A Phage for Generalized Transduction in *Bacillus thuringiensis* and Mapping of Four Genes for Antibiotic Resistance

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A phage ($\phi 63$) for generalized transduction in *Bacillus thuringiensis* was isolated from a soil sample. The diameter of the head was 95 nm and the tail length was 200 nm. The growth cycle required 75 min and the burst size was 330. Phage $\phi 63$ was stabilized by Ca^{2+} ions and phage stocks could be stored cold without loss of titre. The frequency of transduction of Leu^+ into an auxotrophic strain was 2×10^{-6} . The frequency of transduction of genes for resistance to nalidixic acid, rifampicin, streptomycin and spectinomycin was generally an order of magnitude lower. Phage $\phi 63$ could cotransduce these four genes for antibiotic resistance. Crosses were performed which established the gene order as *nalA*–*rifA*–*strA*–*spcA*. Heterologous cotransduction of *rifA* and *strA* at reasonable frequencies was obtained in three of six subspecies.

INTRODUCTION

A number of insect pathogens have been used for biological control of pests (Briggs, 1977; Bulla *et al.*, 1975). The most useful of these organisms is *Bacillus thuringiensis*, which produces an infective spore and a crystalline protein toxin. *Bacillus thuringiensis* is known to produce five or six different toxins (Faust, 1977) and also specific inhibitors of the immune system of insects (Edlund *et al.*, 1976; Sidén *et al.*, 1979). About 20 subspecies of *B. thuringiensis* are recognized (Bulla *et al.*, 1975; Ren *et al.*, 1975; Tyrell *et al.*, 1979) and some of them differ in their pathogenicity to various insects. Systems for genetic exchange in *B. thuringiensis* could be of potential use for the construction of tailor-made insect pathogens.

Several phages of *B. thuringiensis* have been described, chiefly emphasizing morphology, physiological properties, host resistance or phage typing (Bogush *et al.*, 1977; Chapman & Norris, 1966; Colasito & Rogoff, 1969 *a, b*; Sha *et al.*, 1975; Van Tassel & Yousten, 1976). More recently, Thorne described two *B. cereus* phages that could perform generalized transduction in *B. thuringiensis* (Thorne, 1978). However, both phages were reported to be cold sensitive (Thorne, 1978; Van Tassel & Yousten, 1976) and in our hands the better one, CP-54, proved difficult to handle. This prompted us to isolate a set of phages from soil. We now report that one of these phages, $\phi 63$, can perform generalized transduction in *B. thuringiensis*. We have used this phage to determine the map order of four antibiotic resistance genes of which three are part of a ribosomal cluster. We also demonstrate that phage $\phi 63$ mediates heterologous cotransduction in five of the six subspecies of *B. thuringiensis* tested.

METHODS

Bacterial strains and isolation of mutants. The strains of *Bacillus thuringiensis* used and their sources are given in Table 1. Strains Bt2 and Bt3 were isolated as clones from samples of the commercial insecticides Biotrol XK (Nutralite Products, Lakeview, Calif. 92664, U.S.A.) and Entobacterin 3 (Moscow, U.S.S.R.). Strain Bt4 was isolated in the same way from a commercial powder of the international reference strain E61, originating from

