Five Unique Temperate Phages from a Polylysogenic Strain of *Bacillus thuringiensis* Subsp. *aizawai*

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(Received 16 November 1987; revised 28 January 1988)

Five temperate phages were isolated from strain 4042B of *Bacillus thuringiensis* subsp. *aizawai*. The phages, which were heteroimmune, could also be distinguished by their host ranges, plaque and particle morphologies, serological specificities, and locations of restriction endonuclease cleavage sites on their chromosomes. Besides maintaining a stable lysogenic relationship with the 4042B host strain, each phage formed a stable lysogen with *Bacillus cereus*.

INTRODUCTION

Strains of *Bacillus thuringiensis* have elicited a special interest because they produce a proteinaceous parasporal crystal that is toxic to susceptible insect larvae. Since this commercially important species has only limited means of genetic exchange, temperate phages could be important genetic tools, serving as mediators of generalized and specialized transduction and as potential cloning vehicles. We have thus been interested in the characterization of temperate phages from this species.

Past surveys of strains of *B. thuringiensis* have suggested that a significant proportion harbour one or more temperate phages (Colasito & Rogoff, 1969; Ackermann & Smirnov, 1978; Jones *et al.*, 1983). However, the physical characteristics of these phages, as well as their relationships with the host strains, have not been examined in detail. Several phages that mediate generalized transduction in *B. thuringiensis* (Thorne, 1978; Landen *et al.*, 1981; Heierson *et al.*, 1983) and one that converts spore-negative, crystal-negative cells to spore-positive, crystal-positive cells (Perlak *et al.*, 1979) have been described. More recently we have utilized transducing phages to link genetic markers on the *B. thuringiensis* chromosome (Barsomian *et al.*, 1984) and to transfer plasmids among strains of *B. thuringiensis*, *B. cereus* and *B. anthracis* (Ruhfel *et al.*, 1984).

During our investigation of strain 4042B of *B. thuringiensis* subsp. *aizawai*, we isolated five unique temperate phages whose characteristics are described in this report. The phages are heteroimmune and they can be distinguished on the basis of plaque and particle morphology, host range, antigenicity and restriction endonuclease digestion patterns of their DNAs.

METHODS

Bacterial strains. Derivatives of *B. thuringiensis* 4042B and other strains that were used for detection or propagation of phages are listed in Table 1. Strains that were used only for the determination of phage host ranges are included in Table 2.

Bacteriophages. TP-15, TP-16, TP-17, TP-19 and TP-20 were isolated from *B. thuringiensis* 4042B as described in Results. CP-55 was isolated from a culture filtrate of *B. cereus* 13472. TP-15c, TP-16c, TP-17c, TP-20c and TP-19v were spontaneous clear-plaque (c) or virulent (v) mutants.

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Abbreviation: MC, mitomycin C.

Table 1. *List of strains*

Strain	Characteristics	Origin/reference
<i>Bacillus thuringiensis</i>		
4042B	Subsp. <i>aizawai</i>	Barsomian <i>et al.</i> (1984)
4042B UM50	<i>trp-2</i>	UV mutagenesis of 4042B
<i>Bacillus cereus</i>		
4415	Wild-type	ATCC
4415 UM1	<i>trp-1</i>	UV mutagenesis of 4415
4415 UM1(TP-19)	<i>trp-1</i> , carries TP-19	This study
4415 UM1(TP-20)	<i>trp-1</i> , carries TP-20	This study
13472	Wild-type, carries CP-55	ATCC
13472 UM1	<i>thr-1</i>	UV mutagenesis of 13472
13472 UM8	<i>ile-1</i>	UV mutagenesis of 13472
13472(TP-15)	Carries TP-15	This study
13472(TP-16)	Carries TP-16	This study
13472(TP-17)	Carries TP-17	This study
13472 UM1(TP-17)	<i>thr-1</i> , carries TP-17	This study

Media and growth conditions. L broth, PA broth and agar, and peptone diluent were prepared as described previously (Thorne, 1978). BHI-L broth was made by adding 12 ml sterile double strength Difco brain-heart infusion broth (BHI) to 88 ml sterile L broth. CAY broth contained 12 g N-Z Case Peptone (Sheffield Chemical Co.) and 3 g Difco yeast extract per litre. Difco nutrient broth (N broth) and BHI broth were prepared according to instructions from the manufacturer. N agar and soft N agar were prepared by adding 15 and 5 g Difco agar, respectively, per litre of N broth. Cultures were incubated at 37 °C on a rotary shaker (250 r.p.m.) unless otherwise described. Spores were prepared from cultures grown on potato agar as described (Thorne, 1962).

