Transducing Bacteriophage for *Bacillus cereus*

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A phage, designated CP-51, that carries out generalized transduction in *Bacillus cereus* 569 was isolated from soil. All auxotrophic mutants tested, those requiring tryptophan, histidine, leucine, isoleucine, methionine, or phenylalanine, were transduced to prototrophy. The phage was extremely unstable when stored at 2 to 4 C, but stability was enhanced by storage at higher temperatures. The optimal temperature of those tested for maintenance of plaque-forming units was 15 C.

Generalized transducing phages for members of the genus *Bacillus* have been described for *B. subtilis* and *B. licheniformis*. Those that have been best characterized are PBS-1(5) for *B. subtilis* and SP-10 and SP-15 (6, 7) for both *B. subtilis* and *B. licheniformis*. Welker and Campbell (9) recently showed that SP-10 also transduces *B. amyloliquefaciens*. *B. cereus* is not a host for any of these transducing phages. The present report deals with transduction in *B. cereus* 569 by a phage, designated CP-51, that was isolated from soil. Presumably, this is the first example of a transducing phage for *B. cereus*.

MATERIALS AND METHODS

Organisms. *B. cereus* NRRL 569 and mutants derived from it were used in this study. The mutants, listed in Table 1, were isolated by the procedure of Goldberg et al. (3) in which diethyl sulfate is the mutagen.

Media and cultural conditions. The minimal medium, M10, described previously (8) was usually used for scoring transductants. In addition to salts, glucose, and glutamic acid, this medium contains alanine, valine, leucine, isoleucine, serine, and threonine. When transductants for leucine or isoleucine were being scored, the appropriate amino acid was omitted from M10. For scoring transductants for tryptophan, M10C was usually used. In M10C, the six amino acids listed above were omitted, and 5 g of vitamin-free acid-hydrolyzed casein (Nutritional Biochemicals Corp., Cleveland, Ohio) per liter was added. NBY medium was composed of 8 g of Difco Nutrient Broth and 3 g of Difco yeast extract per liter. PA medium contained the following (g/liter): Difco Nutrient Broth, 8; NaCl, 5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.05; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.15. The pH of PA was adjusted to 5.9 with hydrochloric acid. For solid media, 15 g of agar per liter was added.

Spores of the auxotrophic mutants were grown on slants of potato-agar (7) in screw-cap tubes (20 × 150 mm). They were incubated at 37 C for 3 to 5 days, and

the growth from a single slant was suspended in 5 ml of water. The suspensions were heated at 65 C for 30 min. Such suspensions usually contained about 10^9 spores per ml. Larger batches of spores of wild-type *B. cereus* 569, for use as indicator in phage assays, were grown in PA broth. Erlenmeyer flasks (250 ml) containing 50 ml of broth were inoculated with 10 ml of a 16-hr shaken culture grown in the same medium. These were incubated at 37 C on a rotary shaker (250 rev/min) for 4 days. Spores from five flasks were collected by centrifugation and suspended in about 30 ml of water. After storage at 4 C for 3 days, to allow cell lysis to continue, they were collected again by centrifugation and finally suspended in 30 ml of water. They were then held at 65 C for 30 min.

Recipient cells for transduction were grown, usually for 6 hr, in 250-ml Erlenmeyer flasks containing 25 ml of NBY broth. Such flasks were inoculated with 2.5 ml of a 16-hr culture grown in NBY broth from a loop of spores. All cultures were grown at 37 C on the shaker described above. Viable counts were made by plating on NBY agar after diluting cultures appropriately in Difco peptone (1%, w/v). Since the organisms grew in chains of varying lengths, the values for viable counts (colony-forming units) represent numbers of chains rather than numbers of individual cells. No attempts were made to enumerate actual numbers of cells.