Table 1. *Bacillus thuringiensis* strains used

Strain	Parent	<i>B. thuringiensis</i> subspecies	Genotype (alleles)				Other properties*			Source/reference†
			<i>nal</i>	<i>rif</i>	<i>str</i>	<i>spc</i>	Exo	Cry	Spo	
Bt1	—	<i>gelechiae</i> ‡	+	+	+	+	+	—	+	Sebesta & Horska (1970)
Bt11	Bt1	<i>gelechiae</i>	+	+	<i>A1</i>	+	+	—	+	Edlund <i>et al.</i> (1976)
Bt12	Bt11	<i>gelechiae</i>	+	<i>A1</i>	<i>A1</i>	+	+	—	+	Edlund <i>et al.</i> (1976)
Bt13	Bt1	<i>gelechiae</i>	<i>A1</i>	+	+	+	+	—	+	Sp, this paper
Bt14	Bt1	<i>gelechiae</i>	+	<i>A3</i>	+	+	+	—	+	Sp, this paper
Bt101	Bt1	<i>gelechiae</i>	+	+	+	<i>A2</i>	+	—	+	Sp, this paper
Bt103	Bt101	<i>gelechiae</i>	+	<i>A2</i>	+	<i>A2</i>	+	—	+	Sp, this paper
Bt131	Bt13	<i>gelechiae</i>	<i>A1</i>	+	<i>A2</i>	+	+	—	+	Sp, this paper
Bt1101	Bt11	<i>gelechiae</i>	+	+	<i>A1</i>	<i>A3</i>	+	—	+	Sp, this paper
Bt1134	Bt12	<i>gelechiae</i>	+	<i>A1</i>	<i>A1</i>	+	—	—	—	NG, T. Edlund (unpublished)
Bt1140	Bt1134	<i>gelechiae</i>	+	<i>A1</i>	<i>A1</i>	<i>A1</i>	—	—	—	Sp, this paper
Bt1314	Bt131	<i>gelechiae</i>	<i>A1</i>	<i>A4</i>	<i>A2</i>	+	+	—	+	Sp, this paper
Bt2	—	<i>kurstaki</i>	+	+	+	+	—	+	+	Biotrol XK
Bt3	—	<i>galleriae</i>	+	+	+	+	—	+	+	Entobacterin 3
Bt4	—	<i>thuringiensis</i>	+	+	+	+	+	+	+	E61
Bt5w	—	<i>alesti</i>	+	+	+	+	—	+	+	H. J. Somerville, 1979
Bt5	Bt5w	<i>alesti</i>	+	+	r§	+	—	+	+	H. J. Somerville, 1975
Bt8	—	<i>finitimus</i>	+	+	+	+	+	+	+	H. J. Somerville, 1975
Bta1	—	<i>israelensis</i>	+	+	+	+	+	+	+	H. de Barjac, 1977; identical to 1897-10
Bta3	—	<i>ostrinae</i>	+	+	+	+	+	+	+	Ren <i>et al.</i> (1975)
Bta40	—	<i>thompsoni</i>	+	+	+	+	+	+	+	Thorne (1978); identical to NRRL4060
Bta43	Bta40	<i>thompsoni</i>	+	+	+	+	+	+	+	Thorne (1978); Leu ⁻ , identical to NRRL4060 M52
Bta4301	Bta43	<i>thompsoni</i>	+	+	+	r§	+	+	+	Sp, Leu ⁻ as in Bta43
HD131	—	<i>aizawai</i>	+	+	+	+	+	+	+	H. T. Dulmage, 1977

* Exo, production of β -exotoxin; Cry, crystal formation; Spo, ability to sporulate.

† Sp, spontaneous mutants; NG, mutants obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment. Where only a name and year are given, this indicates from whom and when the strains were received.

‡ *gelechiae* is the designation used by Sebesta; the strain is not a generally recognized subspecies.

§ r, resistance, without further determination of locus.

the Institut Pasteur, Parix XV, France. Other strains used were *Bacillus cereus* Bc11, a Str^r mutant from ATCC 11778 and *Bacillus subtilis* 168 obtained from L. Rutberg. Spontaneous antibiotic-resistant mutants were isolated by spreading about 10⁹ bacteria from an overnight culture on plates containing the appropriate antibiotic: 100 µg streptomycin ml⁻¹, 100 µg spectinomycin ml⁻¹, 25 µg nalidixic acid ml⁻¹ and 5 µg rifampicin ml⁻¹.