Propagation and assay of phages. TP-15 and TP-15c were propagated on cells of *B. cereus* 13472 in soft N agar overlays on N agar plates. The overlays were seeded with phage from a plaque suspended in 1% (w/v) peptone. TP-16 and TP-16c were propagated from phage lysates by mixing 1×10^9 p.f.u. and approximately 3×10^8 spores in 3 ml soft N agar and overlaying on N agar plates. Overlay plates were incubated for 20 h and the soft agar layer was suspended in 6 ml N broth. TP-17 and TP-17c were grown by lytic infection of L broth cultures of *B. cereus* 13472 UM1. Three to five plaques of phage were suspended in 1 ml 1% peptone and added to 25 ml culture in the exponential phase of growth. Following removal of debris by slow speed centrifugation, phage lysates were filtered through Millipore DA membranes.

TP-19 and TP-20 were propagated by induction of their *B. cereus* 4415 UM1 lysogens during exponential growth with mitomycin C (MC) at $0.7 \mu\text{g ml}^{-1}$ final concentration. The MC was removed after 15 min by pelleting the cells and resuspending them in 25 ml fresh BHI broth. TP-19v and TP-20c were propagated from plaques on cells of *B. cereus* 4415 in soft PA agar overlays on PA agar plates. Overlays were incubated 16 h before the phage was harvested as described for TP-15 and TP-16.

To induce *B. cereus* 13472 lysogens carrying TP-15 or TP-16, a 10% (v/v) transfer of a 12 h L broth culture was made into BHI-L broth. After 135 min, 10 ml was transferred to 15 ml BHI-L broth containing MC at a final concentration of $0.4 \mu\text{g ml}^{-1}$. Induction of *B. cereus* 13472 UM1(TP-17) was by the same procedure except that CAY broth was substituted for BHI-L broth.

Lysates of TP-17, TP-19, TP-20 and CP-55 were stored at 15 °C after filtration and those of TP-15 and TP-16 were stored at 4 °C.

All phages were assayed by the soft agar overlay technique (Adams, 1959). TP-15 and TP-16 and their clear-plaque mutants were assayed in N agar with *B. cereus* 13472 spores as the indicator. Spores of *B. cereus* 13472 were also used for TP-17 and TP-17c but PA agar was substituted for N agar. TP-19, TP-19v, TP-20, TP-20c and CP-55 were assayed with *B. cereus* 4415 cells as the indicator in PA agar.

Purification of phages. Phage lysates were incubated with DNAase ($1 \mu\text{g ml}^{-1}$) for 30 min at 37 °C. The phages were concentrated by centrifugation and purified in CsCl density gradients as described previously (Perlak *et al.*, 1979). TP-17, TP-19, TP-20 and CP-55 were centrifuged at 15 °C, and the other phages were centrifuged at 4 °C.

Phage antiserum. Purified phage suspensions were homogenized with an equal volume of Freund's incomplete adjuvant (Difco) and injected into rabbits. Each rabbit received 3 ml intramuscularly and 1 ml subcutaneously. Rabbits were bled after 5 weeks and serum was heated at 56 °C for 30 min. The neutralization rates (*K* values) were determined by the procedure of Eisenstark (1967).

Electron microscopy. Purified phage stocks were negatively stained with 2% (w/v) aqueous uranyl acetate, 2% (w/v) sodium phosphotungstate or 2% (w/v) ammonium molybdate, pH 7.0, on carbon-coated grids and examined in an RCA EMU-4 or Siemens 102A electron microscope.