Phage assay. Phage CP-51 was assayed on PA medium by the agar-layer technique (1). The bottom agar (25 ml) contained 1.5% agar, and the soft agar for overlaying contained 0.5% agar. Phage was diluted in 1% peptone and 0.1 ml was mixed with 0.1 ml of *B. cereus* 569 spores (2×10^7) in 2 ml of soft agar. Plates were incubated at 37 C and plaques were counted after 16 to 20 hr.

Isolation of phage. A bit of soil was added to a flask of NBY broth and this was incubated with shaking at 37 C for 7 hr. A 5-ml sample, along with 1 ml of a 16-hr culture of RM20 in NBY broth, was transferred to 50 ml of NBY broth containing 400 μg of streptomycin per ml (4). This flask was incubated on the shaker at 37 C for 10 hr. The culture was centrifuged at low speed to sediment the cells, and the super-

TABLE 1. *Characterization of the organisms used*^a

Designation	Properties ^b
569.....	Wild-type, prototrophic
RM1.....	His ⁻
RM9.....	Ile ⁻ Gly/Ser ⁻
RM20.....	Trp ⁻ Str ^R
RK3.....	Phe ⁻
RK36 D11.....	Met ⁻ Leu ⁻
RK36 D60.....	Met ⁻ Ile ⁻

^a All auxotrophs were derived from the wild-type *B. cereus* 569.

^b Abbreviations: His, histidine; Ile, isoleucine; Gly, glycine; Ser, serine; Trp, tryptophan; Phe, phenylalanine; Met, methionine; Leu, leucine; Str^R, resistance to streptomycin.

nant solution was tested for plaque-forming units (PFU) by the phage assay procedure. A colony-centered plaque was selected for further study and, after purification by repeated single plaque isolations, it was designated CP-51.

Propagation of phage. Phage CP-51 was propagated in NBY agar. A toothpick that was used to pick a plaque from an assay plate was dropped into 1 ml of 1% peptone, and the adhering phage and cells were suspended by mixing on a Vortex mixer. A 0.5-ml amount of the suspension was added to 3 ml of soft NBY agar (0.5%) containing about 10⁸ spores of *B. cereus* 569, and the mixture was poured onto a freshly prepared plate containing 25 ml of NBY with 1.5% agar. Plates were incubated at 37 C for 20 hr. The soft-agar layer was collected in 5 to 10 ml of NBY broth and centrifuged at room temperature at about 3,000 rev/min to sediment the agar and cell debris. The supernatant fluid was filtered through a Millipore AA membrane (Millipore Corp., Bedford, Mass.). All phage suspensions were tested for bacterial contamination by adding 0.2 ml to an NBY-agar plate and incubating at 37 C for 24 hr.

Preparation of *B. cereus* 569 spores infected with CP-51. Soft-agar plates with plaques were prepared as in the phage assay procedure, except that NBY agar was used instead of PA. The soft-agar layer from a plate estimated to have 1,000 to 1,500 plaques was collected in 5 ml of NBY broth, and the material was passed through the tip of a pipette several times to break up the agar lumps. The suspension was transferred to a 250-ml Erlenmeyer flask containing 20 ml of NBY broth, and the flask was incubated on the shaker at 37 C for 6 days. The culture was held in a 65 C bath for 30 min, and samples were stored in screw-cap tubes in the refrigerator. Such suspensions had titers of 3×10^7 to 5×10^7 PFU/ml, representing 30 to 100% infection of spores based on viable spore counts. It is possible, however, that not all viable spores formed colonies; lysis probably occurred in some instances after spores germinated.

Transduction. Transductions were usually carried out by adding 0.9 ml of recipient culture and 0.1 ml of phage to 18-mm Pyrex tubes and incubating in a 37 C water-bath shaker for 30 min. Controls for

spontaneous reversion were always run by substituting 0.1 ml of NBY broth for the phage. After incubation, samples (usually 0.1 ml) were spread on appropriate minimal agar plates. When samples were plated with phage antiserum, the standard procedure was to mix an equal part of serum with the sample when spreading on the plate. Plates were incubated at 37 C. When M10C was the plating medium, colonies could be scored after 18 to 20 hr; with medium M10, growth was considerably slower and colonies could not be scored accurately before 36 to 40 hr. The numbers of transductants recorded in the tables are averages derived from two or more plates.