Growth conditions and media. All bacteria used were grown at 37 °C and liquid cultures were incubated on rotary shakers. Minimal medium (MIN3) and phage assay medium (PA) were prepared as described by Thorne (1978). MIN4 medium was MIN3 supplemented with lysine, methionine and aspartic acid, each at 10 µg ml⁻¹. Solid medium (LAG) was LB medium (Bertani, 1951) supplemented with 0.2% (w/v) glucose and, when appropriate, antibiotics, and solidified with 1% (w/v) agar. Liquid medium (LEG) was LB medium supplemented with mineral medium E (Vogel & Bonner, 1956) and 0.2% (w/v) glucose. All dilutions were performed with dilute nutrient broth (0.1%, w/v) containing NaCl (0.5%, w/v). For transduction of auxotrophic markers, the soft agar contained only 0.6% (w/v) agar.

Isolation of phage ϕ 63. Three different soil samples were used for enrichment cultures with LEG medium and strain Bt1134. Five different phages were isolated by single plaque purification. Preliminary experiments showed that two phages, ϕ 42 and ϕ 63, could perform generalized transduction. However, ϕ 63 gave higher transduction frequencies and was selected for further study.

Preparation of phage stocks and assay methods. Phage stocks for transductions were prepared as follows. LAG plates were seeded with about 10⁷ plaque-forming units (p.f.u.) of ϕ 63 and about 10⁷ colony-forming units (c.f.u.) from cultures of late-exponential phase bacteria. Plates were incubated at 37 °C overnight and then overlaid with 3 ml of dilution medium. After standing at room temperature for 4 h, the phage suspension was recovered and centrifuged at 12000 g for 10 min at 4 °C. Bacteria in the supernatant were killed by shaking with chloroform.

The supernatant usually contained 10^{10} to 10^{11} p.f.u. ml⁻¹. Phage were assayed by the soft agar overlay technique (Adams, 1959) with strain Bt1 as indicator on PA plates incubated at 37 °C overnight. Phage stocks were regularly tested for bacterial sterility.

One-step growth curve. The one-step growth curve experiments were performed as described by Clowes & Hayes (1968), except that cells were lysed with chloroform instead of streptomycin.

Phage antiserum. A 0.5 ml portion of a crude phage stock (about 10^{10} p.f.u. ml⁻¹) was injected intravenously into the ear of a rabbit. Injections were repeated after 2 and 10 d, and the first serum was collected after 3 weeks. Later serum samples were taken 1 week after further boosters. The *K* values of the antiserum varied from 200 to 500 when assayed by the method of Clowes & Hayes (1968).

Transduction experiments. A 0.8 ml portion of an overnight culture of the recipient bacteria was added to 40 ml LEG medium. The bacteria were allowed to grow for 5 h, then centrifuged and resuspended in 2 to 4 ml LEG medium containing 2 mM-CaCl₂ to give a final concentration of 2×10^9 c.f.u. ml⁻¹. Samples of this bacterial suspension and phage $\phi 63$ (inactivated to 1 to 5% survival by u.v. irradiation) were mixed to give a multiplicity of infection (m.o.i.) of 1.5 and a final volume of 1 ml (m.o.i. was calculated on p.f.u. before u.v. irradiation). Such transduction mixtures were incubated with gentle shaking for 30 min at 37 °C. After incubation the bacteria were washed with 10 ml medium – MIN3, if the experiment concerned auxotrophic markers, otherwise LEG. The bacteria were resuspended in 1 ml medium, an equal volume of undiluted phage antiserum was added, and the mixture was incubated for 10 min at 37 °C.

In experiments with the leucine marker, all bacteria were then spread directly on MIN4 plates using 0.2 ml samples in soft agar. Experiments with antibiotic resistance genes required growth for phenotypic expression; this was achieved by diluting the suspension with 40 ml LEG medium to a turbidity corresponding to less than 3×10^7 viable cells ml⁻¹ and incubating at 37 °C. After 3 h growth, the bacteria were washed with 40 ml LEG medium, concentrated 40-fold and incubated as before with 1 ml of undiluted phage antiserum. Finally, 0.2 ml samples were spread on the appropriate antibiotic plates. Transductants were scored on MIN4 plates after 48 h, and on antibiotic plates after 36 h. Control experiments were performed in the same way as the transductions, except that dilution medium was used instead of phage.

