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Lynn Ann Anderson
San Jose State University

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GENETIC AND BIOCHEMICAL CHARACTERIZATION OF BACTERIOPHAGES
HARBORED BY *BACILLUS THURINGIENSIS* SUBSPECIES *KURSTAKI*

A Thesis
Presented to
the Faculty of the Department of Biological Sciences
San Jose State University

In Partial Fulfillment
of the Requirements of the Degree
Master of Science

by
Lynn Ann Anderson
December, 1994

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APPROVED FOR THE DEPARTMENT OF BIOLOGICAL SCIENCES

_____

Dr. Robert Fowler

_____

Dr. Jean-Christophe Piot

_____

Dr. Ruth Ann Kibler

APPROVED FOR THE UNIVERSITY

_____

Abstract

GENETIC AND BIOCHEMICAL CHARACTERIZATION OF BACTERIOPHAGES HARBORED BY *BACILLUS THURINGIENSIS* SUBSPECIES *KURSTAKI*

by Lynn Ann Anderson

Bacillus thuringiensis is used against specific crop pests and a few products containing this bacterium are manufactured by various companies. In at least some strains bacteriophage or bacteriocin-related problems such as spontaneous lysis have been observed. These problems are of significant importance in industrial scale fermentation and have prompted a study of the bacteriophages.

Four unique bacteriophages were isolated from *Bacillus thuringiensis* subspecies *kurstaki* HD119 and HD942. The phages could be distinguished by their plaque and particle morphologies, restriction endonuclease cleavage products, host range specificities and plating efficiencies on various strains of *Bacillus thuringiensis*. The apparent molecular sizes of the bacteriophage DNAs ranged from 29.4 to 38.3 kb. Each of the four bacteriophages formed a stable lysogenic relationship with *Bacillus cereus* 569.

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Table of Contents

	Page
Abstract	iv
Acknowledgements	v
Table of Contents	vi
List of Tables	viii
List of Figures	x
Introduction	1
Literature Review	2
Materials and Methods	7
Bacterial strains	7
Media and growth conditions	7
DNA and protein manipulations	8
Bacteriophage induction in wild-type strains	9
Purification of various bacteriophages from induced <i>B.thuringiensis</i> HD119 and HD942 strains	10
Lysogenization of <i>B. cereus</i> 569 by <i>B. thuringiensis</i> bacteriophages	10
Preparation of high-titer bacteriophage stocks	11
Purification of bacteriophages	12
Isolation of bacteriophage DNA	13
Determination of host range and plating efficiency	14
Polymerase chain reaction probe preparation	15
Cloning and restriction mapping	16

Bacteriophage protein analysis	19
Electron Microscopy	20
Results	21
Isolation of various bacteriophages from two strains of <i>B.thuringiensis</i> subspecies <i>kurstaki</i>	21
Optimization of induction parameters	22
Isolation of several types of bacteriophages with distinct plaques.	27
Lysogenization of <i>B. cereus</i> 569	28
Phage typing (cross spotting).	37
Biochemical and genetic characterization of bacteriophages isolated from <i>B. thuringiensis</i> subspecies <i>kurstaki</i> HD119 and HD942	39
Large scale purification of Ø6-1, Ø6-2, Ø6-5.1 and Ø12-1	39
Cesium chloride gradient purification of bacteriophage stocks	42
Morphological characterization of the bacteriophages	44
Biochemical characterization of the bacteriophages	46
Analysis of bacteriophage DNA	48
Restriction site mapping of bacteriophage Ø6-2	51
Genetic evidence of the similarity of Ø6-2-like bacteriophages	52
Determination of the host range of the various bacteriophages	54
Determination of the efficiency of plating (eop) on various indicator strains	55
Detection of the bacteriophage Ø6-2 by polymerase chain reaction	59
Discussion	62
Literature Cited	68

List of Tables

Table	Page
1. Bacteriophage yields following induction with ultraviolet irradiation or mitomycin C treatment	24
2. Plaque types obtained from <i>B. thuringiensis</i> HD119 and HD942 induced cultures . .	27
3. Phage titers obtained with various mitomycin C concentrations on <i>B. cereus</i> 569::Ø6-2	29
4. Bacteriophage titers obtained after induction of <i>B. cereus</i> 569 lysogens with 1 µg/ml mitomycin C	33
5. Yields of bacteriophage obtained from cultures infected with Ø6-5.1 at various multiplicity of infections	35
6. Bacteriophage Ø6-5.1 and Ø12-1 titers obtained after induction of <i>B. cereus</i> 569 lysogens with 1 µg/ml mitomycin C	37
7. Phage typing (cross-spotting)	38
8. Phage titers obtained from 500-ml cultures of <i>B. cereus</i> 569 lysogens induced with 1 µg/ml mitomycin C	41
9. Phage titers obtained after induction of <i>B. cereus</i> 569 lysogens grown in 100 ml cultures	42
10. Recovery of bacteriophage applied to cesium chloride gradients	44
11. Dimensions of bacteriophage particles	46
12. Apparent molecular weight of phage proteins	48

13. Determination of the presence of restriction endonuclease sites on bacteriophage DNA	50
14. Sizes of <i>EcoRI</i> restriction fragments and deduced total genome sizes of <i>E.</i> <i>thuringiensis</i> bacteriophages	51
15. Host ranges of four bacteriophages	55
16. Titers of plating bacteriophages Ø6-1, Ø6-2, Ø6-5.1 and Ø12-1 on various <i>B. thuringiensis</i> isolates and <i>B. cereus</i> 569	56
17. Efficiency of plating bacteriophages Ø6-1, Ø6-2, Ø6-5.1 and Ø12-1 on various <i>B. thuringiensis</i> isolates and <i>B. cereus</i> 569	57
18. Influence of the host strain on the efficiency of plating of bacteriophages Ø6-1 and Ø6-2	58
19. Oligonucleotide primers for the detection of Ø6-2	59
20. Amplification of fragments of bacteriophage DNA by PCR	60

List of Figures

Figure	Page
1. Lysis of culture growth of <i>B. thuringiensis</i> subspecies <i>kurstaki</i> HD119	21
2. Induction of HD942 through ultraviolet irradiation or incubation with mitomycin C	23
3. HD942 induction with ultraviolet irradiation for 25 seconds or mitomycin C treatment at 2.5 µg/ml	25
4. HD119 induced with ultraviolet 25 seconds, mitomycin C 2.5 µg/ml	26
5. <i>B. cereus</i> 569 lysogens 6-1, 6-2, 6-3 and 6-5.3 induced with 1 µg/ml mitomycin C	31
6. <i>B. cereus</i> 569 lysogens 12-2, 12-3 and 12-7 induced with 1 µg/ml mitomycin C . . .	32
7. A ₆₀₀ of Ø6-5.1 at various time points, m. o. i. 0.1, 0.01 and 1	34
8. <i>B. cereus</i> 569 lysogens of bacteriophage Ø6-5.1 and Ø12-1 induced with 1 µg/ml mitomycin C	36
9. Induction of <i>B. cereus</i> 569 lysogens, 500 ml cultures	40
10. Cesium chloride gradients of concentrated bacteriophage stocks	43
11. Electron micrographs of bacteriophages	45
12. SDS-PAGE gels of bacteriophage structural proteins	47
13. Agarose gel electrophoresis of bacteriophage DNA digested with <i>Sau</i> 3AI restriction endonuclease	49
14. Physical map of bacteriophage Ø6-2 for restriction endonucleases <i>Hind</i> III and <i>Kpn</i> I	52
15. Restriction patterns of bacteriophages belonging to the Ø6-2 family	53

Introduction

Bacillus thuringiensis (*B. thuringiensis*) is a gram positive, sporulating bacterium characterized by its ability to synthesize a variety of insecticidal proteins during sporulation. These proteins are produced at very high levels, and form cytoplasmic inclusions, often in the form of crystalline structures. *B. thuringiensis* can be used against specific crop pests and a number of commercial products containing this bacterium are manufactured by various companies.

In order to improve the insecticidal activity of *B. thuringiensis* strains, researchers have developed transgenic derivatives of the strains used in commercial products (Thorne, 1978; Perlak *et al.*, 1979; Gamel and Piot, 1992). In at least some of these strains bacteriophage- or bacteriocin-related problems such as spontaneous lysis have been observed. These problems are of significant importance in industrial scale fermentation and have prompted me to initiate a study of the bacteriophages harbored by *B. thuringiensis* strains used in the production of biological insecticides.

During my investigation four unique bacteriophages were isolated from *B. thuringiensis* subspecies *kurstaki* strains HD119 and HD942, whose characteristics are described in this thesis. The phages were distinguished on the basis of their plaque and particle morphologies, location of restriction endonuclease cleavage sites, electrophoresis patterns of their structural proteins, and host range specificities as well as plating efficiencies.

Literature Review

Most of the quite limited literature found on *B. thuringiensis* bacteriophages deals with either the physiological characterization of bacteriophages isolated after induction with genotoxic agents (Auffray and Poutibonnes, 1985; Inal *et al.*, 1990; Kochkina *et al.*, 1978; Rautenshtein *et al.*, 1970; Reynolds *et al.*, 1988), or with generalized transduction promoted by bacteriophages isolated from *Bacillus cereus* or *B. thuringiensis* (Inal *et al.*, 1992; Landén *et al.*, 1981; Lecadet *et al.*, 1980; Thorne, 1978).

In general, bacteriophage are currently classified into 12 families. Tailed phages, all of which contain double-stranded (ds) DNA, constitute 95% of bacteriophages, and fall into three families defined by tail structure. *Myoviridae* are phage with contractile tails, *Siphoviridae* have long, noncontractile tails, and *Podoviridae* have short tails (Francki *et al.*, 1991). When *Bacillus* phages were first classified in 1974, there were 12 species of tailed phages defined by their morphology and the physiochemical properties of the phage particles and their DNA (Ackermann, 1974). More recently, nine new species of *Bacillus* bacteriophage were described, which were novel, based on their morphological and physiochemical properties (Ackermann *et al.*, 1994).

Temperate bacteriophages from *B. thuringiensis* subspecies *galleriae* obtained upon induction with ultraviolet light, hydrogen peroxide and mitomycin C were characterized (Colasito and Rogoff, 1969). In this study, the particle morphology as well as the host ranges of eight bacteriophages were described. Bacteriophages with similar morphology were found to have the same host range. In another study (Kochkina *et al.*, 1978) a polylysogenic strain of *B. thuringiensis* subspecies *galleriae* was examined. It was noted that phage-induced lysis was frequently observed in cultures of this subspecies, which is

widely used in a number of countries for the production of insecticides, and in this case, the lysis was caused by virulent mutants of the temperate phage harbored by this strain. A further study (Kochkina, 1986a) examined the peculiarities between the two phages that were isolated from this culture. The phage differ in particle morphology, but exhibit a cross-reactivity in immunoassay using antiphage sera, and thus appear to be antigenically related. A further study evaluated structural features of the two phages (Kochkina, 1986b). The spectral properties of DNA isolated from the two phages was evaluated. Results point to a double stranded, helical structure of the DNA. The DNAs differ in their sequence, as shown by the molar content of the nitrogenous bases and in the character of their distribution along the molecule.