Phage antiserum. Antiserum to phage CP-51 was prepared as described previously for phage SP-10 (6).

Ultraviolet irradiation. Ultraviolet (UV) light exposure was carried out with two General Electric germicidal lamps (15 w). A petri dish containing 5 ml of phage suspension was placed on a rotating platform 40 cm below the UV source.

RESULTS

Phage. Phage CP-51 formed remarkably distinct plaques on both NBY and PA media. The efficiency of plating was about 30% higher on PA than on NBY and, therefore, PA was used routinely for assays. The plaques normally had a small colony in the center, but mutants occurred spontaneously that gave completely clear plaques. When colony-centered plaques were the source of inoculum for phage propagation, preparations usually had low proportions of clear PFU. Phage yields of 10¹¹ to 2.5×10^{11} PFU/ml were obtained routinely.

Phage CP-51 particles have the form of a head with a contractile tail. Details of the phage structure revealed by the electron microscope will be reported later.

Evidence for transduction. Table 2 shows the results of a transduction experiment in which an auxotroph requiring tryptophan for growth was transduced to prototrophy by phage propagated on wild-type *B. cereus* 569. From the mixture made up of cells and phage, 830 Trp⁺ colonies per ml were obtained, with a transduction frequency of 1.1×10^{-6} per PFU. The mixture containing cells but no phage produced no colonies; the mutant, RM20, used in these tests was quite stable, although spontaneous revertants did sometimes occur in low numbers. When phage was heated to inactivate PFU, transduction was prevented. Similarly, when PFU were inactivated with phage antiserum, no prototrophs were found. Treatment of the phage with preimmune serum did not have a significant effect on the numbers of transductants produced. In other experiments, incubation of phage with deoxyribonuclease (50 μ g/ml) for 15 min at 37 C did not affect the transduction results.

The data in Table 3 provide additional evi-

TABLE 2. *Inhibition of transduction by phage antiserum and heat^a*

Treatment of phage	Trp ⁺ transductants/ml
Phage omitted.....	0
No treatment.....	830
65 C, 30 min.....	0
Incubated with phage anti- serum.....	0
Incubated with normal serum.....	780

^a Transduction mixtures contained 0.8 ml of RM20 cells (5×10^8) and 0.1 ml of phage (7.2×10^8 PFU) propagated on wild-type cells. In the tests with serum, phage was incubated with 0.1 ml of normal serum or phage antiserum for 15 min at 37 C before cells were added. In the other mixtures, 0.1 ml of NBY broth was added so that the final volume of each was 1.0 ml. The treatment with antiserum inactivated 99.99% of the PFU; no PFU could be detected in the heated sample. Transductants were scored on M10C.

TABLE 3. *Specificity of transduction by phage propagated on an auxotrophic mutant^a*

Recipient cells	Colonies/ml		
	Without phage	With phage propagated on	
		Wild-type	RM20 (Trp ⁻)
RM20 (Trp ⁻)	0	620	0
RM1 (His ⁻)	10	400	640
RK36 D60 (Met ⁻ Ile ⁻)	0 (Met ⁺)	470 (Met ⁺)	440 (Met ⁺)

^a Each phage preparation had 1.7×10^{10} PFU/ml before UV treatment that inactivated 99.1% of the PFU. Transduction mixtures consisted of 0.9 ml of cells (about 6×10^8) and 0.1 ml of phage or NBY broth. Transductants for Trp were scored on M10C and those for His and Met were scored on M10.

dence that the change from auxotrophy to prototrophy was indeed mediated by phage CP-51. Phage propagated on a tryptophan-dependent mutant was incapable of inducing prototrophy in cells of that same mutant, although it did transduce other mutants for the histidine and methionine markers. In the same experiment, samples of the same recipient cells were exposed to phage propagated on wild-type cells, and the tryptophan mutant, as well as the histidine and methionine mutants, was transduced.