Antibiotic-resistant clones were transferred to master plates and tested without further purification for resistance to all relevant antibiotics.

Electron microscopy. A high-titre phage stock was dialysed for 48 h against 0.01 M-potassium phosphate buffer, pH 6.4, containing 0.15 M-NaCl. One drop of phage suspension was put on a 300 mesh parlodion-coated copper grid and negatively stained with a fresh solution of 0.5 or 1% (w/v) ammonium molybdate, pH 7. The specimens were examined in a Jeol 100S electron microscope.

Materials. Spectinomycin was kindly donated by Upjohn International, Kalamazoo, Mich. 49001, U.S.A. Other antibiotics were purchased from Sigma.

RESULTS

Characterization of phage $\phi 63$

To be of maximum usefulness, a *B. thuringiensis* transducing phage should have a wide host range. We therefore examined phage $\phi 63$ for its ability to form plaques on 10 different subspecies of *B. thuringiensis* as well as strains of *B. cereus* and *B. subtilis*. The phage grew well on 8 of the 13 strains tested (Table 2). There was a modest restriction on subspecies *finitimus* and no growth at all on subspecies *israelensis*, *aizawai* and on one of the derivatives of *alesti*. There was good growth on *B. cereus* and no growth on *B. subtilis*. Three strains which gave no plaques – Bta1, HD131 and *B. subtilis* 168 – were compared with Bt1 for phage adsorption. Equal evidence for receptors was found in Bt1, Bta1 and HD131 while no adsorption was demonstrated with *B. subtilis*.

The size of the phage was estimated from electron micrographs (Fig. 1): the diameter of the head was 95 nm and the tail length was 200 nm. The one-step growth curve showed that $\phi 63$ had a latent period of 55 min, a rise period of 20 min and a burst size of 330 (average data from three experiments). The phage was stable for many days when kept cold in the presence of CaCl₂, but was destabilized by EDTA (Fig. 2).

Strategy of the genetic work

It is considerably easier to isolate antibiotic-resistant mutants than it is to obtain auxotrophs. Moreover, spontaneous mutations will often give isogenic strains, whereas

Table 2. *Plaque-forming ability of phage $\phi 63$ on different strains of Bacillus*

Strain	<i>B. thuringiensis</i> subspecies	Plaque morphology	E.o.p.*
Bt1	<i>gelechia</i>	Clear	1
Bt2	<i>kurstaki</i>	Turbid	0.4
Bt3	<i>galleriae</i>	Turbid	0.5
Bt4	<i>thuringiensis</i>	Turbid	0.4
Bt5w	<i>alesti</i>	Clear	$<10^{-9}$
Bt5	<i>alesti</i>	Clear	0.6
Bt8	<i>finitimus</i>	Turbid	10^{-3}
Bta1	<i>israelensis</i>	No plaques	$<10^{-8}$
Bta3	<i>ostrinae</i>	Clear	0.4
Bta40	<i>thompsoni</i>	Turbid	0.5
HD131	<i>aizawai</i>	No plaques	$<10^{-9}$
<i>B. cereus</i> Bc11		Clear	0.6
<i>B. subtilis</i> 168		No plaques	$<10^{-7}$

* Efficiency of plating of a stock of phage $\phi 63$, which was propagated on strain Bt1.