In a study by Azizbekyan *et al.* (1980), various strains of *B. thuringiensis* were analyzed for phages, and all of the cultures examined were shown to be able to release phages. The subspecies of *B. thuringiensis* included in this study were *galleriae*, *dendrolimus*, *tolworthi* and *insectus*. *B. thuringiensis insectus* was found to be lysogenic. The phages it released were of an unusual morphology for *B. thuringiensis* phages; they had hexagonal heads and no tails. Reynolds *et al.* (1988) isolated five temperate phages from a polylysogenic strain of *B. thuringiensis* subspecies *aizawai*. These could be distinguished by their host ranges, plaque and particle morphologies, serological specifications, and locations of restriction endonuclease cleavage sites on their chromosomes. The phages all maintain a stable lysogenic relationship with *B. cereus*. Kanda and Aizawa (1988) described the selective induction of two temperate bacteriophages from *B. thuringiensis* strain AF101 with ethidium bromide and ultraviolet irradiation. Both phage are inducible by either agent. The host range of the two phages are identical and include *B. thuringiensis* subspecies *sotto* and *israelensis*, which show a lytic

response to the phages. Comparison of DNA restriction patterns of their genomes show that they differ. In a study of five bacteriophages of the *Myoviridae* family, Jarvis *et al.* (1993) shows that these phages are morphologically identical and have similar protein patterns, yet differ considerably in their burst size. Their genomes, which are 95 to 166 kilobases (kb) in size, are shown to be distinct by DNA-DNA hybridization and restriction endonuclease analysis.

Transmission electron microscopy has been a valuable tool in the characterization of *B. thuringiensis* bacteriophages. One study investigated the fine structures of tailed phages from *B. thuringiensis* subspecies *galleriae* (Rautenshtein *et al.*, 1975). The study revealed the presence, on the tail, of a collar-like structure with teeth-like appendages. Phages of similar morphology were found in many strains of *B. thuringiensis* subspecies *galleriae*. Smirnova *et al.* (1979) examined the interaction between various morphologically distinct phages and R and S variants of *B. thuringiensis* subspecies *galleriae*. The morphological diversity of *B. thuringiensis* phages suggests a multiplicity of adsorption mechanisms and the presence of several types of receptors. A microscopic study of the intracellular development of a phage specific for *B. thuringiensis* subspecies *galleriae* showed the successive stages of phage development, from the absorption of phage particles to the cell wall, to the release of the phage from the cell. The phage adsorbs to the cell wall at the level of the enlarged tip of the tail. The prongs of the “collar,” which is located near the head, appears to contact the surface of the bacterial cell wall upon adsorption and to release lytic enzymes. The burst size of the phage studied varied from several hundred to over one thousand particles per cell.

Phages from industrial cultures of *B. thuringiensis* strains that had spontaneously lysed have been examined in several studies. A biological and physiochemical analysis of

phages isolated from a failed production batch of strain H1-98 was conducted by Koroleva *et al.* (1990). Two morphologically distinct groups of phage were found. One of the phage had a high burst size, a dangerous feature for industrial production. Restriction analysis did not reveal any difference in the genetic makeup between the two morphological groups of phages. Bacterial mutants resistant to these phages were obtained; however, the authors advise against the use of these mutant *B. thuringiensis* strains because of the occurrence of modified phages with a broadened host range. Besaeva *et al.* (1987) presents data on the effect of cultivation conditions on the intensity of spontaneous phage induction, which was shown to take place during the germination stage of the thermoactive spores. Subsequent lytic cycles occur during active growth of the cultures, but the increase in phage titer stops before the end of the vegetative growth phase. Forced aeration was also another factor causing the appearance of bacteriophages (Zamola *et al.*, 1983).

Phages naturally associated with *B. thuringiensis* subspecies *aizawai* were shown to be relevant to strain identification (Jones *et al.*, 1983). Sixteen out of thirty-six *B. thuringiensis* subspecies *aizawai* strains studied were naturally associated with bacteriophages. Host range, along with plaque morphology and differences in cold lability, were used to taxonomically differentiate between strains of this subspecies.

Bacteriophage-resistant mutants of *B. thuringiensis* have been isolated by several groups. In one study, the phage-resistant *B. thuringiensis* shows decreased virulence, resistance to seven phages and the loss of flagella (Heierson *et al.*, 1986). However, decreased virulence was not coupled to phage resistance or the lack of a flagella because other mutants affected in these properties are virulent. Koroleva *et al.* (1992) isolated and characterized thirty-four phage-resistant mutants of *B. thuringiensis* strain H1-177-131.

The development of phages is limited to various degrees in the phage-resistant mutants, which retain normal levels of endotoxin synthesis and spore titers. Resistance is not due to a modification of the phage receptors, but rather to changes in the mechanisms of intracellular development.

In this study data will be presented on the genetic and biochemical characterization of bacteriophages from the two strains of *B. thuringiensis* subspecies *kurstaki*. A better understanding of the biology of the phages harbored by this economically important microorganism will hopefully lead to the development of more reliable strains for the large-scale production of biological insecticides.

Materials and Methods

Bacterial strains

All *Bacillus thuringiensis* (*B. thuringiensis*) strains were obtained from C. Y. Chen, Sandoz Agro. The strains labelled HD originated from the culture collection of Howard Dulmage. *Bacillus cereus* NRRL 569 (Agricultural Research Service, Northern Regional Research Laboratory, U. S. Department of Agriculture, Peoria, Ill.) was a gift from Curtis Thorne. *Escherichia coli* DH5 α (Gibco BRL) was used as a host in cloning experiments.

Media and growth conditions

B. thuringiensis, *B. cereus* and *E. coli* strains were grown in Luria-Bertani (LB) (per liter: Bacto Tryptone (Difco), 10 g; Bacto Yeast Extract (Difco), 5 g; NaCl, 5 g; pH 7.0), unless otherwise stated. For phage assays, PA medium (per liter: Bacto Nutrient broth, 8 g; NaCl, 5 g; MgSO₄·7H₂O, 0.05 g; MnSO₄·H₂O, 0.2 g; CaCl₂·2H₂O, 0.05 g; pH 6.0), was used throughout. PA agar and PA soft agar contained 15 g and 5 g Bacto agar per liter respectively. CYS medium (per liter: Casitone (Difco), 10 g; glucose, 5 g; Bacto Yeast Extract (Difco), 2 g; KH₂PO₄, 1 g; MgCl₂, 500 mM; MnCl₂, 50 mM; ZnSO₄, 50 mM; FeCl₂, 50 mM; CaCl₂, 200 mM) was used in the preparation of *B. thuringiensis* and *B. cereus* 569 spore stocks. SOC medium (per liter: Bacto Tryptone (Difco), 20 g; Bacto Yeast Extract (Difco), 5 g; NaCl, 0.6 g; KCl, 0.5 g; MgCl₂, 10 mM; glucose, 20 mM; pH 7.0) was used for the recovery of *E. coli* after transformation. Where needed ampicillin (Amp), 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal), isopropyl- β -D-thiogalacto-

pyranoside (IPTG) were added to a final concentration of 75 µg/ml, 60 µg/ml, 1 mM respectively. Purified bacteriophage stocks were stored in SM buffer (per liter: NaCl, 5.8 g; MgSO₄·7H₂O, 2 g; Tris·HCl, 50 mM; Gelatin, 0.1 g; pH 7.5). Unless otherwise stated, the liquid cultures were grown at 30°C in a shaking incubator running at 300 rpm.

DNA and protein manipulations

Restriction enzymes were obtained from Pharmacia Biotech or New England Biolabs and used under the conditions described by the manufacturer. DNA was stored in TE buffer (Tris·HCl, 10 mM; EDTA, 1 mM; pH 8.0) or sterile NANOpure (Barnstead) water. Oligonucleotides were synthesized on a PCR-Mate synthesizer (Applied Biosystem, 391 DNA Synthesizer). PCR reactions were performed with AmpliTaq (Perkin-Elmer) in PCR buffer (Tris·HCl, 10 mM; KCl, 50 mM; MgCl₂, 1.5 mM; deoxyribonucleoside triphosphates (dNTPs), 200 µM; gelatin, 0.1% (wt/vol); pH 8.3). Primer concentration was 1 µM. The reactions were run for 20-25 cycles (94°C, 0.25 minutes; 52°C, 0.5 minutes; 72°C, 1 minute) in a Perkin-Elmer Thermal Cycler (GeneAmp 9600), using 0.2 ml PCR microtubes (StripEase, Robbins Scientific). DNA was analyzed by agarose gel electrophoresis run in TBE buffer (per liter: Tris-borate, 89 mM; EDTA, 2.5 mM; pH 8.3). Preparative agarose gels were run in TAE buffer (per liter: Tris-base, 40 mM; EDTA, 1 mM; sodium acetate, 5 mM; pH 7.6).

Proteins were analyzed by SDS-PAGE, performed according to the method of Laemmli (1970). Proteins were denatured by boiling for 5 minutes in SDS-PAGE loading buffer (63 mM Tris·HCl (pH 6.8), 10% glycerol, 2% SDS, 0.05% bromophenol blue, 5% 2-Mercaptoethanol) and were analyzed on an 8-16% Tris-Glycine gradient gels, in a discontinuous system.

Bacteriophage induction in wild-type strains

B. thuringiensis HD119 and HD942 were grown on LB agar overnight at 30°C. A single colony was used to inoculate 10 ml LB in a 125-ml shake flask. The culture was grown to an absorbance at 600 nm (A_{600}) of 0.2, and then induced by ultraviolet (UV) irradiation or treatment with mitomycin C (Sigma), according to the method of Inal *et al.* (1990).

For UV induction, the culture was centrifuged (10,000 rpm, 1 minute, 20°C) and the pellet was resuspended in 10 ml of $MgSO_4$ (10 mM; pH 7.0). The 10-ml suspension was placed in a 5-cm (diameter) open petri dish and swirled gently under a sterilizing UV light placed at a distance of 73 cm for a period of time ranging from 25 to 100 seconds. The dose rate for UV irradiation equaled 1.3 J/m² per second. The irradiated sample was centrifuged (10,000 rpm, 1 minute, 20°C), the pellet was resuspended in 10 ml of LB pre-warmed at 30°C, and transferred to a foil-covered, 125-ml shake flask. An unirradiated culture was used as a control. The A_{600} was measured at regular intervals, and once it had dropped below a value of 0.09, the culture was centrifuged (10,000 rpm, 10 minute, 20°C). The supernatant, containing the phages, was filtered through a cellulose acetate filter (Whatman) with a pore size of 0.45 μ m. The phage stocks were stored at 4°C, over a drop of chloroform.