Scoring of transductants. Several factors affected the yield of transductants; among them were: (i) the medium used for scoring, (ii) the multiplicity of infection in transduction mixtures, (iii) the proportion of clear PFU in the phage preparation, and (iv) differences in suscepti-

bility of recipient cells of various mutants to lysis by the phage. Some examples are shown in Table 4. Minimal medium (M10) fortified with hydrolyzed casein (M10C) could be used for scoring cells transduced from Trp⁻ to Trp⁺. Usually, more Trp⁺ transductants were found on M10C than on M10; the difference in numbers scored on the two media became more pronounced with increasing proportions of clear PFU (compare lysates A and B in Table 4). Plating of transduction mixtures with phage antiserum resulted in greater yields of transductants and a diminution in the difference between the numbers found on M10 and M10C. In the absence of phage antiserum, lysis of cells (as determined by observation of the background growth) appeared to be much more extensive on M10 than on M10C.

The data for phage lysate B (Table 4) emphasize the difference in behavior of different mutants with respect to the yields of transductants on M10 with and without phage antiserum. With RM20 (Trp⁻) on M10, the yields of transductants in the absence of antiserum were 50 to 70% of those in the presence of antiserum. On the other hand, with RK36 D60, the yield of Met⁺ transductants in the absence of antiserum was less than 2% of the yield obtained with antiserum.

Transduction by UV-irradiated phage. Data on transduction by phage preparations after treatment with UV are shown in Table 5. When transduction mixtures were plated without antiserum, the yield of transductants was greater with treated than with untreated phage. An exposure time of 60 sec was the optimum of those times tested; this treatment usually inactivated 99.1% of the PFU. In some but not all instances, the yield of transductants was increased further by plating in the presence of antiserum. Treatment of phage suspensions with UV for 60 sec has been adopted as standard procedure.

Effect of multiplicity of infection on yields of transductants. Table 6 gives the results of transduction experiments in which the amount of phage added to a series of tubes containing the same number of cells was varied to result in multiplicity of infection ranging from 0.13 to 4.2. It should be pointed out that the multiplicity-of-infection values are based on numbers of colony-forming units or chains of cells rather than on actual cell-numbers.

Isolation of transductants. Transductants could be isolated in pure culture and freed from any detectable phage CP-51 by streaking on minimal agar. Prototrophic transductants isolated in this manner could not be distinguished from wild-type *B. cereus* 569. Met⁺ transductants derived

TABLE 4. *Influence of plating medium and post-transduction treatment with antiserum on yields of transductants^a*

Phage lysate	Recipient	Transduction mixture		Colonies/ml on			
		Cells	MOI ^b	M10	M10 + antiserum	M10C	M10C + antiserum
A	RM20 (Trp ⁻)	4.5 × 10 ⁸	0	0	0	10	0
		4.5 × 10 ⁸	1.6	0	750	110	780
A	RM1 (His ⁻)	4.0 × 10 ⁸	0	0	10		
		4.0 × 10 ⁸	1.8	70	380		
A	RK3 (Phe ⁻)	4.5 × 10 ⁸	0	20	20		
		4.5 × 10 ⁸	1.6	0	510		
B	RM20 (Trp ⁻)	3.3 × 10 ⁸	0	10	0	30	20
		3.3 × 10 ⁸	4	230	440	470	520
		3.3 × 10 ⁸	1.3	760	1,120	920	1,200
B	RK36 D60 (Met ⁻ Ile ⁻)	2.3 × 10 ⁸	0	0 (Met ⁺)	0 (Met ⁺)		
		2.3 × 10 ⁸	3	10 (Met ⁺)	620 (Met ⁺)		

^a Of the plaques produced by lysate A, 10% were clear; less than 1% of those produced by lysate B were clear. Plating of transduction mixtures in the presence of normal serum did not influence the yield of colonies.

