

# Growth and Cultivation of the Unusual Generalized Transducing *Bacillus* Bacteriophage SP-15

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Additional properties of SP-15, a generalized transducing bacteriophage notable for the ability to transfer an unusually large fragment of deoxyribonucleic acid (DNA) to *Bacillus subtilis* and *B. licheniformis*, are presented together with improved methods that enhance its utility. Simple means have been found to provide the rigid control over moisture that is necessary for the assay of plaque-forming units (PFU). Reproducible procedures for propagating transducing phage, which depend upon an appropriate mixing of PFU with uninfected bacteria, have replaced less reliable methods that utilized infected spores. Transduction of *B. subtilis* W-23 increased linearly when  $MgSO_4$  in recipient cell-SP-15 mixtures was increased from 0.005 to 0.03 M. Methods have been developed that protect SP-15 from the damaging effects of CsCl and of osmotic shock subsequent to dilution. Evidence that the PFU and transducing particles of lysates decay at the same slow rate during extended storage suggests that the decay is a result of damage to protein rather than to DNA. One-step growth experiments, in which SP-15 was propagated on *B. subtilis* W-23-S<sup>r</sup>/1 mg, indicated a latent period of 100 min, a rise period of 60 min, and a burst size of 25 to 34 PFU per infected cell. These findings suggest explanations for some of the technical difficulties SP-15 has presented.

Evidence (12) that bacteriophage SP-15 (9) can transfer a larger fragment of the *Bacillus* genome than any phage heretofore described established its unique value for mapping the genome of *B. licheniformis* 9945A and provided a valuable addition to the generalized transducing phages already available for the chromosome mapping of *B. subtilis* W-23. Further, the buoyant density in CsCl of SP-15 deoxyribonucleic acid (DNA) was found to be greater than that of either SP-10 or PBS-1, two well-known *Bacillus* transducing phages. SP-15, therefore, should be especially suited for experiments involving density gradient separation of plaque-forming units (PFU) containing phage DNA from transducing particles (TP) containing host DNA (7). Such experimentation with SP-15 was not practical until new techniques were devised for handling this phage that would be more reliable and less cumbersome than the techniques reported earlier (9, 12). This report describes our improved methods for the preparation and assay of SP-15 lysates, the stability of such lysates during storage, and a procedure for protecting

particles of this phage from the damaging effects of CsCl. Estimates of latent period and burst size, obtained from one-step growth experiments, will be correlated with certain unusual properties of SP-15.

## MATERIALS AND METHODS

**Organisms.** The strains of *Bacillus* used for this study are listed in Table 1. Heat-shocked spores of all strains were stored in water at 4 C and reheated for 30 min at 65 C within 48 hr of use to inoculate broth cultures for phage propagation. Strain W-23-S<sup>r</sup>/1 mg served both as host and indicator strain for the one-step growth experiments. Unless indicated otherwise, the work for this report was with a lysate of SP-15 derived from a single plaque to eliminate any mutant phage that might have accumulated during the repeated propagation of SP-15 since its isolation 8 years earlier. This lysate, 922GL, exhibited the same activities of PFU and TP as did lysates derived from the parent strain.

**Media.** The media were those used by Tyeryar et al. (12) or Goldberg and Bryan (3), except that minimal 1-yeast extract-glycerol broth contained 0.4 g of  $MgSO_4 \cdot 7H_2O$  per liter and PA soft agar was usually prepared with 0.45% agar. PA<sup>++</sup> broth indicates additions to PA broth of 40  $\mu g$  of  $FeCl_3 \cdot 6H_2O$  and 1  $\mu g$  of  $ZnSO_4 \cdot 7H_2O$  per ml (12). The usual diluent

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TABLE 1. Description of *Bacillus* cultures

Designation	Tentative genotype	Origin or reference
<i>Bacillus licheniformis</i> 9945A	Wild type	Thorne (11)
<i>Bacillus subtilis</i> W-23	Wild type	Ft. Detrick
W-23-S <sup>r</sup>	<i>str</i> <sup>a</sup>	Thorne (11)
W-23-S <sup>r</sup> /1 mg	<i>str</i> /1 mg	Ft. Detrick <sup>b</sup>
W-23-S <sup>r</sup> M26	<i>ile-01</i>	Ft. Detrick <sup>c</sup>
W-23 M9	<i>ile-1</i>	Ft. Detrick <sup>c</sup>
W-23 TD-G8	<i>lys-1 tyr-1</i>	Ft. Detrick <sup>d</sup>

<sup>a</sup> Abbreviations: *ile*, isoleucine; *lys*, lysine; *tyr*, tyrosine; *str*, resistance to streptomycin.

<sup>b</sup> This strain was isolated from W-23-S<sup>r</sup> by a method previously described (3).

<sup>c</sup> Both *ile*<sup>-</sup> mutants were obtained by ultraviolet treatment (4).

<sup>d</sup> This double auxotroph was derived from W-23 *lys-1 tyr-1 ile-2* (12) by transduction with SP-15 propagated on W-23-S<sup>r</sup>.

for cells and phage was 1% Difco peptone in water (w/v). All media were made with triple glass-distilled water.

**Phage assay.** The most accurate method for the assay of SP-15 PFU is the spotting technique previously described (9). We used that technique, with incubation of plates at 30 C instead of 37 C, for evaluating the more rapid assay methods to be described below.

**Phage propagation.** Details regarding media and numbers of PFU and colony-forming units (CFU) for propagating SP-15 will be found below. Lysates from broth cultures of *B. subtilis* W-23-S<sup>r</sup> or W-23-S<sup>r</sup>/1 mg infected with phage were processed and stored as before (12). When phage was concentrated by centrifugation of filtrates, the pellet was resuspended in supernatant fluid.