For mitomycin C (MMC) induction, MMC was added at concentrations ranging from 0.1 to 10 μ g/ml and the culture was incubated for 15 minutes at 30°C in a shaking incubator running at 300 rpm. The culture was then centrifuged (10,000 rpm, 1 minute, 20°C), the cells were washed by resuspending the pellet in 20 ml LB, centrifuging once more as above, and resuspending the pellet in 10 ml of LB pre-warmed to 30°C. The cultures were then processed as the UV-induced cultures. A non-induced culture was used

as a control.

Purification of various bacteriophages from induced *B.thuringiensis* HD119 and HD942 strains

Lysates obtained by induction of *B. thuringiensis* strains HD119 and HD942 were diluted and aliquots were mixed with 5 ml soft PA agar containing approximately 10^8 spores of *B. cereus* 569 and plated on PA agar. The plates were incubated overnight at 30°C, with the overlay on top. For each plaque type, based on plaque morphology, an agar plug was taken with a sterile Pasteur pipette and macerated in 100 µl 1% peptone to release the bacteriophages. The suspension was then serially diluted and aliquots were plated on PA plates as described above. The various phages were purified by repeating these steps until one single type of plaque was obtained. The phages were harvested by scraping and macerating the soft PA agar overlay in 5 ml SM buffer by repeated pipetting followed by centrifugation at 6,000 rpm for 10 minutes at 4°C to remove the agar and bacterial debris. The lysates were filtered and stored as described above.

Lysogenization of *B. cereus* 569 by *B. thuringiensis* bacteriophages

A PA soft agar overlay containing 1×10^8 spores of a phage-free *B. cereus* 569 strain was plated on a PA agar plate (Ruhfel *et al.*, 1984). Once set, a drop of lysate containing 6×10^3 to 1.8×10^5 pfu of each bacteriophage lysate was spotted on the overlay (primary plate). The plate was incubated at 30°C overnight. Colonies growing in the zone cleared by the bacteriophage were streaked onto LB agar plates and incubated at 30°C overnight in order to obtain isolated colonies. Twenty isolated colonies from each spot were individually resuspended in 100 µl of 1% peptone. About 1 µl of the cell suspension

was plated on a LB plate for storage and the remainder was added to 4 ml molten PA soft agar and plated on PA agar. Once set, the same phage lysate used to infect the primary plate was spotted on the overlay, and the plate was incubated overnight at 30°C. The isolates that showed no clearing from the phage lysate were supposed to be either immune to superinfection due to lysogenation, or resistant to phage infection, and were further characterized. Lysogeny was tested by determining the ability of the isolates to produce phage particles upon induction by UV irradiation or treatment with MMC.

Preparation of high-titer bacteriophage stocks

One isolated colony of each *B. cereus* 569 lysogen was resuspended in 10 ml LB in a 125-ml shake flask and incubated overnight with shaking at 300 rpm at room temperature. After measuring the A_{600} of the overnight culture, a fresh culture was prepared by inoculating 500 ml to 2000 ml of LB with an aliquot of the overnight culture in order to obtain a starting A_{600} of 0.02. The culture was shaken at 300 rpm and 30°C until the A_{600} reached a value of 0.2, and was then induced by adding MMC to a final concentration of 1 µg/ml. After a 15 minute incubation with shaking at 300 rpm and 30°C, the MMC was removed by centrifuging the culture (10,000 rpm, 5 minutes, 25°C), resuspending the pellet in 20 ml LB pre-warmed at 30°C, centrifuging again as above and resuspending the pellet in the original volume of pre-warmed LB. The induced culture was then incubated in the same conditions as above, and the A_{600} was measured at regular intervals. After lysis, the culture was stored at 4°C. The titer of the phage suspensions was determined by diluting and plating aliquots of the lysates as described above.

For two of the bacteriophages, *B. cereus* 569 lysogens could not be obtained immediately. Therefore, the following alternate method for preparing high-titer stocks was

used. An overnight culture of *B. cereus* 569 was diluted in 20 ml of LB to a starting A_{600} of 0.02, and grown shaking at 30° C until the A_{600} reached a value of 0.2 (approximately 1×10^7 cfu/ml). Aliquots of the culture were mixed with the bacteriophage stocks in order to obtain a multiplicity of infection (m. o. i.) of 0, 0.01, 0.1 or 1. The mixtures were incubated at 30°C without shaking for 16 hours to allow bacteriophage adsorption and multiplication. To further increase the phage titer, the overnight culture was mixed with molten soft PA agar and plated on a 150-mm (diameter) PA agar plate. If the overnight culture was judged too clear, 1×10^8 colony-forming units (cfu) *B. cereus* 569 spores were added prior to plating. The plates were incubated at 30°C overnight. Phages were harvested from the plates by scraping off and macerating the overlay in 5 ml of SM buffer. Bacterial debris were removed by centrifugation (6,000 rpm, 10 minutes, 20°C) and the lysate was filtered and stored as described above.

Purification of bacteriophages

Bacteriophages were purified as described by Maniatis *et al.* (1985). Culture lysates were incubated with deoxyribonuclease I (DNase I) and ribonuclease A (RNase A) at 1 µg/ml each for 1 hour at room temperature. The phage particles were disassociated from the bacterial debris by the addition of NaCl to a final concentration of 1 M, followed by a 1-hour incubation on ice. The bacterial debris were removed by centrifugation (10,000 rpm, 10 minutes, 4°C). The phage were precipitated by the addition of polyethylene glycol (PEG, molecular weight 8000 g/mol) to a final concentration of 10% (wt/vol) followed by a 1-hour incubation on ice. The precipitated bacteriophages were recovered by centrifugation (11,000 rpm, 10 minutes, 4°C). The supernatant was decanted and the pellet was soaked in 4 ml of SM buffer to allow resuspension of the bacteriophage

particles. The concentrated phage stocks were extracted once with chloroform and further purified on discontinuous CsCl density gradients made of 5-ml steps of CsCl solutions of densities 1.3, 1.4, 1.5 and 1.7 g/ml respectively. The phage stocks were layered on top of the CsCl gradients and centrifuged for 2 hours at 22,000 rpm (109,000 xg) at 4°C in a Beckman SW28 rotor. The opalescent phage bands were collected through the wall of the tube with a syringe fitted with a 18-gauge hypodermic needle. The CsCl was diluted either by the dropwise addition of SM buffer or by delicately overlaying the collected gradient fraction with SM buffer and allowing diffusion of the CsCl overnight at 4°C. Phages were pelleted by centrifugation at 22,000 rpm (109,000 xg), for 2 hours at 4°C in a Beckman SW28 rotor. The pellet was overlaid with 0.5 ml SM buffer and left for 16 hours at 4°C to allow resuspension.

Isolation of bacteriophage DNA

Bacteriophage DNA was extracted as described by Maniatis *et al.* (1985), as follows. To a solution containing 2×10^9 to 9×10^{12} pfu contained in 500 µl SM in a microfuge tube, disodium ethylenediaminetetra-acetate-2H₂O (EDTA), proteinase K and sodium dodecyl sulfate (SDS) were added to final concentrations of 20 mM, 5 g/l and 0.05 g/l respectively. The mixture was incubated at 56°C for 1 hour, and cooled to room temperature. An equal volume of phenol/chloroform mixture (1/1 vol/vol) equilibrated with 50 mM Tris (pH 8.0), was added to the treated bacteriophage suspension. The mixture was homogenized by shaking for 30 seconds on a vortex mixer and incubated at 45°C for 15 minutes. Phases were separated by centrifugation at 14,000 rpm for 5 minutes, the aqueous layer was transferred to a new tube, and was extracted once with an equal volume

of chloroform/isoamyl alcohol mixture (24/1, vol/vol). The phases were separated as described above, and the aqueous phase was transferred to a new tube. Sodium acetate (pH 6.0) was added to a final concentration of 0.3 M, and the DNA was precipitated by the addition of 2 volumes of ice-cold 100% ethanol and pelleted by centrifugation at 14,000 rpm for 30 minutes at 4°C in a microfuge. The pellet was washed twice with 70% ethanol, air dried for 5 minutes, and resuspended in 50 to 100 µl TE buffer.

Determination of host range and plating efficiency

Spore stocks of *B. cereus* 569 and representative strains belonging to several subspecies of *B. thuringiensis* were prepared as follows. An isolated colony of each strain was inoculated in 100 ml CYS, and incubated with shaking at 300 rpm, 30°C for 48 hours. The sporulated cultures were centrifuged (6,000 rpm, 10 minutes, 4°C). The pellets were washed once with 20 ml sterile distilled water, centrifuged and resuspended in 10 ml distilled water. The spore suspensions were heat-treated for 45 minutes at 65°C. Spore titers were determined by plating aliquots of various dilutions of the spore stocks on LB agar which were incubated overnight at 30°C.

The plating efficiencies were determined as follows. PA agar overlays containing 1×10^8 spores were plated on PA agar plates. Once set, a 2 µl aliquot of each bacteriophage suspension was spotted onto the overlay, and the plate was incubated, overnight, at 30°C with the overlay on top. Plating efficiency was determined by mixing aliquots of each serially diluted phage stocks with 1×10^8 spores of each host in 4 ml PA soft agar and plating on PA agar plates. The plates were incubated overnight as indicated above.

Polymerase chain reaction probe preparation

Bacteriophage DNA and pUC18 plasmid vector (Pharmacia) were digested with *Hind*III (Gibco BRL, REact 2, 1 hour, 37°C). The vector was dephosphorylated with Shrimp Alkaline Phosphatase (United States Biochemical; 1 hour, 37°C). The digested, dephosphorylated vector was run on a preparative 0.8% agarose gel and the vector band was excised. The agarose was cleaved to neoagro-oligosaccharides (Yaphe, 1957) with Agarase (Boehringer Mannheim Biochemicals) as follows. The gel slice was placed in a tared tube to determine its weight, 0.04 volumes of 25x agarase buffer (Boehringer Mannheim Biochemicals) was added and the agarose was melted by incubating at 65°C for 15 minutes. The molten agarose was cooled to 45°C, 1 unit of agarase per 100 mg of agarose was added, and the mixture was incubated at 45°C for 1 hour. The agarose oligomers were precipitated by the addition of sodium acetate to a final concentration of (300 mM) followed by a 1-hour incubation at 0°C. The precipitate was pelleted by centrifugation (14,000 rpm, 15 minutes, 4°C), the supernatant was transferred to a new tube and the DNA was precipitated by the addition of 2.5 volumes of ethanol.

The ligation was set up as follows. The linearized, dephosphorylated vector and the digested bacteriophage DNA were ligated overnight at 16°C with T4 DNA ligase (Pharmacia) in 20 µl of One-Phor-All buffer (Pharmacia; containing: 10 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate) and 1 mM ATP. The ligase was heat-inactivated at 65°C for 15 minutes.