^b Multiplicity of infection.

TABLE 5. *Transduction by UV-irradiated phage*

Recipients	UV treatment of phage		Transduction mixtures		Colonies/ml when plated	
	Time	PFU in-activated	Cells	MOI ^a	Without antiserum	With antiserum
RM20	<i>sec</i>	<i>%</i>	3.0 × 10 ⁸	0	10	0
	0	0	3.0 × 10 ⁸	1.4	880	1,200
	30	93	3.0 × 10 ⁸	1.4	1,080	1,250
	60	99.1	3.0 × 10 ⁸	1.4	1,140	1,060
	90	99.9	3.0 × 10 ⁸	1.4	780	840
RK36 D60			2.3 × 10 ⁸	0	20 Met ⁺ , 20 Ile ⁺	5 Met ⁺ , 20 Ile ⁺
	0	0	2.3 × 10 ⁸	3	10 Met ⁺ , — ^b	620 Met ⁺ , — ^b
	60	99.1	2.3 × 10 ⁸	3	120 Met ⁺ , — ^b	640 Met ⁺ , — ^b
	60	99.1	2.3 × 10 ⁸	1.4	570 Met ⁺ , 0 Ile ⁺	1,100 Met ⁺ , 680 Ile ⁺
RK36 D11			3.2 × 10 ⁸	0	5 Met ⁺ , 20 Leu ⁺	25 Met ⁺ , 25 Leu ⁺
	60	99.1	3.2 × 10 ⁸	1.0	690 Met ⁺ , 250 Leu ⁺	690 Met ⁺ , 530 Leu ⁺
RM9			3.9 × 10 ⁸	0	50 Ile ⁺ — ^b	60 Ile ⁺ , — ^b
	60	99.1	3.9 × 10 ⁸	0.8	200 Ile ⁺ — ^b	500 Ile ⁺ , — ^b

^a Multiplicity of infection based on phage titer before UV treatment.

^b Transductants for the second marker were not scored. Transductants for Trp were scored on M10C; the others were scored on M10. When Leu or Ile was the selective marker, the amino acid was omitted from M10.

from RK36 D60 retained the requirement for Ile. Similarly, Ile⁺ transductants derived from the double mutant retained the requirement for Met.

Stability of CP-51. When I first began working with phage CP-51, the standard procedure was to keep phage preparations cold as much as

possible. During the process of harvesting phage lysates, material from soft-agar plates was centrifuged at 4 C to pellet the agar and cells, and immediately after filtration at room temperature, preparations were stored in the refrigerator. Under these condition, the phage was extremely unstable; preparations would often lose 90%

of their PFU in 24 hr. Attempts to stabilize the phage led to the following findings (Table 7).

(i) The addition of 10% (v/v) of dimethyl sulfoxide increased the stability somewhat but, after 5 days in the refrigerator, such samples retained less than 40% of their initial PFU. (ii) Samples of preparations that were diluted 1:10 in NBY broth retained a greater proportion of their initial PFU than undiluted samples, and addition of dimethyl sulfoxide to diluted samples further enhanced their stability. (iii) Preparations were more stable when stored at room temperature (24 to 28 C) than when stored in the refrigerator (2 to 4 C).

When samples were tested for viability after storage at several temperatures, the results shown for lysate B (Table 7) were obtained. Of the temperatures tested, 15 C was the most satisfactory. As a result of these findings, the routine procedure now used for phage CP-51 is

to store samples at 15 C after diluting 1:10 in NBY broth.

Because the phage is very unstable, a primary source of it is maintained in the form of infected *B. cereus* 569 spores. When plated for infected centers by the phage assay procedure, such spores serve as a convenient source of plaques for preparation of lysates. Suspensions of *B. cereus* 569 spores infected with phage CP-51 have thus far been stored for 7 months at 2 to 4 C without any detectable changes in titers of PFU.