Isolation and analysis of DNA. The procedure of isolating DNA from purified phage stocks was described previously (Perlak *et al.*, 1979). Phage DNAs were digested by restriction endonucleases according to procedures from BRL. Lambda DNA was obtained from BRL. DNA fragments were electrophoresed in horizontal 1% (w/v)

Table 2. Host ranges of the five phages

Bacterial strain	Phage				
	TP-15c	TP-16c	TP-17c	TP-19v	TP-20c
<i>B. thuringiensis</i>					
NRRL* B-4040, subsp. <i>finitimus</i>	+	—	—	—	—
NRRL B-4041, subsp. <i>alesti</i>	+	—	—	+	—
NRRL B-4043, subsp. <i>dendrolimus</i>	+	—	+	—	—
NRRL B-4044, subsp. <i>kenyae</i>	—	—	—	+	—
NRRL B-4047, subsp. <i>entomocidus-limassol</i>	+	—	—	—	—
NRRL B-4049, subsp. <i>morrisoni</i>	—	+	—	+	+
NRRL B-4050, subsp. <i>tolworthi</i>	—	—	—	—	+
NRRL B-4055, subsp. <i>kurstaki</i>	+	+	+	—	—
NRRL B-4058, subsp. <i>darmstadiensis</i>	—	—	—	+	+
NRRL B-4059, subsp. <i>toumanoffi</i>	+	+	+	—	—
4042A, subsp. <i>thuringiensis</i> †	—	—	+	—	—
4042B, subsp. <i>aizawai</i> †	—	—	—	+	—
BIS, subsp. <i>israelensis</i> ‡	+	+	+	—	—
<i>B. cereus</i>					
NRRL B-569	+	—	+	+	—
ATCC 4415	+	—	+	+	+
ATCC 7064	—	—	+	—	—
ATCC 9139	—	+	—	—	—
ATCC 9592	+	—	+	+	—
ATCC 9620	—	—	—	+	—
ATCC 13472	+	+	+	+	—

+, lysis of test strain; —, no lysis of test strain.

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† Barsomian *et al.* (1984).

‡ Obtained from H. de Barjac.

or 0.7% agarose gels containing Tris/borate buffer (0.089 M-Trizma base, 0.089 M-boric acid and 0.0025 M-EDTA, pH 8.3). The gels were stained with ethidium bromide (1.5 µg ml⁻¹) and photographed over a short wave UV light source.

RESULTS

Isolation of the phages

When filtrates of *B. thuringiensis* 4042B cultures were assayed with *B. cereus* 13472 as indicator, three different pure phage clones were isolated, designated TP-15, TP-16, and TP-17. Each of the phages formed morphologically distinct turbid plaques. Subsequently, two new types of morphologically distinct turbid plaques were observed when filtrates were obtained in the presence of antisera to the three known phages and assayed on *B. cereus* 4415. These phages were named TP-19 and TP-20.

After repeated single plaque isolations, lysates of the different plaque types still contained a low proportion of clear-plaque-forming units. These spontaneous clear-plaque or virulent mutants of TP-15, TP-16, TP-17, TP-19 and TP-20 were used for the determination of host ranges. Purified suspensions of TP-15c, TP-16c and TP-17c were also used for physical characterization of the different phage types. Propagation of these clear-plaque mutants by lytic infection of *B. cereus* 13472 produced the large quantities of phages required for the purification procedure and also avoided the problem of significant contamination of lysates with the resident CP-55 phage that is carried by strain 13472. TP-19 and TP-20 phages for the purification procedure were obtained by MC induction of their respective *B. cereus* 4415 UM1 lysogens isolated from colony-centred plaques.

Host ranges, particle morphologies and antigenic uniqueness

Table 2 shows that each of the five phage types had a unique host range when tested with strains of *B. thuringiensis* and *B. cereus*. *B. cereus* 13472 was chosen as the host for routine assays

Fig. 1. Electron micrographs of (a) TP-15c, (b) TP-16c, (c) TP-17c, (d) TP-19, (e) TP-20 and (f) CP-55 stained with uranyl acetate. The bar represents 100 nm in (a), (c), (d), (e) and (f) and 60 nm in (b).

and propagation of TP-15, TP-16 and TP-17 because that strain gave more distinct plaques and higher efficiencies of plating than any of the other sensitive strains tested. For the same reasons *B. cereus* 4415 was used for assay and propagation of TP-19 and TP-20.

With the exception of TP-20, the phages isolated from *B. thuringiensis* 4042B had similar particle morphologies (Fig. 1a-e) and dimensions (Table 3). Phage tails were flexible and

Table 3. *Dimensions of phage particles*

Values are means of measurements of at least 15 particles. SD values are given in parentheses.