The ligation mixture was used to transform competent *E. coli* DH5α cells as follows. Frozen DH5α cells were thawed on ice. The ligation mixture (5-10 µl) were mixed with 100 µl cells, incubated on ice for 30 minutes. The mixture was then heat-shocked at 42° for 90 seconds and returned to ice for 15 minutes, 900 µl SOC was added

and the suspension was incubated for 1 hour at 37°C with shaking. Aliquots were plated on LB plates containing Amp, X-Gal and IPTG, which were incubated at 37°C overnight.

The PCR analysis was completed as follows. The recombinant clones were screened for the presence and size of inserts as follows. A portion of each isolated colonies was streaked on an Amp-containing LB plate and the remainder was resuspended in 100 µl of PCR buffer containing 0.1% Triton X-100. The suspension was then boiled for 10 minutes. PCR reactions mixtures, consisting of 1 µl of boiled cells and 9 µl of PCR buffer containing 2.5 units AmpiTaq Polymerase (Perkin-Elmer) and M13 primers (Pharmacia) were prepared and PCR reactions were run as described above. Aliquots of the PCR reactions were analyzed by agarose gel electrophoresis. The plasmid DNA from clones containing inserts 600 bp or larger in size was isolated using Qiagen Tip 20 columns. The inserts were sequenced using the Sequenase version 2.0 Sequencing Kit (United States Biochemical) and M13 primers. Sets of primers for the amplification of bacteriophage DNA were designed based on the sequence information obtained from the clones.

Cloning and restriction mapping

The phage DNA was cloned into a vector in order to facilitate the restriction mapping. Flash Nonradioactive Gene Mapping Kit (Stratagene) was used for restriction mapping, as described by the manufacturer with the following modifications. A *KpnI* restriction site was engineered by inserting an oligonucleotide adapter into the unique *BamHI* site of SuperCos I (Stratagene), yielding the cosmid vector SuperKos. Bacteriophage DNA was partially digested with *KpnI* and ligated into the *KpnI*-digested, gel-purified SuperKos vector. The cosmid was packaged using the Gigapack II Packaging Extracts Kit (Stratagene) to increase the efficiency and representation of the library (Kretz *et*

al., 1989). The cosmid DNA (1-4 µl containing 0.1-5 µg) was added to the freeze-thaw extract, the tube was placed on ice and 15 µl of sonic extract was added. The tube was incubated at room temperature (22°C) for 2 hours, then 500 µl of SM buffer and 20 µl of chloroform were added. The tube was centrifuged for 5 minutes at 14,000 rpm to sediment the debris and the supernatant was titered. Dilutions of the supernatant were prepared in SM buffer (1:10 and 1:50), and added to 25 µl of *E. coli* XL1-Blue MR at A₆₀₀ of 0.5. The mixture was incubated at 37°C for 1 hour, with gentle mixing every 15 minutes. The mixture was then centrifuged at 14,000 rpm for 1 minute and the pellet was resuspended in 50 µl LB. The resuspension was plated on LB plates containing 75 µg/ml Amp. Isolated colonies were inoculated in 5 ml LB containing 75 µg/ml Amp and grown with shaking at 37°C for 8 hours. The cosmid DNA was isolated using Tip-20 columns (Qiagen). The cosmid DNA was digested with *NotI* to verify the presence and size of the insert. Recombinant clones were grown in 500 ml Amp-containing LB at 37°C in a shaking incubator for 8 hours and cosmid DNA was isolated.

For mapping, *HindIII* sites were partially destroyed as follows: 500 ng of each cosmid in REact 2 (Gibco BRL) were aliquotted into 1.6-ml Eppendorf microfuge tubes. The tubes were placed into a Stratalinker UV crosslinker (Stratagene) and irradiated for 15 to 45 minutes. An unirradiated sample was also prepared. The samples were then digested with *HindIII* (Pharmacia) to yield partial and complete digests. The restriction sites were mapped using the Flash Nonradioactive Gene Mapping Kit (Stratagene). Aliquots of 250 ng of the UV-irradiated, *HindIII*-digested samples were loaded in duplicate on a 0.8% TBE agarose gel and run at 50 V for 16 hours. The gel was photographed, and the DNA was transferred to a Nytran membrane (Schleicher and Schuell) in a TurboBlotter (Schleicher and Schuell), using the alkaline blotting procedure (Chomczynski, 1992). The blot was cut

in half and prehybridized in prehybridization buffer (50 μ l of buffer /1.0 cm² of membrane) at 37°C for 10 minutes. The prehybridization buffer was removed and hybridization buffer containing either of the T3 or T7 probes (at 2 nM) was added. Hybridization was performed at 37°C for 50 minutes. The blots were then washed in 100 ml prewarmed wash buffer #1 (300 mM NaCl, 35 mM sodium citrate, 1% (w/v) SDS) for 10 minutes at 37°C, then in wash buffer #2 (300 mM NaCl, 35 mM sodium citrate, 1% Triton X-100) for 10 minutes at 37°C, then in 2x SSC buffer (350 mM NaCl, 35 mM sodium citrate) for 10 minutes at room temperature. The blots were rinsed twice in assay buffer (from the kit) for 5 minutes. Substrate buffer (5 ml assay buffer, 30 μ l CSPD chemiluminescent substrate) was added to the membranes and incubated at room temperature for 5 minutes. The membranes were then placed in Saran wrap and autoradiographed. The autoradiograph was photographed and compared to the photograph of the original gel to determine band sizes. Neighboring inserts were positioned by labelling fragments located at the ends of each insert with the ECL DNA labelling kit (Amersham) and using these fragments as probes to determine the identity of adjacent fragments in hybridization experiments run with digests of either the complete phage genome or the other inserts cloned in SuperKos.

Alternatively, where possible, the complete phage genome was cloned into the SuperCos vector. Phage DNA was digested with a single-cutting restriction endonuclease and blunted if necessary (see below). SuperCos was digested with *Xba*I and *Bam*HI, and blunted using the Klenow fragment of DNA Polymerase I (PolKI) as follows: one unit of PolKI was added per μ g of DNA and the mixture was incubated at 25°C for 15 minutes in NEB 2 buffer (New England Biolabs) containing 33 μ M of each dNTP. The enzyme was inactivated by heating to 75°C for 10 minutes. The phage and vector DNA were blunt-end

ligated using T4 DNA Ligase (Pharmacia) in the presence of 1 mM ATP, and the reaction was carried out at 16°C for 16 hours. The ligation products were packaged in bacteriophage Lambda DASH II using the Gigapack II Packaging Extracts Kit (Stratagene).

Recombinant clones were screened for inserts corresponding to the complete phage genome by growing each isolated colony at 37°C and 300 rpm for 8 to 12 hours in 5 ml LB supplemented with 0.2% maltose, 10 mM MgSO₄ and 75 µg/ml ampicillin. The cosmid DNA was isolated using Tip-20 columns (Qiagen) and digesting the DNA with *NotI* to verify the insert size. Restriction sites of appropriate clones were mapped as described above.

Bacteriophage protein analysis

The CsCl-purified bacteriophages were boiled for 5 minutes in SDS loading buffer and loaded onto a 8-16% SDS-PAGE gradient gel (Novex) which was run at 150 V for 2.5 hours. Gels were stained either with Coomassie Brilliant Blue R250 (Sigma) or with the Bio-Rad Silver Stain Kit as follows. After gel electrophoresis, gels were placed in the Fixative Enhancer Solution (methanol, 50% (vol/vol); acetic acid, 10% (vol/vol); Fixative Enhancer Concentrate, 10% (vol/vol); deionized water, 30% (vol/vol)) and fixed for 20 minutes with gentle agitation. The Fixative Enhancer Solution was decanted from the staining vessel, and the gels were rinsed twice with 400 ml deionized water for 10 minutes with gentle agitation. The staining solution (5 ml Silver Complex, 5 ml Reduction Moderator Solution, 5 ml Image Development Reagent and 50 ml room temperature Development Accelerator Solution) was prepared within 5 minutes of use. Gels were stained for approximately 20 minutes or until desired staining intensity was reached. The gels were placed in 5% acetic acid for 15 minutes to stop the reaction and rinsed in

NANOpure water for 5 minutes. The gels were then dried and photographed.

Electron Microscopy

A 50 μ l drop of CsCl-purified bacteriophages (1×10^7 pfu) was deposited on the Formvar-coated, carbon-reinforced copper grid for 2 minutes. The excess liquid was wicked off with filter paper (Whatman), and a 1% aqueous uranyl acetate solution was deposited onto the grid for 1 minute, the excess was wicked off with filter paper, and air dried for 5 minutes. The grids were examined and photographed in a Zeiss transmission electron microscope, at 80 kV. Dimensions were measured on 25 particles per bacteriophage.

Results

Isolation of various bacteriophages from two strains of *Bacillus thuringiensis* subspecies *kurstaki*

In this study, two strains of *B. thuringiensis* subspecies *kurstaki*, which is probably the most widely used *B. thuringiensis* subspecies in large-scale fermentation, were selected. The two strains used were HD119, originally isolated by H. D. Burges, and HD942 isolated by N. R. Dubois, and were chosen due to their propensity to lyse on nutrient plates in areas of high cell density. Lysis was even more apparent on plates inoculated with recombinant derivatives of these two strains (data not shown), containing either plasmid vectors (pSB909.5, Gamel and Piot, 1992) harboring additional *cry* genes, or derivatives containing *cry* genes integrated into the chromosome (Sue Kalman, personal communication). Such a phenomenon can be seen in Figure 1.

Figure 1. Lysis of culture growth of *B. thuringiensis* subspecies *kurstaki* HD119

Optimization of induction parameters

Exponentially growing cultures of HD119 and HD942 were induced with either UV irradiation or incubation with MMC. Initially, several irradiation times or MMC concentrations were used with HD942 in order to determine the optimal conditions for bacteriophage development. Those times and concentrations which gave the most reproducible results were selected for further experiments with HD119 and HD942. Growth and lysis curves of cultures of HD942 induced with various doses of UV or MMC are shown in Figure 2.

Figure 2. Induction of HD942 through ultraviolet irradiation or incubation with mitomycin C. Exponentially growing cultures were exposed to UV light for the time indicated or to MMC at the final concentration given in the legend. The A_{600} was measured at 20- minute intervals. 0 minutes : Time of induction.

As shown in Figure 2, the higher doses of inducing agent slowed growth rates immediately, as shown by the difference in A_{600} at the time when the induced (time 0) and non-induced cells were resuspended in culture media, shown as the first data point obtained after time 0. The effect was most evident with the UV treatment, and the cultures irradiated with the higher doses took longer to resume growth, maybe because of high mortality. In most cases, lysis occurred between 100 and 120 minutes after induction. On the other hand, for the MMC-treated cultures, the effect on growth was not as severe and lysis was not as rapid as with UV. The general shape of the induction curve was, however, quite different from the shape of the curve obtained when inducing other prophages such as PBSX and related phages from *Bacillus subtilis* (Manuel and Karamata, 1984). Indicating either an asynchronous development of the induced bacteriophage(s), or other unknown problems.