Fig. 1. Electron micrographs of phage $\phi 63$, negatively stained with 0.5% ammonium molybdate (pH 7): *left*, sheath extended; *right*, sheath contracted. The bar marker represents 0.1 μm .

mutagen treatments always give multiple mutations and sometimes chromosome alterations. Our first decision was, therefore, to use spontaneous antibiotic-resistant mutants for the development of a transduction system in *Bacillus thuringiensis*. During the isolation of streptomycin-resistant mutants, we found that the spontaneous mutation frequency was as high as 10^{-7} to 10^{-8} . Such a high frequency made it difficult to distinguish between a low order of transduction and mutation. However, the expected frequency of double mutations would be 10^{-14} or lower while the frequency of cotransduction would remain around 10^{-7} to 10^{-8} . Our second decision was, therefore, to screen directly for cotransduction because this would theoretically eliminate the possibility that a cotransduction was confused with a double mutation. In addition, such an approach might lead directly to a phage useful in further genetic work.

In *Escherichia coli*, resistance to streptomycin and spectinomycin are known to be

Fig. 2. Stability of phage $\phi 63$ at different temperatures. A high-titre phage stock containing 2 mM- CaCl_2 was propagated on strain Bt1 as described in Methods. The stock was divided into four parts: two portions were stored at 37 °C, one with 4 mM-EDTA (○) and one without EDTA (●); the other two portions were stored at 4 °C, one with 4 mM-EDTA (△) and one without EDTA (▲). At intervals, samples were withdrawn and assayed for phage titre.

Table 3. *Phage $\phi 63$ -mediated transduction using single and double selection*

Donor	Recipient	Selection	No. of colonies*
Bt1140	Bt1	Str ^r Spc ^r	164
Control without phage	Bt1	Str ^r Spc ^r	0
Bt131	Bt1	Str ^r	443
Control without phage	Bt1	Str ^r	214

* Before plating the number of colony-forming units was $6 \times 10^9 \text{ ml}^{-1}$. The multiplicity of infection was 0.9 in the first experiment with double selection, and 1.7 in the second.

alterations in ribosomal proteins P10 and P4 of the 30S subunit (Taylor & Trotter, 1972). Since ribosomal genes are known to be clustered (Hoch, 1978; Taylor & Trotter, 1972; Young & Wilson, 1975), we screened our phages for their ability to cotransduce Str^r and Spc^r. For comparison we also included an experiment with selection for only one of the markers used. From the results in Table 3 we conclude that phage $\phi 63$ can perform cotransduction and that the phage would be potentially useful for the development of the genetics of *B. thuringiensis*.

Optimizing the conditions for transduction with phage $\phi 63$

Using a leucine auxotroph we investigated some parameters found to influence the overall yield of transductants. Table 4 shows that an important step was the u.v. inactivation of the phage. In this experiment the reversion rate of the marker was low as indicated from the two controls. Before plating, antiserum was added to all of the samples in Table 4 (see Methods). If antiserum against $\phi 63$ was omitted the number of colonies obtained was of the same order but the colony morphology indicated heavy lysis. Such transductants could be tested for cotransduction only with great difficulty. Thus, it is important to prevent phage multiplication after the first infection.

Table 4. *Parameters affecting the transduction frequency with phage ϕ 63 carrying Leu⁺ from strain Bta40*

Transduction conditions*		No. of Leu ⁺ colonies
Addition	U.v. inactivation (%)	
None	98	3791‡
None	0	1179
4 mM-EDTA	98	607
Preincubation with antiserum†	98	28
Control without phage	0	16

* The multiplicity of infection in all experiments was 1.25. All samples contained 2 mM-CaCl₂. The recipient was strain Bta4301.

† Antiserum against ϕ 63 was added before the phage were allowed to adsorb to the recipient strain.

‡ This number corresponds to a transduction frequency of 2×10^{-6} per p.f.u.

