HELPER PHAGE-DEPENDENT TRANSFECTION IN BACILLUS SUBTILIS

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This report deals with a bacteriophage, SP-18, that renders competent cells of Bacillus subtilis 168 (ind$^-$) susceptible to transfection by DNAs from apparently unrelated phages. We use the term transfection as defined by Foldes and Trautner (1964), i.e., "infection of cells by the isolated nucleic acid from a virus, resulting in the production of a complete virus." SP-18 DNA was active in transfection, but SP-10 DNA or LP-19 DNA was inactive when tested alone. However, if SP-10 DNA or LP-19 DNA was added to competent cells along with intact SP-18 phage, infective centers of the respective phage, SP-10 or LP-19, were produced. DNA from SP-18 was also active as helper, but it was less effective than the intact phage. SP-18 appeared to be unrelated to SP-10 or LP-19; the three phages were serologically distinct, they had different host ranges, and their DNAs gave different Tm values. The mechanism of the helper effect of SP-18 has not been elucidated.

MATERIALS AND METHODS

Phages SP-18 and LP-19 were isolated from soil by Dr. Ivan D. Goldberg in this laboratory. SP-10 is the transducing phage described previously (Thorne, 1962). All phage suspensions were assayed on phage assay agar (PA) seeded with spores of the appropriate indicator as described by Thorne (1962). SP-10 was propagated on B. subtilis W-23-S^r according to Taylor and Thorne (1966). Shaken cultures in TY broth (Romig, 1962) were used for preparing SP-18 lysates on B. subtilis W-23-S^r and LP-19 lysates on B. licheniformis 9945A.

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Transforming DNA was prepared from B. subtilis W-23-S^r as described by Gwinn and Thorne (1964). Phage DNA was extracted by a modification of the phenol technique of Mandell and Hershey (1960). It was dialyzed for 22 hours at 5 C against 0.15 M NaCl-0.015 M citrate at pH 8.0 to remove the phenol and stored at 5 C. DNA was determined by the method of Burton (1956). The procedure of Marmur and Doty (1962) was used for determining the Tm of DNA and calculating the G-C content. All DNA preparations were tested for contamination with bacteria or viable phage by spreading samples on nutrient agar and by plating samples (after treatment with 50 μg of deoxyribonuclease) in PA agar seeded with the appropriate indicator.

Competent cells of B. subtilis 168 (ind$^-$) were grown by essentially the procedure of Anagnostopoulos and Spizizen (1961). Transformations and transfections were carried out in 18 x 100 mm tubes containing 0.9 ml of recipient culture (usually 2 x 10^8 cells) and 0.1 ml of DNA. The tubes were incubated, usually for 60 min, in a slanted position on a reciprocal shaker at 37 C. Pancreatic deoxyribonuclease (1 X crystallized, Worthington) was added (50 μg in 0.05 ml) and incubation was continued for 15 min. Prototrophic transformants were scored on minimal agar (Thorne and Stull, 1966) and infective centers were scored against the appropriate indicator by the soft agar overlay method in PA agar or minimal 10 agar. The minimal 10 agar was the medium of choice because it contained no tryptophan and therefore did not support growth of the recipient cells of 168 (ind$^-$); however, the indicator cells, B. subtilis W-23-S^r for SP-10 and B. licheniformis 9945A for LP-19, grew well. The efficiencies of plating for SP-10 or LP-19 were the same on PA agar and minimal 10 agar.

RESULTS AND DISCUSSION

Some characteristics of the three phages are shown in Table 1. SP-10 adsorbs to and transduces B. subtilis 168 but does not form plaques or propagate on it (Thorne, 1962). LP-19 neither forms plaques nor propagates on 168; it adsorbs to this strain very poorly if at all. The three phages can be distin-

guished from each other not only by the characteristics shown in Table 1, but also by their distinctive plaque types.

Results typical of those from many transfection experiments are given in Table 2. SP-18 DNA produced infective centers when incubated with competent cells of 168; under the same conditions DNA from SP-10 or LP-19 failed to produce detectable numbers of infective centers. The helper effect associated with SP-18 was first demonstrated in an experiment in which SP-18 DNA and LP-19 DNA were mixed and infective centers for each phage were formed. With mixed DNAs the number of SP-18 infective centers was reduced from that obtained with SP-18 DNA alone; however, this could be interpreted as a result of competition between the two DNAs because in other tests equivalent amounts of bacterial DNA caused a similar reduction in the number of infective centers. When viable SP-18 phage was substituted for the SP-18 DNA in transfection experiments with LP-19 DNA, the number of LP-19 infective centers was increased 100-fold. Infective centers of SP-10 also were produced when competent cells were incubated with SP-10 DNA and SP-18 DNA or SP-18 phage. However, with a given preparation of competent cells, the yield of infective centers from SP-10 DNA was never as great as that from LP-19 DNA. The possibility that SP-18 DNA "helps" because it produces phage which "helps" is unlikely because in an experiment in which SP-18 DNA, LP-19 DNA and SP-18 antiserum were mixed LP-19 infective centers were formed.