Phage	Head diameter (nm)	Tail length (nm)
TP-15c	51 (1.6)	184 (6.2)
TP-16c	53 (1.6)	248 (6.3)
TP-17c	47 (1.3)	135 (7.3)
TP-19	48 (1.6)	170 (7.1)
TP-20	35 (0.5) × 70 (2.4)	128 (3.3)

Table 4. *Neutralization of phages by antisera*

Phage antiserum	K value for phage				
	TP-15c	TP-16c	TP-17c	TP-19	TP-20
TP-15c	261 000	67	2	3	3
TP-16c	49	497 000	2	3	3
TP-17c	3	17	2710	4	3
TP-19	2	1	1	164	27
TP-20	2	1	1	2	186

noncontractile. Four or five short projections were visible at the ends of TP-15c and TP-16c tails when the particles were stained with sodium phosphotungstate (not shown).

The *K* value of a phage antiserum is a measure of its neutralizing potency, a high *K* value indicating a high titre. Thus, the *K* values obtained when a particular antiserum is tested against different phages are indicative of the degrees of serological relatedness of the phages (Adams, 1959). As shown in Table 4, the *K* values indicate that each of the five phages was serologically unrelated to the others.

Under the described conditions less than one active CP-55 particle per 10³ p.f.u. was present in the *B. thuringiensis* phage lysates (data not shown). CP-55 had a negligible effect on the determination of TP-15c, TP-16c, and TP-17c host ranges in Table 2 since it was able to infect only the *B. cereus* 4415 strain of those listed. The relatively low proportions of CP-55 virions in lysates of TP-15c, TP-16c and TP-17c did not significantly affect the physical characterization of these phages. Examination of numerous fields of stained TP-15c, TP-16c and TP-17c particles did not reveal any virions resembling CP-55 (Fig. 1*f*), but CP-55-like particles could be found among phage purified from a filtrate of an MC-induced culture of *B. cereus* 13472 (TP-16). Dilutions of TP-15c-, TP-16c- or TP-17c-specific antiserum which completely neutralized the homologous phage did not significantly neutralize CP-55 activity (data not shown).

Phage DNAs

Comparison of fragments from *Eco*RI and *Hind*III endonuclease digests of DNA extracted from purified TP-15c, TP-16c, TP-17c, TP-19 and TP-20 particles indicated that the different phage chromosomes were physically unique (Table 5). The *Eco*RI-cut fragments of TP-15c, TP-16c and TP-17c DNA were not the same size as those from an *Eco*RI digest of CP-55 DNA thus demonstrating that only insignificant amounts of CP-55 DNA could have been present in those preparations. None of the *B. thuringiensis* phage DNAs could be digested by *Bam*HI, and TP-17c DNA was not cut by *Hind*III.

The sizes of the *Eco*RI and *Hind*III fragments in Table 5 were estimated by comparing their relative migration distances in agarose gels with those of fragments from *Eco*RI, *Hind*III and *Eco*RI-*Bam*HI digests of lambda DNA. The size of each phage DNA, as determined from the sum of the sizes of its DNA fragments, is given in Table 5.

Table 5. *Restriction fragments of phage DNAs*

Fragments from <i>Eco</i> RI digestion of phage (MDa)					Fragments from <i>Hind</i> III digestion of phage (MDa)				
	TP-15c	TP-16c	TP-17c	TP-19	TP-20	TP-15c	TP-16c	TP-19	TP-20
6.70		14.0	3.90	5.30	5.60	3.85	3.50	6.70	5.40
5.50		3.70	2.50	3.45	5.35	3.04	2.92	3.78	4.55
4.50		2.38	2.25	3.00	4.85	2.68	2.39	2.15	4.35
2.33 (2)		1.88	1.57	2.44	1.87	2.64	2.24	1.75	2.80
0.92		1.30	1.35	2.33	1.60	2.42	2.22	1.58	2.73
0.87		1.20	1.32	1.25	1.45	1.51	2.04	1.50 (2)	1.65
		1.00	1.30	1.06 (2)	1.36	1.20	1.45	1.20	1.43
			1.18	0.84	1.16	1.01	1.42	1.07	0.74
			0.98	0.75	0.74	0.98	1.18	1.03	0.58
			0.93	0.73		0.90	1.15	0.57	0.47
			0.80	0.68		0.53	1.05	0.50 (2)	
			0.70	0.52		0.49	0.92	0.45	
			0.56	0.49 (2)		0.46	0.84		
			0.54			0.39	0.80		
			0.51				0.64		
			0.47 (2)				0.45		
			0.42						
Total	23.15	25.45	21.84	24.42	23.98	22.19	25.21	24.28	24.70

Table 6. *Sensitivity of B. cereus lysogens to the five phages*

Sensitivity of strains was tested in phage assays and in cross-streaks.