Table 5. *Phage ϕ 63-mediated cotransduction between strA1 and two other genes in the ribosomal cluster*

Donor	Recipient	No. of Str ^r colonies		Cotransduction of unselected markers (%)†		
		Obtained*	Tested	rifA1	spcA ⁺	rifA1 spcA ⁺
Cross A						
Bt1134 (rifA1 strA1 spcA ⁺)	Bt101 (rifA ⁺ strA ⁺ spcA2)	651	502	17	8	54
Control without phage	Bt101 (rifA ⁺ strA ⁺ spcA2)	64	18	0	0	0
Cross B				rifA1	spcA1	rifA1 spcA1
Bt1140 (rifA1 strA1 spcA1)	Bt1 (rifA ⁺ strA ⁺ spcA ⁺)	497	355	28	13	50
Control without phage	Bt1 (rifA ⁺ strA ⁺ spcA ⁺)	40	32	0	0	0

* When the number of spontaneous mutants was subtracted from the number of colonies obtained with ϕ 63, the calculated transduction frequency was 4×10^{-7} for cross A and 2×10^{-7} for cross B.

† Not shown are the transductants which received only the strA1 marker. For cross A this number (40) was calculated as the difference between the number of colonies which were only Str^r and spcA2 (104) and the number of spontaneous Str^r colonies (64). This recombinant class is of the same order as the one which received spcA⁺ together with strA1.

Table 6. *Three-factor crosses with selection for crossover between rifA and strA*

Donor	Recipient	Donor marker selected	No. of Rif ^r Str ^r colonies		Colonies with rifA strA spcA	
			Obtained	Tested	No.	%*
Cross C						
Bt103 (rifA2 strA ⁺ spcA2)	Bt11 (rifA ⁺ strA1 spcA ⁺)	rifA2	810	676	10	8
Control without phage	Bt11 (rifA ⁺ strA1 spcA ⁺)	—	689	460	0	0
Cross D						
Bt1101 (rifA ⁺ strA1 spcA3)	Bt14 (rifA3 strA ⁺ spcA ⁺)	strA1	125	124	26	66
Control without phage	Bt14 (rifA3 strA ⁺ spcA ⁺)	—	86	76	0	0

* The total number of rifA strA transductants was calculated by subtracting the number of spontaneous mutants obtained in the controls. This number was used to calculate the percentage of rifA strA spcA recombinants.

Mapping of genes rifA, strA and spcA in the ribosomal cluster

To determine the order of these genes we performed a number of transduction experiments. Table 5 shows two crosses with primary selection for only *strA*. The genotype of the donor and the recipient were chosen so that the transductants could be tested for the cotransfer of *rifA1* and of *spcA1* or their wild-type alleles. The results in Table 5 show that three genes were cotransduced at a higher frequency than two, indicating a close linkage of all three genes. Moreover, *rifA* was cotransduced at a frequency higher than observed for *spcA*⁺. If the gene order had been *rif*–*spc*–*str* such a result would have required a double crossover for the *rifA1* recombinants. This gene order can thus be excluded and we conclude that *spc* is not located in the centre of the cluster.

To determine that the *str* gene was located between *rif* and *spc* we performed two inverted-marker crosses with *rifA* and *strA* in different strains and selected for crossover between these genes (Table 6). We then compared the frequency of transductants which were resistant to all three antibiotics. The results in Table 6 show that cross C gave only 8% of recombinants with this phenotype, while in cross D the frequency was 66%, numbers indicating a double crossover in cross C and a single crossover in cross D. Since *spc* must be in a distal position (see Table 5) the results in Table 6 are only compatible with the gene order *rifA*–*strA*–*spcA*.

Cotransduction between nalA and the ribosomal cluster

Two crosses were performed to demonstrate that *nalA* and *strA* were cotransducible. Table 7 shows that with double selection for Rif^r and Str^r it was possible to demonstrate a low frequency of cotransduction between *nalA* and *spcA*. Since only 5% of the transductants were *nalA1* while 69% were *spcA*⁺, *nalA1* must be outside the region *rifA*–*strA*–*spcA*. In a similar cross with double selection for Nal^r and Str^r, 52% of all clones were found to carry all four donor markers (Table 7). Of the remaining clones 42% received *rifA4* but only 6% *spcA*⁺. These results indicate that *nalA* is on the *rif* side of the *rif*–*spc* cluster.