The effect of multiplicity of infection (MOI) with SP-18 on transfection with LP-19 DNA is shown in Table 3. As the MOI decreased below 0.5, fewer infective centers were produced. In other experiments there was no significant effect of increasing the MOI within the range of 0.5 to 20. That the helper effect was contributed by the viable SP-18 particles is evidenced by the fact that specific antiserum rendered phage preparations ineffective and by the demonstration that the helper activity of the supernatant fluid after high-speed centrifugation of an SP-18 lysate was consistent with the small number of SP-18 particles remaining.

Table 1. Some characteristics of phages SP-10, SP-18, and LP-19.

Phage	Per cent neutralized* by antiserum to			Tm	Per cent G + C	Forms plaques and propagates on		
	SP-10	SP-18	LP-19			B. subtilis 168	B. subtilis W-23-S^r	B. licheniformis 9945A
SP-10	100	2	4	80.3	43**	no	yes	yes
SP-18	4	100	5	85.3	39	yes	yes	yes
LP-19	18	8	86	88.2	46	no	no	yes

* A sample of each phage was exposed for 5 min to a 1:10 dilution of each antiserum (prepared in rabbits) and the numbers of survivors were determined.

** The per cent G + C for SP-10 is taken from Bott and Strauss (1965); this phage contains an unusual base resulting in a lowered Tm. The others are calculated from the Tm values according to Marmur and Doty (1962).

Table 2. Helper effects of SP-18 phage and its DNA on transfection with DNA from phages SP-10 and LP-19.

	Plaque-forming units of SP-18 added per cell	DNA (μg/ml)			Infective centers/ml		
		SP-18	SP-10	LP-19	SP-18	SP-10	LP-19
Exp. 1	0	0		0	0		0
	0	58		0	2.0×10^6		0
	0	0		181	0		0
	0	58		181	5.0×10^4		1.2×10^5
	16	0		181	*		1.7×10^5
Exp. 2	0	0	0	181	0	0	0
	17	0	0	181	*	0	8.1×10^4
	0	0	151	0	0	0	0
	0	58	151	0	6.6×10^3	9.0×10^2	0
	17	0	151	0	*	3.4×10^3	0

Recipient cells (2×10^8), DNA, and SP-18 phage as given were incubated together in a final volume of 1 ml for 60 min. Following treatment with deoxyribonuclease the mixtures were assayed for infective centers in PA agar seeded with spores of B. subtilis W-23-S^r (for SP-10) or B. licheniformis 9945A (for SP-18 and LP-19). To assist in scoring SP-10 and LP-19 infective centers SP-18 antiserum was added to the plating mixtures to inactivate residual free SP-18 as well as that released from SP-18 infective centers. Controls in which phage DNA was treated with deoxyribonuclease before it was added to recipient cells produced no infective centers. The frequencies of transformation obtained with the recipient cells were 0.9% in Exp. 1 and 0.7% in Exp. 2.

* SP-18 was not determined.

Table 3. Effect of multiplicity of infection with SP-18 helper phage on transfection with LP-19 DNA.

MOI (SP-18)	LP-19 DNA (μg/ml)	LP-19 infective centers/ml
0	0	0
0	112	0
5×10^{-1}	112	2.5×10^{5}
5×10^{-2}	112	2.3×10^{4}
5×10^{-3}	112	1.0×10^{3}
5×10^{-4}	112	1.0×10^{2}
5×10^{-5}	112	10
5×10^{-6}	112	0
8×10^{-4}*	112	3.7×10^{2}
5×10^{-1}**	112	0

The procedure was the same as that given in the footnote to Table 2.

* Most of the SP-18 particles were removed from a sample of lysate by centrifugation, and the supernatant fluid was tested undiluted for helper effect. The number of SP-18 PFU remaining in the sample gave a MOI of 8×10^{-4}; the concentration of supernatant fluid in this test was equivalent to that in the sample in which the MOI was 0.5.

** The SP-18 was treated with specific antiserum before it was added to the cells.

The data in Table 4 demonstrate the effect of time of addition of SP-18 helper phage to mixtures of recipient cells and LP-19 DNA. Incubation of cells with LP-19 DNA prior to the addition of SP-18 phage appeared to be ineffective. When the phage was added 5 min before the DNA the results were no different from those obtained when phage and DNA were added at the same time. The critical factor appeared to be the time of incubation of cells in the presence of helper phage and transfecting DNA together. Experiments to be reported elsewhere show that the majority of LP-19 plaques formed after incubation of cells in the presence of DNA and helper phage for 60 min were produced by infected cells rather than by free phage. Disruption of cells from transfection mixtures by treatment with lysozyme and chloroform showed that after 60 min fewer than 10 per cent of the infective centers could be accounted for as mature phage. After 90 min all infective centers could be accounted for as mature phage and the average burst size was calculated to be about six. Although in

the experiments reported here 100 μg or more of LP-19 DNA per ml was used, maximum yields of infective centers were obtained with 50 to 60 μg of DNA per ml and detectable numbers were produced with as little as 0.5 μg.