Strain tested	Phage				
	TP-15c	TP-16c	TP-17c	TP-19	TP-20c
13472	+	+	+	+	W
4415 UM1	+	W	+	+	+
13472(TP-15)	-	+	+	+	W
13472(TP-16)	+	-	+	+	W
13472(TP-17)	-	-	-	+	W
4415 UM1(TP-19)	-	W	+	-	+
4415 UM1(TP-20)	+	W	+	+	-

+, Strain was sensitive; -, strain was not sensitive; W, wild-type strain was not sensitive.

Lysogenizing characteristics of the phages

Stable *B. cereus* lysogens of each of the five phages isolated from *B. thuringiensis* 4042B were obtained by isolation from the centres of turbid plaques. Attempts to cure such lysogens, as well as *B. thuringiensis* 4042B, by repeated single colony isolations were unsuccessful. All of the five phages were reisolated from 4042B UM50 after the strain was serially subcloned by single colony isolation for a total of 21 times. Furthermore, all five phages were reisolated from strain 4042B UM50 spores prepared by allowing a PA broth culture to sporulate in the presence of sufficient phage-specific antiserum to completely neutralize all plaque-forming activity and then heating the resulting spores at 80 °C for 30 min.

From the results shown in Table 6, it appears that each of the phages possessed a distinctive immunity, although TP-16 and TP-20 immunities could not be compared in this experiment due to their mutually exclusive host ranges. Although TP-15c was unable to lyse the TP-19 lysogen, TP-19 could lyse the TP-15 lysogen. Similarly, neither TP-15c nor TP-16c could lyse the TP-17 lysogen, but TP-17c could lyse TP-15 and TP-16 lysogens. Strain 4042B was immune to each of the five phages and their clear-plaque mutants but was lysed by the virulent mutant of phage TP-19.

Fig. 2. MC induction of (a) *B. thuringiensis* 4042B UM50 and (b) *B. cereus* 13472(TP-16). Each strain was induced by the procedure described for *B. cereus* 13472(TP-16), i.e. the culture was transferred at 0 min to broth containing MC (see Methods). Optical density (given in Klett units) was determined with a Klett-Summerson colorimeter equipped with a red (no. 66) filter. O, Optical density; X, TP-16 titre.

Table 7. Phage yields following mitomycin C induction of *B. cereus* lysogens

Strain	Untreated culture (p.f.u. ml ⁻¹)	MC-induced culture (p.f.u. ml ⁻¹)
13472(TP-15)	4.85×10^7	4.60×10^9
13472(TP-16)	6.61×10^7	1.09×10^{10}
13472(TP-17)	6.00×10^1	1.03×10^3
4415 UM1(TP-19)	2.00×10^6	7.20×10^9
4415 UM1(TP-20)	1.80×10^6	4.00×10^9

Treatment of lysogenized *Bacillus* strains with DNA-damaging agents such as MC or UV light will often greatly increase the rate of induction and release of phage particles (Warner *et al.*, 1977). Treatment of *B. thuringiensis* 4042B cultures with MC at several different concentrations caused massive culture lysis but unexpectedly did not bring about any significant increase in the titres of the five phages (data not shown). In an example of this phenomenon (Fig. 2a), an MC-treated culture of strain 4042B did not release an increased amount of TP-16 during lysis. However, MC treatment of a culture of the *B. cereus* 13472 (TP-16) lysogen under the same conditions did significantly increase the TP-16 titre (Fig. 2b). Table 7 shows that *B. cereus* lysogens prepared from each of the five phages isolated from *B. thuringiensis* 4042B released increased amounts of phage upon induction with MC.

Transduction

Each of the five phages was examined for the ability to mediate generalized transduction. Mutants of *B. cereus* 13472 or 4415 were used as recipients for phage grown on wild-type strains. TP-17 was the only one of the five phages able to effect generalized transduction. The *thr-1* and

ile-1 markers of strain 13472 were transduced at an average frequency of 6×10^{-7} transductants per p.f.u. Other markers were not tested. TP-17 was temperate enough so that inactivation of p.f.u. was not necessary for survival of transductants.

