysed the *B. thuringiensis* serovar *Thuringiensis* HER1410 strain but not the HER1047 strain listed as *B. megaterium* serovar *Tiberius*. The infected cell lines obtained were shown

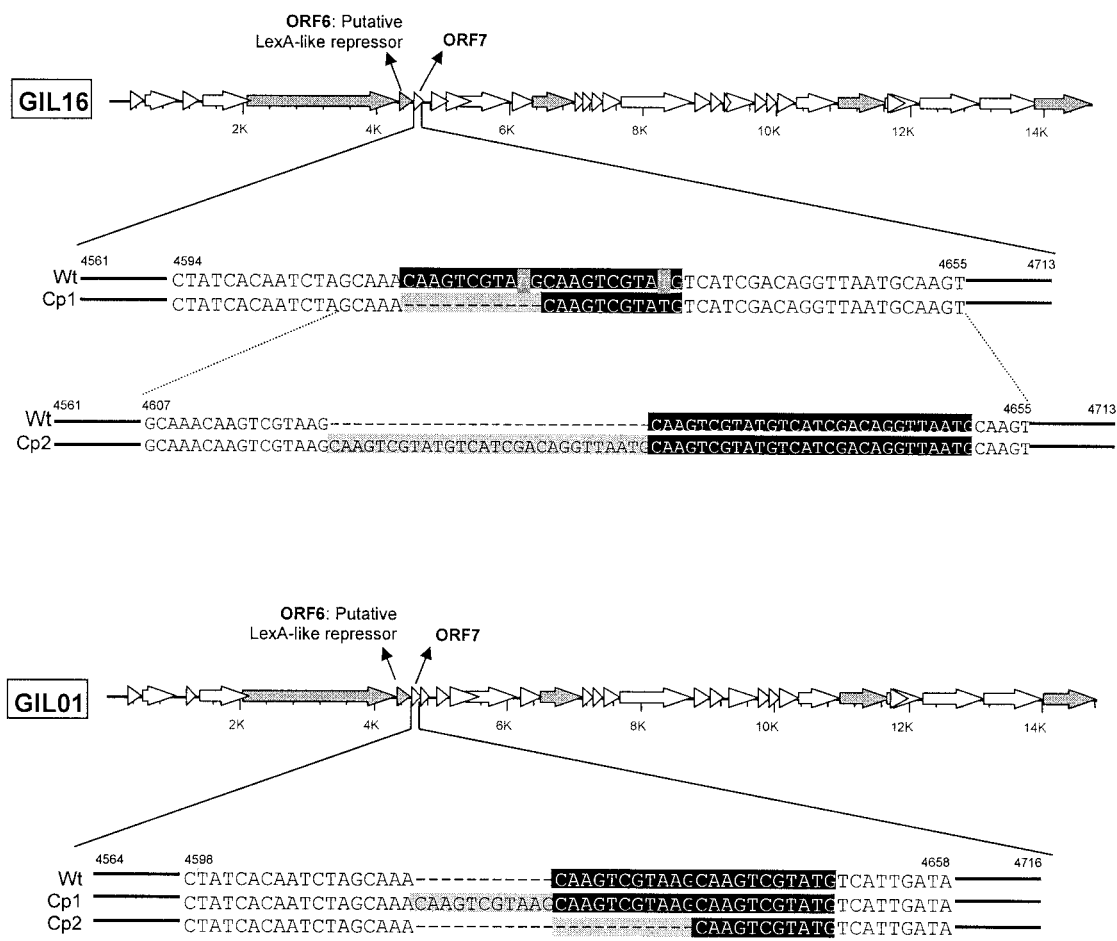


FIG. 5. Mutations observed in the ORF6-ORF7 region of GIL16 and GIL01 clear plaque (cp) mutants compared to the wt sequence. Motifs affected by the mutations are indicated by white letters over a black background (mismatches are indicated by gray boxes), and mutations observed in each cp mutant are indicated by black letters over a gray background.

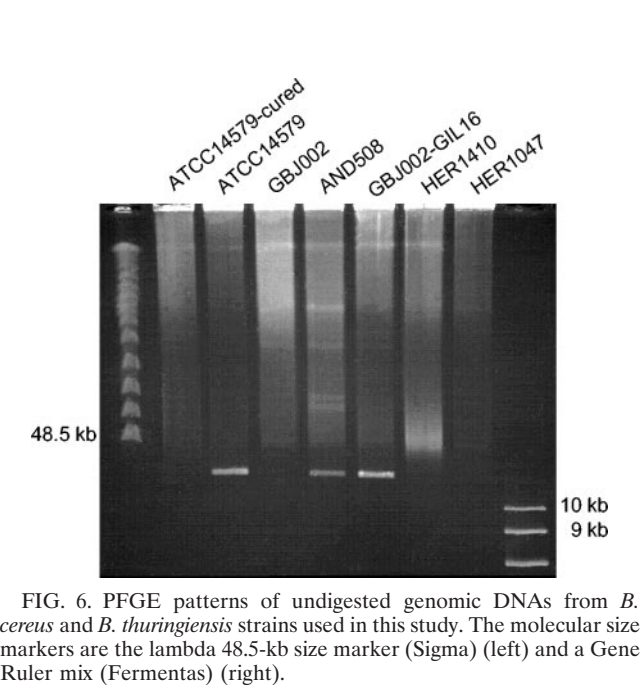


FIG. 6. PFGE patterns of undigested genomic DNAs from *B. cereus* and *B. thuringiensis* strains used in this study. The molecular size markers are the lambda 48.5-kb size marker (Sigma) (left) and a Gene Ruler mix (Fermentas) (right).

to contain GIL01- and GIL16-specific sequences by PCRs with single bacterial colonies (data not shown).