In order to evaluate the efficiency of the induction process, as represented by the bacteriophage titers, the culture lysates obtained above were serially diluted and aliquots were mixed with spores of *B. cereus* 569 and plated. Results are shown in Table 1.

Table 1. Bacteriophage yields following induction with ultraviolet irradiation or MMC treatment.

Treatment		pfu/ml
Control		4.7×10^6
UV	25 s	7.5×10^7
	50 s	4.6×10^7
	100 s	7.0×10^5
MMC	2.5 $\mu\text{g/ml}$	3.8×10^7
	10 $\mu\text{g/ml}$	1.6×10^7

The results in Table 1 show that the highest titers were obtained when the cultures were induced with 2.5 $\mu\text{g/ml}$ MMC or irradiated for 25 seconds with UV light, and these conditions were selected for experiments with HD942 and HD119 (Figures 3 and 4). Noteworthy is the fact that even the non-induced culture contained significant numbers of phages, consistent with the original observation that spontaneous lysis could take place on nutrient plates.

Figure 3. HD942 induction with ultraviolet irradiation for 25 seconds or mitomycin C treatment at 2.5 $\mu\text{g/ml}$.

Figure 4. HD119 induced with ultraviolet 25 seconds, mitomycin C 2.5 $\mu\text{g/ml}$.

Isolation of several types of bacteriophages with distinct plaque morphologies

When the lysates obtained in the previous experiment were plated as described, several morphologically distinct plaque types were obtained. These morphologies are presented in Table 2.

Table 2. Plaque types obtained from *B. thuringiensis* HD119 and HD942 induced cultures.

<i>B. thuringiensis</i> strain induced	Plaque morphology	Bacteriophage designation
HD942	small, clear	Ø6-5.1
	small, turbid	Ø6-2
	small, turbid, clear center	Ø6-1
	large, turbid	Ø6-3
	turbid, raised center	Ø6-4
	large, turbid, derivative Ø6-5	Ø6-5.3
HD119	small, clear	Ø12-1
	small, turbid	Ø12-2
	large, turbid	Ø12-3
	large, turbid, raised center	Ø12-7

The types of plaques obtained reflect the major subdivision among the bacteriophages, namely the existence of virulent and temperate phages. Virulent phages

lyse the bacteria they infect, producing a clear plaque. On the other hand, temperate phage often produce turbid plaques since some cells that are infected are not lysed.

Each type of plaque was further purified on a lawn of *B. cereus* 569 until a single plaque morphology could be detected; at this point the bacteriophage suspension was considered pure. Interestingly, one phage, Ø6-5, gave rise to morphological variants after purification. When the clear plaque-producing bacteriophage was purified through subculturing, variants producing large turbid plaques could be detected at a frequency of about 10^{-4} . This phenotype was stable upon subculturing, and the clear plaque phage and the turbid plaque mutant were given the designations Ø6-5.1 and Ø6-5.3 respectively.

In order to obtain sufficient numbers of bacteriophages, the pure suspensions were plated on a lawn of *B. cereus* 569. Upon plaque development, the overlay was scraped off the plates and macerated. The dense mixture of *B. cereus* 569 cells, agar, and bacteriophages was centrifuged, and the supernatant containing the bacteriophages was collected and titered on a lawn of *B. cereus* 569.

Lysogenization of *B. cereus* 569

A droplet containing 1×10^3 to 1.8×10^5 pfu was deposited ("spotted") on a lawn of *B. cereus* 569 spores. As expected, the suspensions containing plaque-forming units (pfu) which originally yielded turbid plaques also produced turbid spots, indicating the temperate nature of the bacteriophage. On the other hand, the two clear plaque-producing suspensions yielded clear spots, but some islands of apparently resistant bacteria were observed on the edge of the spots. The presence of the islands could indicate either growth of cells resistant to the bacteriophages or cells that were immune to them and hence lysogenic. In order to determine whether lysogens had been obtained, cells were taken

from the center of the turbid spots or from the islands described above and streaked on a nutrient plate. A number of isolated colonies were obtained and examined for their sensitivity to bacteriophage infection. The colonies were individually resuspended in a small volume of media which was then overlaid on a nutrient plate. Once set, the same bacteriophage suspension which was originally used to obtain these isolates was spotted on the lawn. Although a number of isolates still were susceptible to the bacteriophage, as shown by the presence of a spot, several isolates did not show any clearing and were further examined for their ability to yield bacteriophages upon induction. The induction procedure was essentially the same as the one used for *B. thuringiensis*, except that only MMC was used as an inducing agent. Three concentrations of MMC were first evaluated in order to optimize pfu yields. Results of these preliminary experiments run with *B. cereus* 569::Ø6-2 are presented in Table 3.

Table 3. Phage titers obtained with various mitomycin C concentrations on *B. cereus* 569::Ø6-2.

MMC treatment	pfu/ml
control	1.1×10^7
0.1 µg/ml	3.9×10^{10}
1 µg/ml	2.5×10^{10}
10 µg/ml	1.0×10^8

As shown in Table 3, the highest yields were obtained when the lysogens were induced with 0.1 µg/ml or 1 µg/ml MMC; however, 1 µg/ml gave more reproducible results (not shown), probably due to the instability of MMC. This concentration was used throughout the rest of the study.

For the purpose of biochemical characterization and bacteriophage typing, significant amounts of the various bacteriophages were needed. To that end, each of the *B. cereus* 569 lysogens was induced with MMC. The induction curves are presented in Figures 5 and 6.

Figure 5. *B. cereus* 569 lysogens 6-1, 6-2, 6-3 and 6-5.3 induced with 1 $\mu\text{g/ml}$ mitomycin C.

Figure 6. *B. cereus* 569 lysogens 12-2, 12-3 and 12-7 induced with 1 μ g/ml mitomycin C.

In contrast with what was observed when *B. thuringiensis* was induced, the lysis pattern of many of the lysogenic strains was reminiscent of that obtained in other systems such as PBSX in *Bacillus subtilis* (Manuel and Karamata, 1984). However, the notable exception was the strain harboring Ø6-1, which did not lyse as well as the others.

The titers of the crude lysates were determined by plating aliquots on *B. cereus* 569. These results are presented in Table 4.

Table 4. Bacteriophage titers obtained after induction of *B. cereus* 569 lysogens with 1 µg/ml mitomycin C.

Bacteriophage	pfu/ml
Ø6-1	4.7×10 ⁶
Ø6-2	9×10 ¹⁰
Ø6-3	1.2×10 ¹¹
Ø6-4	6.7×10 ¹⁰
Ø6-5.3	4.5×10 ¹⁰
Ø12-2	2.5×10 ¹⁰
Ø12-3	2.1×10 ¹⁰
Ø12-7	3.1×10 ¹⁰

Since no *B. cereus* 569 lysogens could be obtained with the two virulent bacteriophages, Ø6-5.1 and Ø12-1, an alternative method was used for propagation of these two phages (see Materials and methods). Several multiplicities of infection (m. o. i., ratio of bacteriophage/bacteria) were tested in order to determine the conditions giving the highest yields of bacteriophages. Results of experiments run with Ø6-5.1 are shown in

Figure 7 and Table 5.

Figure 7. A_{600} of *B. cereus* 568 lysogen of Ø6-5.1 at various time points, m. o. i. 0.1, 0.01, and 1.

Table 5. Yields of bacteriophage obtained from cultures infected with Ø6-5.1 at various m. o. i.

m.o.i	titer liquid (a)	titer plate (b)
1	6×10^8	1.95×10^{10}
0.1	1×10^8	3.4×10^{10}
0.01	1×10^8	1.5×10^{10}

(a): titers obtained from cultures after an overnight incubation in liquid medium. (b): titers obtained after a further round of multiplication in soft PA agar, where the lysates from (a) were plated on the PA plates, with the addition of 1×10^8 *B. cereus* 569 spores if the culture was cleared.

A m. o. i. of 1 gave confluent lysis of the *B. cereus* 569 lawn and gave high, consistent titers for both Ø6-5.1 and Ø12-1. The highest titers were obtained when a phage-host mixture is used which gives almost confluent lysis at the time of maximum growth of the host. The results presented in Figure 7 and Table 5 show that although m. o. i. ranging from 0.01 to 1 were used, the titers obtained from liquid cultures infected with bacteriophages Ø6-5.1 vary only by a factor of about five, and that titers obtained from the plates by a factor of about two.

Genetic and biochemical characterization were carried out on the bacteriophages obtained by this method. Eventually, *B. cereus* 569 lysogens were acquired for bacteriophages 6-5.1 and Ø12-1 and the biochemical and genetic characterizations were

repeated with these bacteriophages. The induction graphs and titers are shown in Figure 8 and Table 6.

Figure 8. *B. cereus* 569 lysogens of bacteriophage Ø6-5.1 and Ø12-1 induced with 1 µg/ml mitomycin C.

Table 6. Bacteriophage Ø6-5.1 and Ø12-1 titers obtained after induction of *B. cereus* 569 lysogens with 1 µg/ml mitomycin C.

Bacteriophage	pfu/ml
Ø6-5.1	4×10 ⁹
Ø12-1	1.6×10 ¹⁰

However, the prophage obtained from the induction of lysogenic *B. cereus* 569 of bacteriophage Ø6-5.1 and Ø12-1 differed from the original bacteriophage obtained from the m. o. i. method (see Biochemical and genetic characterization section).

Phage typing (cross spotting)

In order to determine whether the various types of bacteriophages were related to each other, each phage suspension was spotted on a lawn containing spores of each of the lysogens. Results of this phage typing experiment are presented in Table 7.

Table 7. Phage typing (cross-spotting).

Phage suspension	Indicator strain									
	Bc 569	Ø6-1	Ø6-2	Ø6-3	Ø6-4	Ø6-5.1	Ø6-5.3	Ø12-1	Ø12-2	Ø12-7
Ø6-1	+	-	+	+	+	-	+	-	+	+
Ø6-2	+	+	-	-	-	+	-	-	-	-
Ø6-3	+	+	-	-	-	+	-	-	-	-
Ø6-4	+	+	-	-	-	+	-	-	-	-
Ø6-5.1(a)	+	+	+	+	+	+	+	+	+	+
Ø6-5.3	+	+	-	-	-	+	-	-	-	-
Ø12-1(a)	+	+	+	+	+	-	+	-	+	+
Ø12-2	+	+	-	-	-	+	-	-	-	-
Ø12-3	+	+	-	-	-	+	-	-	-	-
Ø12-7	+	+	-	-	-	+	-	-	-	-
Ø6-5.1(b)	+	+	+	+	+	+	+	+	+	+
Ø12-1(b)	+	+	+	+	+	+	+	-	+	+

(+): lysis of indicator; (-): no lysis of indicator; (a): lysates obtained from plates; (b): lysates obtained from induced *B. cereus* 569 lysogen.