Plasmid profiles of lysogens

A comparison of plasmid profiles of *B. thuringiensis* 4042B, *B. cereus* strains 13472 and 4415, and *B. cereus* lysogens carrying the five phages indicated there were no new plasmids in the lysogens. Four plasmids with estimated molecular masses of 5.0, 9.0, 10.2 and >100 MDa were present in 4042B UM50. Hybridization experiments with TP-15c and TP-16c DNA probes confirmed a lack of homology with any of the 4042B plasmids (Reynolds, 1982). Similar hybridization experiments using DNA from the three other phages as probes were not done.

DISCUSSION

B. thuringiensis 4042B is a polylysogen that is able to maintain a stable relationship with five different prophages. Although Jones *et al.* (1983) isolated 11 possibly temperate phages in a survey of 36 strains of the *aizawai* subspecies, no strain carrying more than one phage was identified. Other investigators have previously identified *B. thuringiensis* strains that carry more than one phage (Ackermann & Smirnov, 1978).

Previous investigations of temperate phages inhabiting *B. subtilis* have placed them into four or five major groups based on similar antigenicity, homoimmunity and phage adsorption sites (Dean *et al.*, 1978). Phages within several of these groups share extensive ($>80\%$) DNA homology according to endonuclease analysis and heteroduplex mapping (Rudinski & Dean, 1979). Even the dissimilar SPO2 and $\phi 105$ temperate phages share 14% DNA homology and are serologically related (Chow *et al.*, 1972). In contrast to these examples, the five temperate phages carried by strain 4042B were unrelated by every criterion examined. The uniqueness of the TP-15c, 16c and 17c genomes demonstrated by restriction endonuclease analysis was confirmed by Southern blotting experiments (Reynolds, 1982). Heterogeneity, in at least some functions (e.g. immunity), is most likely essential for maintenance of different temperate phages in a single host.

The absence of new plasmids in lysogens of *B. cereus* carrying the five phages suggests that the five phages integrate into the host chromosome. Assuming that the mass of the *B. thuringiensis* chromosome is equivalent to that estimated for the chromosome of *B. subtilis*, i.e. $2.0 \times 10^9 - 2.5 \times 10^9$ Da (Henner & Hoch, 1980), the integrated prophages would amount to a total of 4.8 to 6% of the 4042B chromosome.

The results presented here suggest that when the prophages in strain 4042B are simultaneously induced by treatment with MC, they interfere with each other's induction or replication (Fig. 2). Of the five phages carried by strain 4042B, TP-17 was notable for its strikingly low titres (1×10^3 or fewer p.f.u. ml $^{-1}$) released spontaneously or by MC-induced cultures of *B. thuringiensis* 4042B and the *B. cereus* 13472 (TP-17) lysogen. Each of the strains in question harbours one or more additional heteroimmune phages which may have somehow interfered with TP-17 induction and/or replication. Also, the inability of TP-15c or TP-16c to lyse the *B. cereus* 13472(TP-17) lysogen and of TP-15c to lyse the 4415 UM1(TP-19) lysogen (Table 6) suggests that several of the phages carried by strain 4042B have specific mechanisms of interference with phage infection or replication.

Based on the size of its genome (21.8 MDa; Table 5) and its ability to mediate generalized transduction, TP-17 may be valuable for fine mapping of linked chromosomal markers in *B. thuringiensis* and *B. cereus*.

While characterizing TP-16, we isolated a spontaneous deletion mutant which we designated TP-16-1. Restriction endonuclease digestions of TP-16 and 16-1 DNAs showed that TP-16-1 has about 1.13 MDa DNA deleted from the adjacent 1.3 and 2.38 MDa *EcoRI* fragments of TP-16. Since this deletion of TP-16 DNA did not affect phage immunity or its essential replication functions, insertion of foreign DNA into this region may form a nondefective specialized

transducing phage. This method may prove useful for the construction of cells diploid for the *B. thuringiensis* crystal protein gene and hence, it may result in the improvement of *B. thuringiensis* strains for agricultural use.

We thank Christopher Woodcock for taking the electron micrographs of TP-17 and TP-20 and Judith A. Siess for her assistance in preparing the manuscript.

This work was supported in part by Grant PCM79-21851 from the National Science Foundation.

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