The mechanism of the helper effect observed with SP-18 will be the subject of further investigation. Some of the possibilities we have considered are: (1) The phage "helps" the SP-10 DNA or LP-19 DNA enter the cell. This seems unlikely because SP-18 DNA as well as intact phage acts as helper. If the helper function is related to entry of DNA into the cell, one might expect non-competent cells to be effective. However, experiments not reported here show that helper-dependent transfection correlates with competence of the recipient cells for transformation. Furthermore, the fact that SP-10 adsorbs to cells of 168 and injects DNA, resulting in transduction, also implies that the function of helper phage is not concerned with entry of transfecting DNA into the

Table 4. Effect of time of addition of SP-18 phage on transfection with LP-19 DNA.

LP-19 DNA (μg)	Minutes of incubation with LP-19 DNA: Before addition of SP-18	Minutes of incubation with LP-19 DNA: After addition of SP-18	LP-19 infective centers/ml
100	-5*	65	1.8×10^5
100	0	60	1.9×10^5
100	30	60	1.9×10^4
100	30	30	1.3×10^4
100	60	30	1.0×10^4
100	0	30	1.8×10^4

Recipient cells (2×10^8 in 0.9 ml) and LP-19 DNA (0.1 ml) were combined in tubes and incubated on the shaker. At the times indicated SP-18 phage (0.1 ml) was added and incubation was continued as shown. The MOI with SP-18 was 0.5. Each sample was treated with deoxyribonuclease after its final incubation period. The LP-19 infective centers were scored in minimal 10 agar seeded with spores of B. licheniformis. SP-18 antiserum (0.1 ml) was included in each indicator plate to inactivate residual SP-18 phage as well as that released from SP-18 infective centers. A sample of recipient cells was tested for competence by transformation to tryptophan independence; the frequency of transformation was 0.5%. In control tubes LP-19 infective centers were not produced in the absence of SP-18 phage, or with SP-18 phage that had been treated with specific antiserum, or with LP-19 DNA that had been treated with deoxyribonuclease.

* In this tube phage was added 5 min before the DNA.

cell. (2) The function of the helper is in lysis of the cell and release of the heterologous phage. This seems unlikely because no phage was detected when competent cells were exposed to LP-19 DNA alone and disrupted at various times by treatment with lysozyme and chloroform. This treatment was not harmful to mature LP-19. In LP-19 transfection experiments with helper SP-18 DNA in which infective centers were scored in the absence of SP-18 antiserum, LP-19 plaques were picked and tested for the presence of SP-18 by plating on W-23-S^r; none was detected, indicating that mixed bursts did not occur. (3) The DNA from the helper phage and the transfecting DNA undergo recombination. No evidence for recombination has been uncovered. LP-19 produced by transfection retained its serological specificity and distinctive plaque morphology, it did not plaque on 168 or W-23-S^r, and its DNA was ineffective in transfection unless helper SP-18 was added. (4) The genome of the transfecting phage becomes incorporated in an SP-18 protein coat. This seems unlikely because one would expect such a particle to be inactivated by SP-18 antiserum and unable to initiate plaque formation in the presence of the antiserum. (5) The helper phage interferes with restriction of the heterologous phage DNA. At the present stage of the investigation no evidence against this possibility is apparent to us.

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REFERENCES

Anagnostopoulos, C. and J. Spizizen, J. Bacteriol. 81, 741 (1961).
Bott, K. and B. Strauss, Virology 25, 212 (1965).
Burton, K., Biochem. J. 62, 315 (1956).
Foldes, J. and T. A. Trautner, Z. Vererbungsl. 95, 57 (1964).
Gwinn, D. D. and C. B. Thorne, J. Bacteriol. 87, 519 (1964).
Mandell, J. D. and A. D. Hershey, Anal. Biochem. 1, 66 (1960).
Marmur, J. and P. Doty, J. Mol. Biol. 5, 109 (1962).
Romig, W. R., Virology 16, 452 (1962).
Taylor, M. J. and C. B. Thorne, J. Bacteriol. 91, 81 (1966).
Thorne, C. B., J. Bacteriol. 83, 106 (1962).
Thorne, C. B. and H. B. Stull, J. Bacteriol. 91, 1012 (1966).