From the results presented in Table 7, it can be concluded that phages Ø6-2, Ø6-3, Ø6-4, Ø6-5.3, Ø12-2, Ø12-3, and Ø12-7 are closely related, if not identical, since the lysogens harboring those phages are immune to superinfection with the other phages in that group. Therefore, Ø6-2 was selected as the prototype. Other groups comprised phages Ø6-5.1(a), Ø6-5.1(b) and Ø12-1(a), Ø12-1(b) and Ø6-1, respectively and were selected for further studies.

Biochemical and genetic characterization of bacteriophages isolated from *B. thuringiensis* subspecies *kurstaki* HD119 and HD942

Large scale purification of Ø6-1, Ø6-2, Ø6-5.1 and Ø12-1

In order to obtain large concentrations of each phage for protein and DNA analysis each lysogen was grown in a 2 liter flask containing 500 ml of medium. The induction curves and titers obtained in these experiments are shown in Figure 9 and Table 8.

Figure 9. Induction of *B. cereus* 569 lysogens, 500 ml cultures. The cultures were induced at time=0 with 1 µg/ml MMC.

Table 8. Phage titers obtained from 500-ml cultures of *B. cereus* 569 lysogens induced with 1 µg/ml mitomycin C.

Bacteriophage	pfu/ml
Ø6-1	4.7×10 ⁶
Ø6-2	2.8×10 ¹⁰
Ø6-5.1	2×10 ⁷
Ø12-1	2×10 ⁶

Due to the relatively low titers for all of the phage lysates obtained from the large-scale cultures, except Ø6-2, as opposed to those obtained from small scale inductions, the induction experiments were repeated with 100-ml cultures grown in 500-ml flasks. Titers obtained in these experiments were higher (Table 9).

Table 9. Phage titers obtained after induction of *B. cereus* 569 lysogens grown in 100 ml cultures.

Bacteriophage	pfu/ml
Ø6-1	7×10^7
Ø6-2	9×10^{10}
Ø6-5.1	5×10^9
Ø12-1	3×10^9

The cultures were induced with 1 µg/ml MMC at T=0.

The decrease in the culture size resulted in all the cases in an increase in the phage titers. The increase ranged from about 3-fold for Ø6-2 to over a 1000-fold for Ø12-1, indicating a possible effect of the growth rate limitation due to insufficient aeration on the outcome of bacteriophage development. Following this finding, the lysogens were grown in 100 ml cultures for all subsequent induction experiments.

Cesium chloride gradient purification of bacteriophage stocks

After concentration from the crude lysates, the phage stocks were loaded onto preformed, discontinuous cesium chloride (CsCl) gradients as described in the Materials and Methods section. After centrifugation, an iridescent bluish band, characteristic of concentrated bacteriophage stocks could be seen at different locations in the gradients depending on the phage (Figure 10).

lane   1   Ø6-1
          2   Ø6-2
          3   Ø6-5.1(a)
          4   Ø6-5.1(a)
          5   Ø12-1(a)
          6   Ø12-1(a)

Figure 10. CsCl gradients of concentrated bacteriophage stocks. 1). Ø6-1, 2). Ø6-2, 3 and 4). Ø6-5.1, and 5 and 6). Ø12-1. The pictures were taken after a 4 hour centrifugation. (a): phage obtained by infection of *B. cereus* 569 spores.

Since there were major differences in the appearance of the bands in the gradients, the recovery yields were determined. The results are shown in Table 10.

Table 10. Recovery of bacteriophage applied to cesium chloride gradients.

Bacteriophage	pfu loaded	pfu recovered	yield pfu loaded/pfu recovered
Ø6-1	1×10^6	8×10^5	1.25
Ø6-2	2×10^{13}	3.2×10^{12}	6.25
Ø6-5.1	2.7×10^9	2.4×10^6	1125
Ø12-1	2.4×10^{10}	1.3×10^6	18462

Morphological characterization of the bacteriophages

After purification on CsCl gradients, the bacteriophages were visualized by transmission electron microscopy (TEM), using the negative contrast staining technique. Pictures of the representative samples are shown in Figure 11. The dimensions of the phages were also measured and are given in Table 11.

A.

B.

C.

D.

Figure 11. Electron micrographs of bacteriophages A.) Ø6-1, B.) Ø6-2, C.) Ø6-5.1 and D.) Ø12-1.

Table 11. Dimensions of bacteriophage particles. Values are the average of 25 particles.

Bacteriophage	Dimensions	
	Head diameter (nm)	Tail length (nm)
Ø6-1	21	42
Ø6-2	22	60
Ø6-5.1	22	N/A
Ø12-1	17	56

As shown in Figure 11, bacteriophages Ø6-1, Ø6-2 and Ø12-1 have similar particle morphologies and dimensions. They are composed of a hexagonal head and a flexible and non-contractile tail. On the other hand, Ø6-5.1 had no visible tail and circular shaped head.

Biochemical characterization of the bacteriophages

In order to determine the sizes of the structural proteins of the bacteriophages, aliquots corresponding to 6×10^3 pfu were subjected to SDS-PAGE (Figure 12). The apparent molecular weights of the structural proteins were calculated based on the relative mobilities of the standard proteins. These values are given in Table 12.

lane

1	Bio-Rad broad mol. standard	6	Ø6-5.1(b)
2	Ø6-1(a)	7	Ø12-1(a)
3	Ø6-1(b)	8	Ø12-1(b)
4	Ø6-2	9	Bio-Rad broad mol. wt. standard
5	Ø6-5.1(a)	10	Bio-Rad high mol. wt. standard

Figure 12. SDS-PAGE gels of bacteriophage structural proteins. (a): designates propagation of phage by multiplicity of infection (plate method). (b): designates propagation of phage by induction of *B. cereus* 569 lysogen. The gel was silver stained.

Biorad molecular weight marker bands:

molecular weight (daltons)	high range	broad range
200,000	X	X
116,500	X	X
97,400	X	X
66,200	X	X
45,000	X	X
31,000		X
21,500		X
14,400		X
6,500		X

Table 12. Apparent molecular weight of phage proteins.

Phage	Molecular weight (kDa)	
	Major proteins	Minor proteins
Ø6-1(a)	17, 15	
Ø6-1(b)	11, 15	265, 228
Ø6-2	21, 18	15
Ø6-5.1(a)	26, 15	34, 28
Ø6-5.1(b)	17, 16	300, 197, 172
Ø12-1(a)	22	17, 17
Ø12-1(b)	20	197, 151, 39, 35

(a): bacteriophages obtained by infection of *B. cereus* 569 cells. (b): bacteriophages obtained from induced *B. cereus* 569 lysogens on plates denote phage propagated by the multiplicity of infection method.

Analysis of bacteriophage DNA

In order to compare the patterns of the various phage DNA restriction sites, DNA was extracted from crude bacteriophage stocks by two phenol extractions performed at 45°C, followed by two chloroform extractions. The DNA was subsequently digested with a number of restriction endonucleases, and the digests were analyzed by agarose gel electrophoresis, the *Sau3AI* digest is shown in Figure 13. Results on the occurrence of restriction sites are presented in Table 13.

lane	1	BRL 1 kb ladder	5	Ø12-1(b)
	2	Ø6-1(b)	6	Ø6-2(a)
	3	Ø6-2(b)	7	Ø6-5.1(a)
	4	Ø6-5.1(b)	8	Ø12-1(a)

Figure 13. Agarose gel electrophoresis of bacteriophage DNA digested with *Sau3AI* restriction endonuclease. (a): bacteriophages obtained from induced *B. cereus* 569 lysogens. (b): bacteriophages obtained by infection of *B. cereus* 569 cells on plates. The BRL 1 kb ladder:

fragment size (kb)	1	12.2	9	4.07	17	0.34
	2	11.2	10	3.05	18	0.29
	3	10.2	11	2.04	19	0.22
	4	9.16	12	1.64	20	0.20
	5	8.14	13	1.02	21	0.15
	6	7.13	14	0.52	22	0.13
	7	6.11	15	0.51	23	0.08
	8	5.09	16	0.39		

Table 13. Determination of the presence of restriction endonuclease sites on bacteriophage DNA.

Restriction enzyme	Bacteriophage					
	Ø6-1	Ø6-2	Ø6-5.1(a)	Ø12-1(a)	Ø6-5.1(b)	Ø12-1(b)
<i>Bam</i> HI	+	-	+	+	+	-
<i>Eco</i> RI	+	+	-	+	+	+
<i>Hind</i> III	+	+	-	-	-	-
<i>Kpn</i> I	-	+	+	+	-	-
<i>Nde</i> I	+	+	-	-	-	-
<i>Sac</i> II	-	-	-	-	+	-
<i>Sau</i> 3AI	+	+	+	+	+	+
<i>Xba</i> I	+	+	-	-	+	+

(a): bacteriophages obtained by infection of *B. cereus* 569 cells on plates. (b): bacteriophages obtained from induced *B. cereus* 569 lysogens. (+): presence of restriction sites. (-): absence of restriction sites.

Based on the restriction patterns observed, the bacteriophage genomes were digested with *Eco*RI, due to the limited number and size range of the restriction fragments obtained with this enzyme. The fragment sizes were carefully determined (Table 14), and the genome sizes (Table 14) were calculated by summing up the sizes of the *Eco*RI fragments. These ranged from 29,400 to 38,270 kb. The DNA for phage Ø6-5.1 degraded upon digestion with all restriction endonucleases and was therefore not included in Table 14.

Table 14. Sizes of *EcoRI* restriction fragments and deduced total genome sizes of *B. thuringiensis* bacteriophages.

Ø6-1	Ø6-2	Bacteriophage		
		Ø6-5.1(b)	Ø12-1(a)	Ø12-1(b)
9550	10,580	7930	10,580	6830
6860	7620	5900	7620	4860
5710	6500	5110	6500	4430
3830	2840	3680	2840	3210
2960	1860	3070	1860	2810
2220		2340		2570
2050		2340		2570
1680		2160		2070
1030		1820		1900
890		1170		710
630		1000		590
590		710		
		660		
		380		
Total genome (bp)				
38,000	29,400	38,270	29,400	32,550

(a): bacteriophages obtained by infection of *B. cereus* 569 cells on plates. (b): bacteriophages obtained from induced *B. cereus* 569 lysogens.

Restriction site mapping of bacteriophage Ø6-2

In order to obtain a partial genetic map of bacteriophage Ø6-2, its genome was partially or completely digested with *KpnI* or *HindIII*, which cut only a limited number of times. The mapping was performed using the Flash Nonradioactive Gene Mapping Kit from Stratagene. The order of restriction fragments was determined based on the pattern obtained from the restriction site mapping kit results (Figure 14).