Heterologous transduction of ribosomal genes mediated by phage ϕ63

As demonstrated in Table 3, double selection eliminated the background of spontaneous mutations and it was therefore the method of choice for the investigation of heterologous transduction, a phenomenon which would be expected to be rare. Table 8 shows that *rifA1* and *strA1* from subspecies *gelechiae* could be cotransduced with a reasonable frequency to three other subspecies and with a reduced frequency to an additional two subspecies. No transfer of the ribosomal genes was obtained to subspecies *ostrinae*.

DISCUSSION

The spontaneous mutation frequency to antibiotic resistance. The mutation frequency to Str^r was always one or two orders of magnitude higher than the reversion rate for the leucine auxotroph Bta43 (Tables 3 and 4). Moreover, the spontaneous mutation rate to Rif^r was one order of magnitude higher than the rate for Str^r (Table 6). Whether these fluctuating frequencies can be traced to transposons or other unorthodox genetic events remains to be determined.

Phage ϕ63 as a transducing vector. As preincubation of phage ϕ63 with antiserum reduced the number of Leu⁺ colonies to less than 1%, the genetic events recorded in Table 4 must be mediated by ϕ63. Altogether (Tables 4 and 7) five different genes were transferred. Moreover, when transductants with 17 different genotypes were investigated for sensitivity to reinfection with ϕ63, no case of immunity was detected (results not shown). Thus, ϕ63 must be a phage for generalized transduction of auxotrophic markers and antibiotic resistance genes. We have so far no evidence for a helper phage or for conversion (Perlak *et al.*, 1979) but we intend to study the mechanism of ϕ63 transduction further.

Table 7. Phage $\phi 63$ -mediated cotransduction between *nalA* and genes in the ribosomal cluster

The results given are the sums of two separate experiments.

Donor	Recipient	Selected phenotype	No. of colonies tested*	Cotransduction of unselected markers (%)		
				<i>nalA1</i>	<i>spcA</i> ⁺	<i>nalA1 spcA</i> ⁺
Cross E						
Bt1314 (<i>nalA1 rifA4 strA2 spcA</i> ⁺)	Bt101 (<i>nalA</i> ⁺ <i>rifA</i> ⁺ <i>strA</i> ⁺ <i>spcA2</i>)	Ri ^r Str ^r	152	5	69	2
Control without phage	Bt101 (<i>nalA</i> ⁺ <i>rifA</i> ⁺ <i>strA</i> ⁺ <i>spcA2</i>)	Ri ^r Str ^r	0	0	0	0
Cross F						
Bt1314 (<i>nalA1 rifA4 strA2 spcA</i> ⁺)	Bt101 (<i>nalA</i> ⁺ <i>rifA</i> ⁺ <i>strA</i> ⁺ <i>spcA2</i>)	Nal ^r Str ^r	36	42	6	52
Control without phage	Bt101 (<i>nalA</i> ⁺ <i>rifA</i> ⁺ <i>strA</i> ⁺ <i>spcA2</i>)	Nal ^r Str ^r	0	0	0	0

* The total number of colonies isolated were 175, 0, 43, 0, reading from top to bottom. In the cross E, 76% of the *rifA4 strA2* transductants had also received unselected markers; the remaining 24% had no additional marker cotransduced. In cross F, all *nalA1 strA2* transductants had also received unselected markers.

Table 8. *Phage ø63-mediated heterologous cotransduction of rifA1 strA1*

The stock of ø63 used was propagated on strain Bt1134. The data shown are from two separate experiments. The number of colonies isolated with Bt1 as recipient varied between 414 and 1722.