DISCUSSION

The results presented here indicate that phage CP-51 carries out generalized transduction in *B. cereus* 569. Mutants representing six different auxotrophic markers were all transduced; other markers have not yet been tested.

Difficulties were encountered in scoring transductants; no single method was found that was optimum for all mutants tested. The phage, even though it forms colony-centered plaques, is quite virulent; its lytic activity seemed to vary with different mutants, different ages of recipient cells, and different plating media. The yield of transductants was enhanced generally by inactivation of a large proportion of PFU with UV. But even with UV-treated phage, plating of transduction mixtures with phage antiserum often resulted in an increased yield of transductants. With the Trp⁻ mutant RM20, much less lysis was apparent on the medium with casein hydrolysate (M10C) than on M10. In some instances with UV-treated phage, plating with antiserum resulted in a decreased yield of Trp⁺ transductants on M10C. A possible explanation for this is that transduction (adsorption of phage) continued to occur after transduction mixtures were plated, and the presence of antiserum would prevent such further transduction.

The fact that, under the conditions used in this

TABLE 6. *Effect of multiplicity of infection (MOI) on yield of transductants^a*

MOI	Trp ⁺ transductants	
	Per ml	Per PFU
0	0	0
4.2	310	1.1×10^{-7}
2.1	670	4.9×10^{-7}
1.0	730	1.0×10^{-6}
0.53	610	1.8×10^{-6}
0.26	440	2.6×10^{-6}
0.13	350	4.1×10^{-6}

^a Transduction mixtures contained 6.4×10^8 cells of mutant RM20 and appropriate amounts of phage in a final volume of 1 ml. The phage was treated with UV to give 99.9% inactivation of PFU. MOI values are based on PFU before UV treatment. Transductants were scored on medium M10C.

TABLE 7. *Stability of phage CP-51 under various conditions*

Lysate A			Lysate B				
Treatment ^a	Original PFU (%) remaining after 5 days at		Treatment ^a		Original PFU (%) remaining after		
	2-4 C	24-28 C	Dilution	Storage temp (C)	4 days	15 days	58 days
None	0.25	30	1:10	2-4	20	2.5	0.2
10% DMSO added	38	50	1:10	15	100	100	33
Diluted 1:10	7	50	1:10	21	100	92	2
Diluted 1:10 and 10% DMSO added	60	59	1:10	29	52	16	0.02
			1:10	37	15	0.001	

^a Dilutions (1:10) were made with NBY broth, pH 6.6.

study, *B. cereus* 569 grows in the form of chains of variable length rather than individual cells, probably contributes to the difficulties in quantitation.

I am not aware of any other reported example of a phage that is less stable at refrigerator temperatures (2 to 4 C) than at higher temperatures. However, such a stability characteristic might easily remain undetected, since it is a common practice among investigators isolating phages to maintain them at low temperatures. It seems possible, therefore, that phages of this nature may not necessarily be rare. Dilution in NBY broth and storage at 15 C offered a convenient way of sufficiently stabilizing the phage for the transduction studies, and, therefore, further research on the problem of stability was postponed. A detailed investigation of phage CP-51 with respect to its stability characteristics is planned for the future.

Presumably, this is the first example of transduction and genetic exchange in *B. cereus*. The report by Felkner and Wyss (2) on transformation in *B. cereus* 569 has been shown to be erroneous; the organism used as *B. cereus* 569 in that study was later shown to be *B. subtilis* (C. B. Thorne and I. D. Goldberg, *unpublished results*; I. D. Goldberg and D. D. Gwinn, *in preparation*). Studies are in progress on the susceptibility of other strains of *B. cereus* and related organisms to transduction by phage CP-51. Preliminary results indicate that the phage is

active on several other strains of *B. cereus* and inactive on *B. subtilis* and *B. licheniformis*.

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