Figure 14. Physical map of bacteriophage Ø6-2 for restriction endonucleases *Hind*III and *Kpn*I. H: *Hind*III sites. K: *Kpn*I sites. Fragments sizes are not drawn to scale.

Genetic evidence of the similarity of Ø6-2-like bacteriophages

It was assumed earlier that several bacteriophages which did not plate on the *B. cereus* 569 strain lysogenic for Ø6-2 must have shared similar genetic determinants. In order to verify this hypothesis, the restriction patterns of the genome of these bacteriophages were compared (Figure 15). Each bacteriophage DNA in the Ø6-2 family was digested with *Dra*I and analyzed by agarose gel electrophoresis.

lane	1	BRL 1 kb ladder	5	Ø6-4
	2	Ø12-7	6	Ø6-5.3
	3	Ø6-2	7	Ø12-2
	4	Ø6-3	8	Ø12-3

Figure 15. Restriction patterns of bacteriophages belonging to the Ø6-2 family. Bacteriophage DNA was digested with *DraI* and the digests were analyzed by agarose gel electrophoresis. The amount of Ø12-7 DNA was too low to be visualized on the gel. The BRL 1 kb ladder:

fragment size (kb)	1	12.2	9	4.07	17	0.34
	2	11.2	10	3.05	18	0.29
	3	10.2	11	2.04	19	0.22
	4	9.16	12	1.64	20	0.20
	5	8.14	13	1.02	21	0.15
	6	7.13	14	0.52	22	0.13
	7	6.11	15	0.51	23	0.08
	8	5.09	16	0.39		

As shown in Figure 15, the identity of the restriction patterns supports the hypothesis that all of these bacteriophages belonged to the same family.

Determination of the host range of the various bacteriophages

The host ranges of the bacteriophages were determined in order to assess their potential distribution within the *B. thuringiensis* species. Each of the four bacteriophage was spotted on a lawn of indicator spores belonging to representative isolates of various subspecies of *B. thuringiensis*. Each has a unique host range (Table 15) when tested with strains of *B. thuringiensis* and *B. cereus* 569.

Table 15. Host ranges of four bacteriophages.

Bacterial strain		Bacteriophage			
strain	subspecies	Ø6-1	Ø6-2	Ø6-5.1	Ø12-1
<i>B. thuringiensis</i>					
HD73	<i>kurstaki</i>	+	-	+	+
HD2	<i>thuringiensis</i>	-	-	-	-
HD229	<i>aizawai</i>	+	+	+	-
HD1	<i>kurstaki</i>	+	+	+	+
SA3 (a)	<i>israelensis</i>	+	+	+	-
HD263	<i>kurstaki</i>	-	-	+	+
SA10 (a)	<i>tenebrionis</i>	+	+	-	+
Int 15-313 (a)	<i>kurstaki</i>	+	-	+	+
B.t.	<i>sotto</i>	+	+	+	+
B.t.	<i>pakistani</i>	-	-	+	+
HD137	<i>aizawai</i>	+	+	-	+
B.t.	<i>kyushuensis</i>	-	-	+	-
Cutlass™ (b)		-	-	-	-
B.t.	<i>ostriniae</i>	-	-	-	-
Foil™ (b)		-	-	-	+
<i>B. cereus</i>					
NRRL B-569		+	+	+	+

(a): Sandoz strains; (b): isolates obtained from registered products from Ecogen Inc. (Langhorne, PA); (+): lysis of test strain; (-): no lysis of test strain. Bacteriophages Ø6-5.1 and Ø12-1 were obtained from plates containing infected lawns of *B. cereus* 569.

Determination of the efficiency of plating (eop) on various indicator strains

The titer of the phages was determined by plating various concentrations of Ø6-1 and Ø6-2 lysate in 1×10^8 spores of each susceptible *B. thuringiensis* indicator strain. The titers obtained are presented in Table 16, and the plating efficiencies are calculated in Table 17.

Table 16. Titters of plating bacteriophages Ø6-1, Ø6-2, Ø6-5.1 and Ø12-1 on various *B. thuringiensis* isolates and *B. cereus* 569.

Bacterial spores strain subspecies	Titer (pfu/ml)			
	Ø6-1	Ø6-2	Ø6-5.1	Ø12-1
HD73 <i>kurstaki</i>	-	-	1.6×10 ³	-
HD2 <i>thuringiensis</i>	-	-	8×10 ²	-
HD229 <i>aizawai</i>	-	-	3×10 ³	2.3×10 ¹⁰
HD1 <i>kurstaki</i>	-	-	1×10 ²	2×10 ⁵
SA3 (a) <i>israelensis</i>	-	7.7×10 ⁸	3×10 ²	1.8×10 ⁸
HD263 <i>kurstaki</i>	-	-	-	-
SA10 (a) <i>tenebrionis</i>	-	2.7×10 ⁹	3.2×10 ³	-
Int 15-313 (a) <i>kurstaki</i>	-	-	3.5×10 ³	-
B.t. <i>sotto</i>	-	-	-	-
B.t. <i>pakistani</i>	5×10 ³	1.6×10 ⁹	1×10 ²	1×10 ⁵
HD137 <i>aizawai</i>	6×10 ⁵	-	5.6×10 ³	3×10 ⁵
B.t. <i>kyushuensis</i>	-	-	7×10 ³	-
Cutlass™ (b)	-	-	-	-
B.t. <i>ostriniae</i>	-	-	-	-
Foil™ (b)	-	-	1.5×10 ⁴	-
<i>B. cereus</i>				
NRRL B-569	7.5×10 ⁸	3.2×10 ¹¹	6.9×10 ⁴	1.2×10 ¹²

(a): Sandoz strains; (b): isolates obtained from registered products from Ecogen Inc. (Langhorne, PA) ; (-): phage did not produce a titer.

Table 17. Efficiency of plating bacteriophages Ø6-1, Ø6-2, Ø6-5.1 and Ø12-1 on various *B. thuringiensis* isolates and *B. cereus* 569.

Bacterial spores strain	subspecies	Efficiency of plating			
		Ø6-1	Ø6-2	Ø6-5.1	Ø12-1
HD73	<i>kurstaki</i>	-	-	2.3×10 ⁻²	-
HD2	<i>thuringiensis</i>	-	-	1.2×10 ⁻²	-
HD229	<i>aizawai</i>	-	-	4.3×10 ⁻²	1.9×10 ⁻²
HD1	<i>kurstaki</i>	-	-	1.4×10 ⁻³	1.7×10 ⁻⁷
SA3 (a)	<i>israelensis</i>	-	2.4×10 ⁻³	4.3×10 ⁻³	1.5×10 ⁻⁴
HD263	<i>kurstaki</i>	-	-	-	-
SA10 (a)	<i>tenebrionis</i>	-	8.4×10 ⁻³	4.6×10 ⁻²	-
Int 15-313 (a)	<i>kurstaki</i>	-	-	5.1×10 ⁻²	-
B.t.	<i>sotto</i>	-	-	-	-
B.t.	<i>pakistani</i>	6.7×10 ⁻⁶	5×10 ⁻³	1.4×10 ⁻³	8.3×10 ⁻⁸
HD137	<i>aizawai</i>	8×10 ⁻⁴	-	8.1×10 ⁻²	2.5×10 ⁻⁷
B.t.	<i>kyushuensis</i>	-	-	1.0×10 ⁻¹	-
Cutlass™ (b)		-	-	-	-
B.t.	<i>ostriniae</i>	-	-	-	-
Foil™ (b)		-	-	2.2×10 ⁻¹	-
<i>B. cereus</i>					
NRRL B-569		1	1	1	1

(a): Sandoz strains; (b): isolates obtained from registered products from Ecogen Inc. (Langhorne, PA); (-): phage did not produce a titer.

The results presented above show that the efficiency of plating on the *B. thuringiensis* hosts was 5- to 5×10⁷- fold lower than the efficiency of plating on *B. cereus* 569, which was used to propagate the phages.

In order to determine whether the plating efficiency was influenced by the origin of the bacteriophage (i. e. the strain on which the phage was propagated) bacteriophage Ø6-1 and Ø6-2 were grown on various *B. thuringiensis* strains as follows. Phages from

plaques isolated from *B. cereus* 569 were mixed with various *B. thuringiensis* indicators and plated. From these plates, phages were again isolated from plaques and repurified twice on the same indicator strain. Finally, an isolated plaque was resuspended and dilutions were plated on the host strain used to propagate the phage as well as on the indicator *B. cereus* 569. Results from this experiment are shown in Table 18.

Table 18. Influence of the host strain on the efficiency of plating of bacteriophages Ø6-1 and Ø6-2.

Bacteriophage	Purification bacterial strain origin (a)	Titers on	
		origin strain	B.c. 569
Ø6-1	B.t. HD137	1.9×10 ⁷	2.1×10 ⁷
Ø6-1	B.t. <i>pakistani</i>	3.9×10 ⁷	4×10 ³
Ø6-2	B.t. SA3	1.4×10 ⁶	2×10 ⁴
Ø6-2	B.t. <i>pakistani</i>	1.1×10 ⁷	2.7×10 ⁴

(a): strain on which the phages were propagated. B. t.: *B. thuringiensis* . B. c. : *B. cereus* 569.

With the exception of the phages propagated on strain HD137, which plated with an equal efficiency on both the origin strain and *B. cereus* 569, all other phages plated with 10² to 10⁴ lower efficiency on *B. cereus* 569 than on the strain from which they originated.

Detection of the bacteriophage Ø6-2 by polymerase chain reaction

Polymerase chain reaction (PCR) was used as an alternative to physiological methods for the detection of bacteriophage within *B. thuringiensis*. Two sets of oligonucleotide primers were designed for the detection of phage Ø6-2, based on the sequence data obtained (not shown) in the course of this study (Table 19). The two sets of primers were complimentary to two different fragments of the genome of the bacteriophage Ø6-2. LA19 and LA20 produced a fragment of 126 bp, while LA21 and LA22 produced a product of 800 bp.

Table 19. Oligonucleotide primers for the detection of Ø6-2.

Primer designation	Sequence	G+C (a)	T _m (b)	Amplicon size (c)
LA19	ATCTTTCACAACAACCTTTAGGCTATCCC	41	60.1	126 bp
LA20	GTAAGTTTGGATGTGTTAGATGAAGCAGCC	43	61.8	
LA21	GAGCAAGCAATTGAAGCGTAGGGA	50	60.4	800 bp
LA22	CCAACCTCAATTAACCTTTGAGATACGCCT	40	62.3	

(a): Percent G+C. (b): Melting temperature. (c): Calculated from the sequence data.

PCR amplifications were run using these primer sets and crude DNA isolated from various strains of *B. thuringiensis* in order to determine the presence of phage Ø6-2 (Table 20).