These results prompted us to do a closer analysis of both *B. thuringiensis* serovar Thuringiensis HER1410 and *B. megaterium* serovar Tiberius HER1047. Sequencing of their 16S rRNA genes indicated a 96% identity to the *B. cereus* and *B.*

TABLE 3. Host range of GIL01, GIL16, and their corresponding clear plaque mutants

Phage	Growth on indicated strain ^a		
	<i>B. thuringiensis</i> serovar Israelensis GBJ002 (cured of pGIL01)	<i>B. thuringiensis</i> serovar Thuringiensis HER1410	<i>B. megaterium</i> serovar Tiberius HER1047
GIL01	Tp	Tp	NL
GIL01c	Cp	Cp	NL
GIL16	Tp	Tp	Tp
GIL16c	Cp	Cp	Cp

^a Tp, turbid plaque; Cp, clear plaque; NL, no lysis. Note that strain HER1047, originally identified as *B. megaterium* serovar Tiberius, turned out to be a *B. cereus* isolate (see the text for details).

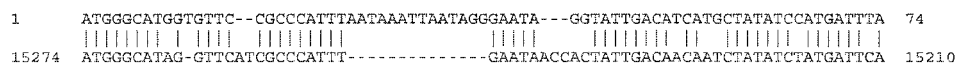


FIG. 7. Inverted terminal repeats of pBClin15. Conserved nucleotides are indicated by vertical lines.

thuringiensis ribosomal genes (data not shown). Since these two members of the *B. cereus* group can only be morphologically distinguished by the presence or absence of toxin crystals in their endospores, both strains were examined by phase-contrast microscopy on five consecutive days. Crystals were detected in the *B. thuringiensis* strain, while the strain that was previously reported to be a *B. megaterium* strain completely lacked these inclusions and presented a different spore morphology. Furthermore, HER1047 cells appeared as threadlike bacilli and not as large rods, ruling out the possibility that they were of the *B. megaterium* species. Further confirmation was obtained by performing PCRs on both strains, using primers hybridizing to the cereolysin O gene (*clo*), a hemolysin specifically found among members of the *B. cereus* sensu lato group (N. Michelet and J. Mahillon, unpublished data). The detection of *clo* in HER1410 and HER1047 definitely enabled to classify these strains in the *B. cereus* cluster.

The recent genome sequencing of Bam35c (21) revealed 99% identity with GIL01, suggesting that these two particles are virtually the same. Nevertheless, the present study revealed that GIL01 does not infect HER1047, a strain which is sensitive to Bam35. It would therefore be pertinent to reexamine the Bam35 host range alongside with GIL01 and GIL16 to rule out the possibility that HER1047 has not mutated since 1978. Surprisingly, GIL16 was able to infect HER1047 and thus displayed a slightly different host spectrum than that of GIL01. While PRD1 and its relatives have a broad host spectrum due to the wide distribution of their receptor-encoding plasmid, what determines the narrow range of gram-positive bacterium-infecting tectiviruses remains to be seen.

pBClin15 is delineated by imperfect ITRs. Sequencing of the *B. cereus* reference strain ATCC 14579 led to the identification of a 15.1-kb linear plasmid, pBClin15 (11). This molecule has a size and genetic organization comparable to those of GIL01, suggesting that it might also be a prophage. Yet the most intriguing distinction of this plasmid relative to tectiviruses remained the absence of ITRs. The hypothesis that these inverted repeats might exist at both extremities prompted us to perform sequencing of both ends. pBClin15 was isolated as described in Materials and Methods, and subsequent runoff sequencing at the extremities gave the whole sequence of this linear molecule. Sequences of 100 and 74 bp were missing from the left and right extremities, respectively, bringing the actual size of pBClin15 to 15,274 bp instead of 15,100 bp as originally reported (11). The pBClin15 sequence has since been corrected (AE016878). The alignment of the resulting sequences revealed the existence of imperfect 74-bp ITRs that share 64.6% identity with each other (Fig. 7). In contrast to those of GIL01 and GIL16, the pBClin15 inverted repeats include several gaps and mismatches, and they only share 51 to 58% identity with those of GIL01 and GIL16 (data not shown).

In order to verify the putative prophage state of pBClin15, we set out to cure the host strain, ATCC 14579. Since GIL01 only infects its cured lysogen, the same scenario could be

considered for pBClin15. One clone devoid of the linear plasmid was isolated after PCR screening with primers specifically hybridizing pBClin15 and a subsequent PFGE control assay (Fig. 6, lane 2). The cured strain was then tested for its sensitivity to pBClin15 viral particles that are putatively released by strain ATCC 14579. The same experiment was done with the *B. thuringiensis* GBJ002 strain as well as with 35 other strains belonging to the *B. cereus* group, including HER1410 and HER1047 (results not shown). No lysis was observed for any of the strains tested, and further PCR analysis failed to detect pBClin15 in single colonies. Since all of the strains were devoid of the linear plasmid, the phage nature of pBClin15 remains to be confirmed. Indeed, even the host strain that was cured of pBClin15 remained uninfected, suggesting that pBClin15 is unable either to form viral particles or to get into the host cell. In order to verify the hypothetical defective prophage state of this plasmid, further studies will focus on the identification of the missing functions.

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