Recipient	<i>B. thuringiensis</i> subspecies	Relative cotransduction frequency (%)
Bt1	<i>gelechia</i>	100
Bt2	<i>kurstaki</i>	10
Bt3	<i>galleriae</i>	50
Bt4	<i>thuringiensis</i>	80
Bt8	<i>finitimus</i>	2
Bta3	<i>ostrinae</i>	<0.2
Bta40	<i>thompsoni</i>	1

We have shown that the background of spontaneous mutations could be reduced to zero by selection for cotransduction (Tables 3 and 7). In Table 5 the background was around 10% while in Table 6 it was 85% and 69% in the two crosses. In the controls there was not a single clone which carried two of the markers involved. These results, together with those presented in Tables 3 and 7, show that cotransduction can be performed even against a high background level of spontaneous mutations.

In our laboratory we have compared phages CP-54 (Thorne, 1978) and ø63 and found the latter to be considerably more stable and easier to work with. Moreover, with ø63 we regularly obtained transduction frequencies for *Str^r* around 5×10^{-7} per p.f.u. compared with 3×10^{-8} with phage CP-54. A paper describing phage TP-13, which mainly concerns conversion of sporulation and crystal formation in *B. thuringiensis* (Perlak *et al.*, 1979), was published while this work was in preparation. Thus, at present we cannot compare the usefulness of phages ø63 and TP-13 for future genetic work. However, in no previous case has cotransduction of more than two genes been reported (Perlak *et al.*, 1979; Thorne, 1978) compared with the four demonstrated here with ø63 (Table 7).

The transduction frequencies obtained for antibiotic resistance genes were always an order of magnitude lower than those found for auxotrophic markers (cf. Tables 4 and 5). One explanation could be that the phenotypic expression required for the ribosomal genes involves a disadvantage compared with when a gene for a missing enzyme converts an auxotroph to a prototroph. Whether this is a phenomenon related only to *Bacillus thuringiensis* or also to ø63 remains to be investigated.

The host range pattern of other *B. thuringiensis* phages described was investigated either by non-quantitative spotting of a concentrated phage suspension on a lawn of bacteria (Ackermann & Smirnoff, 1978; Chapman & Norris, 1966) or by cross-streaking (Perlak *et al.*, 1979). We determined efficiencies of plating (Table 2), which showed that 8 of the 13 strains tested were sensitive to ø63. This broad host range pattern should contribute to the usefulness of the phage.

The map order of the antibiotic resistance markers. The order of genes indicated (Tables 5, 6 and 7) was *nalA-rifA-strA-spcA*, which is the same as in *B. subtilis* (Hoch, 1978; Young & Wilson, 1975). Possible differences in the times needed for phenotypic expression can make our linkage data uncertain. However, the distance *nalA-rifA* is probably about three times longer than the distance *strA-spcA*. It should also be mentioned that we have failed to cotransduce *leu* with *spc*, the only antibiotic resistance marker available to us in subspecies *thomsoni*.

Phage SP-10, which is of the same order of size as ø63, can cotransduce *leu1-arg1* (Tyeryar *et al.*, 1969), a distance corresponding to 3 to 5% of the *B. subtilis* genome (Hoch, 1978; Young & Wilson, 1975). In this organism the distance *nal-spc* is about 6% (Hoch, 1978; Young & Wilson, 1975) but in *B. thuringiensis* it may be considerably shorter because the ribosomal RNA genes are lacking in spacer DNA (Klier *et al.*, 1979). Thus, the

cotransduction of *nalA*–*spcA* reported here may well be compatible with the size of phage $\phi 63$.

Heterologous transduction in *B. thuringiensis*. Heterologous cotransduction was obtained at varying frequencies in five of the subspecies studied (Table 8). Temperate phages and plasmids have been reported in several subspecies of *B. thuringiensis* (Ackermann & Smirnov, 1978; Colasito & Rogoff, 1969*b*; Faust *et al.*, 1977; Stahly *et al.*, 1978; Zakharyan *et al.*, 1979) and some of them could be responsible for the restrictions demonstrated in Table 2. A further study of phage $\phi 63$ as well as characterization of the postulated restricting factors are probably needed in order to obtain a more efficient interstrain transduction system. However, the data presented show that phage $\phi 63$ is very useful for mapping studies in strain Bt1 and its derivatives.

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