Table 20. Amplification of fragments of bacteriophage DNA by PCR.

Source of DNA		Subspecies	PCR amplification (a)	
			LA19/20	LA21/22
Bacteriophage	Ø6-1		-	-
	Ø6-2		+	+
	Ø6-5.1		-	-
	Ø12-1		+	+
<i>B. cereus</i>	569::6-1		-	-
	569::6-2		+	+
	569		-	-
<i>B. thuringiensis</i>	INT 15-313		-	-
	HD1	<i>kurstaki</i>	-	-
	HD12	<i>morrisoni</i>	-	-
	HD52	<i>aizawai</i>	+	+
	HD73	<i>kurstaki</i>	-	-
	HD112	<i>aizawai</i>	+	+
	HD125	<i>tolworthi</i>	+	+
	HD127	<i>aizawai</i>	+	+
	HD131	<i>aizawai</i>	+	+
	HD132	<i>aizawai</i>	+	+
	HD133	<i>aizawai</i>	+	+
	HD134	<i>aizawai</i>	+	+
	HD136	<i>kenyae</i>	-	-
	HD137	<i>aizawai</i>	+	+
	HD147	<i>darmstadiensis</i>	-	-
	HD198	<i>entomocidus</i>	-	-
	HD203	<i>kurstaki</i>	-	-
	HD207	<i>galleriae</i>	-	-
	HD228	<i>aizawai</i>	+	+
	HD229	<i>aizawai</i>	+	-
	HD232	<i>galleriae</i>	-	-
	HD234	<i>galleriae</i>	+	+
	HD245	<i>kurstaki</i>	-	-
	HD248	<i>aizawai</i>	+	+
	HD263	<i>kurstaki</i>	+	+
	HD272	<i>aizawai</i>	+	+
	HD275	not identified	-	+
	HD282	<i>aizawai</i>	+	+
	HD283	<i>aizawai</i>	+	+
	HD301	<i>tolworthi</i>	+	+
	HD567	<i>israelensis</i>	-	-

(+): presence of a band of correct size; (-): absence of a band of correct size.

In most of the cases, the reactions were either positive or negative for both sets of primers, but in two cases (HD229 and HD275), only one of the reactions gave a PCR product of the correct size. It is noteworthy that only one of the subspecies *kurstaki* isolates (HD263) was positive.

Discussion

In this study, bacteriophages isolated from two strains of *B. thuringiensis* subspecies *kurstaki* were characterized at the physiological, biochemical and genetic levels. This subspecies was selected because it is the most widely used in large-scale industrial production of biological insecticides. The two strains used in the investigation, HD119 and HD942, were chosen because of their propensity to lyse on nutrient plates in areas of high cell density. This phenomenon was even more apparent on plates inoculated with recombinant derivatives of these two strains containing, either plasmid vectors such as pSB909.5 harboring additional *cry* genes (Gamel and Piot, 1992), or derivatives containing *cry* genes integrated into the chromosome (Sue Kalman, personal communication). This has important implications for the prospect of future development of transgenic derivatives of *B. thuringiensis* strains, since lysis of the bacterial culture during fermentation can be a major source of yield loss.

A number of bacteriophages, distinguishable mainly by the morphology of their plaques, were isolated. These bacteriophages belonged to either of the two main types of phages, namely virulent phages, which lyse the bacteria they infect after an intracellular round of multiplication, and the temperate phages, which have the alternative of either lysing the infected bacteria in the same way as the virulent phages, or establishing a symbiotic relationship with the host cell. Bacteria that carry phage in this way are known as lysogenic, and the carried phage is known as a prophage. The decision toward a lytic or lysogenic response is largely determined by the physiological state of the bacteria and can be modified by environmental conditions (Calendar, 1988). Plaques produced by virulent phage are nearly always clear, since all the infected bacteria lyse, while infection by

temperate phage yield turbid plaques due to the growth, within the plaque, of the lysogenic bacteria which have become immune to superinfection. Most of the bacteriophages isolated were temperate (Ø6-1, Ø6-2, Ø6-3, Ø6-4, Ø6-5.3, Ø12-2, Ø12-3 and Ø12-7) while only two were virulent (Ø6-5.1 and Ø12-1).

All of the temperate bacteriophages could be separated in two classes by determining their ability to produce a clearing when spotted on lysogenic derivatives of *B. cereus* 569 containing each of the other phages. A bacteriophage which shares identical regulatory functions with a prophage, cannot superinfect a strain lysogenic for that homologous prophage. Thus, the inability of bacteriophages Ø6-2, Ø6-3, Ø6-4, Ø6-5.3, Ø12-2, Ø12-3 and Ø12-7 to produce a clearing upon spotting phage stocks on a lawn of *B. cereus* 569 lysogenic for each of the other bacteriophages allowed them to be grouped in a single immunity class. This classification was further verified at the genetic level by isolating the genomic DNA of all the different bacteriophages in this class and digesting these DNAs with restriction endonucleases. The restriction patterns of all of the various phage genomes were identical, indicating a complete homology within the limits of resolution of this method, and thus showing that the absence of plating capability was indeed due to immunity to superinfection, even though the morphologies of the individual plaques were quite different.

Although the physiological methods used detect the ability of the phages to infect various *B. thuringiensis* strains (spotting, plating efficiency determination) were generally consistent, there were a few instances where equivocal results were obtained. For example when bacteriophages Ø6-1 was spotted on lawns of *B. thuringiensis* HD73, HD229 or HD1, a spot of clearing could be observed. Yet, when dilutions of Ø6-1 were plated on the same *B. thuringiensis* strains, no plaques could be detected, although the range of

dilutions of the bacteriophage stocks was broad enough to exclude the possibility of missing the phage concentration allowing detection of the phages. A possible explanation for these results could be the phenomenon known as lysis from without. In the spot test a highly concentrated stock of the bacteriophage is added to a small area of the plate containing approximately 1×10^6 spores, so that the multiplicity of infection is greater than 1. In such a case, a large number of phages are able to lyse the cell at the time of adsorption, although the cells may be in fact immune to superinfection, as shown by the absence of plating efficiency. The opposite result was also observed. When bacteriophage Ø6-1 was plated on *B. thuringiensis* subspecies *pakistani*, no clearly defined spot was visible, yet when dilutions of the phage stock were plated, small isolated plaques could be seen. This latter result pointed to the limitation of these physiological tests and a more reliable methodology was sought. A likely candidate is the application of PCR analysis to the detection of bacteriophage genetic determinants in the bacterial genome. In order to develop this tool, the nucleotide sequence of several fragments of bacteriophage Ø6-2 was determined, and oligonucleotide primers were derived from the sequence information.

A comparison of the results obtained with PCR detection with those of the spot tests and efficiency of plating experiments showed that the level of sensitivity of the physiological methods were insufficient. The presence of a spot in the spot test was originally attributed to the absence of the corresponding prophage. By contrast, the inability of the bacteriophage to form plaques when titered on the various indicator strains was interpreted as the presence of the corresponding prophage. These observations taken alone did not allow unequivocally ascertainment of the presence or the absence of the prophages. In particular, *B. thuringiensis* subspecies *kurstaki* HD1, and *B. thuringiensis* subspecies *aizawai* HD137 and HD229 gave ambiguous results, since they showed a

clearing in the spot test yet were unable to allow plaque formation. As mentioned above, the opposite case was observed with *B. thuringiensis* subspecies *pakistani*, which did not show a spot but did allow plaquing of Ø6-2.

The implementation of PCR analysis helped to clarify these results. For strains HD137, HD263 and HD229, the absence of plaquing capability correlated with positive PCR reactions, indicating the presence of Ø6-2. The fact that only one of the primer sets gave positive results with HD229 could be due to the larger size of the PCR product and sub-optimal amplification conditions or, alternatively, to some genetic rearrangements taking place within the boundaries defined by the oligonucleotide primers. For strains HD73 and INT-15-313, the absence of spotting and plaquing capability together with the negative PCR results pointed to the resistance of these strains, possibly because of the absence of specific receptors for Ø6-2 on the bacterial surface. Some ambiguity remained in the case of HD1, for which no clear interpretation of the results could be inferred; the strain was susceptible to the phage at high m. o. i., yet was unable to support plaquing and was PCR-negative.

When bacteriophage stocks propagated on *B. cereus* 569 were plated on various *B. thuringiensis* strains, two types of responses were observed. In many cases, no plaques could be seen, pointing to either resistance or immunity to the phages. In the other cases, the titers obtained on *B. thuringiensis* were much lower than those seen on *B. cereus*. In order to determine whether these differences in plating efficiencies were dependent on the origin of the bacteriophages. Two of the phages were propagated on several *B. thuringiensis* strains, and the titers of the stocks were determined on *B. cereus* as well as on the strain used for propagation. The titers of these phage stocks were lower on *B. cereus* than on *B. thuringiensis*, with the notable exception of Ø6-1 propagated on

strain HD137. It thus seems that among the four strains examined, three may have one or more modification/restriction systems that are distinct from those of *B. cereus*. It can be hypothesized that HD137 contains all of the restriction/modification systems of *B. cereus*, since phages propagated on HD137 must contain at least one supplemental restriction/modification system to account for the decrease in plating efficiency of phages propagated on *B. cereus* 569.

Transmission electron microscopy was used to visualize the phage particles. The general morphologies of bacteriophages Ø6-1, Ø6-2 and Ø12-1 were similar. They had a hexagonal head and noncontractile tail. On the other hand, bacteriophage Ø6-5.1 had a circular head and no tail could be detected. The dimensions of the bacteriophages were determined by measuring electron micrographs. Surprisingly, in spite of their extensive homology at the genetic level, bacteriophages Ø6-2 and Ø12-1 displayed significant morphological differences, in the size of the head (22 nm for Ø6-2 and 17 nm for Ø12-1). This is reminiscent of the results of Kochkina *et al.* (1978) and Kochkina (1986a), who made similar observations with a temperate phage of *B. thuringiensis* subspecies *galleriae* and its virulent mutant.

A puzzling result was the difference observed between the two virulent phages Ø6-5.1 and Ø12-1 and their respective temperate counterparts that were isolated at a later date. These phages show considerable differences in both their protein composition, and their DNA sequences, as judged by the differences in their restriction patterns. Clearly, this phenomenon deserves further investigation.

A partial restriction map of bacteriophage Ø6-2 was constructed with the SuperCos Mapping Kit (Stratagene). This cosmid vector was chosen because of its capability for cloning 30-42 kb of DNA, which was well suited to the size of the complete

bacteriophage genome. However, the vast majority of the clones obtained contained inserts that were 10-12 kb in size, which precluded any possibility of mapping the complete genome using this strategy. Nevertheless, by a combination of partial digests and overlap analysis, it was possible to order the restriction fragments and construct the complete restriction map of Ø6-2 for the restriction endonucleases *Hind*III and *Kpn*I.

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