**Transduction.** Transductions were accomplished as described previously (9, 10, 12). A phage diluent used to control Mg<sup>2+</sup> concentration contained, for each milliliter of transduction mixture, 0.075 ml of glycerol, 0.175 ml of peptone diluent, 0.025 ml of 1.2 M MgSO<sub>4</sub>, and 0.025 ml of water containing 50 μg of deoxyribonuclease. For the experiments in Table 2, the concentration of the MgSO<sub>4</sub> to be included was altered appropriately above or below 1.2 M. Transduction mixtures consisted of 0.3 ml of this phage diluent, 0.2 ml of phage lysate or lysate adjusted with heat-inactivated lysate, and 0.5 ml of recipient cells cultured in minimal 1-yeast extract-glycerol broth. When lysates were assayed for their ability to cotransduce two markers (12), strain W-23 *lys-1 tyr-1* was chosen as recipient so that transductant genotypes could be identified by their colonial pigmentation (8).

**Solutions for CsCl studies.** The buffer of Okubo et al. (7) [0.1 M tris(hydroxymethyl)aminomethane, containing 0.005 M MgCl<sub>2</sub>·4H<sub>2</sub>O and adjusted to pH

7 with HCl(Tris-Mg)] was used for control suspensions of phage, for preparation of the CsCl solution required for density gradient banding, and for preparation of the high molarity salt solutions (MgSO<sub>4</sub>, KCl) needed for protection when diluting out of CsCl. A concentrated CsCl stock solution (approximately 98.0% w/v) in Tris-Mg was filtered, and a measured sample then weighed for an empirical determination of its density. Vinograd's equation (13) provided a convenient guide for combining aqueous suspensions of phage with the stock CsCl solution to obtain a density of 1.5 g/cm<sup>3</sup> (approximately 4 M). MgSO<sub>4</sub> (2 M) in Tris-Mg, pH 7 (2 M Mg), was used to protect phage against osmotic shock damage when diluting from 4 M CsCl. This solution was added to the CsCl suspension of phage very gradually for the 10<sup>-1</sup> dilution. Subsequent 10-fold dilutions required for PFU assay were made in dilution blanks containing a series of twofold dilutions of 2 M Mg made in Tris-Mg (e.g., 10<sup>-2</sup> in 1 M Mg<sup>2+</sup>, 10<sup>-3</sup> in 0.5 M Mg<sup>2+</sup>, etc.). Protection of phage for transduction assays was provided by mixing 0.01 ml of phage in 4 M CsCl with 0.01 ml of 2 M Mg and then adding 0.48 ml of a diluent composed of 0.18 ml of PA broth, 0.075 ml of glycerol, 0.175 ml of peptone diluent, and 0.05 ml of water containing 50 μg of deoxyribonuclease. Phage so protected was mixed 1:1 with recipient cells for transduction assay.

**One-step growth experiments.** Spores (2.5 × 10<sup>6</sup>) of *B. subtilis* W-23-S<sup>r</sup>/1 mg, in 50 ml of TY broth in a 250-ml Erlenmeyer flask, were cultured on the reciprocal shaker (10) for 16 hr when the cells were subcultured by adding 5 ml to 45 ml of prewarmed TY broth in a 500-ml flask on the shaker at 37 C. At 30 min, using the general procedure of Adams (1), phage and cells were mixed at an approximate input ratio of 0.25, which achieved a multiplicity of infection (MOI) of approximately 0.1 when adsorption was stopped after 5 min by dilution into antiserum. Further dilutions were made into prewarmed TY broth (final volume of 8 ml) in 50-ml Erlenmeyer flasks for growth tubes which were kept on a water-bath shaker, 3-cm stroke (Eberbach Corp., Ann Arbor, Mich.), set for 37 C and 100 excursions per min. Triplicate or duplicate 0.1-ml samples to be assayed for infected cells and free phage were removed at appropriate intervals to tubes of soft agar containing spores.

**Phage antiserum.** Neutralizing antiserum to SP-15 was that of Taylor and Thorne (9). At a dilution of 1:700, the neutralizing activity of this serum was adequate for these experiments.

**Materials.** Glazed clay culture dish covers were porcelain, Coors 222, inside diameter 97 mm. Polystyrene culture dishes (100 by 15 mm) were supplied by Lab-Tek Products Division, Miles Laboratories, Inc., Westmont, Ill. Deoxyribonuclease was from Worthington Biochemical Corp., Freehold, N.J. Cesium chloride was Trona brand, American Potash and Chemical Corp., New York, N.Y. An optical grade CsCl (Harshaw Chemical Co., Cleveland, Ohio) was also tested. Filter membranes were from Millipore Corp., Bedford, Mass.

## RESULTS AND DISCUSSION

**Assay for plaque-forming units.** Although the spotting method of Taylor and Thorne (9) provided the moisture control necessary to maintain the delicate balance between the growth of the indicator lawn and the replication of SP-15, simpler and more rapid procedures were required for the large-scale experiments in these studies. After testing many variables in the assay technique, we concluded that lawn cell development was controlled satisfactorily when assay plates were prepared as follows. At 1 hr or less before use, 25 ml of melted base agar, cooled to 46 C, was pipetted into each plastic petri plate bottom and covered immediately with a glazed clay lid. Soft agar lawn (46 C) was prepared by mixing, for 10 plates, 9 ml of PA base agar, 21 ml of PA broth, and  $2 \times 10^9$  spores of W-23-S<sup>r</sup> suspended in 1 ml of water. This lawn was layered over each plate, either from a warm test tube in which 3.1 ml of lawn and 0.1 ml of phage (diluted to contain 50 to 100 PFU) had been mixed or by a method suggested by R. M. Zsigray (*personal communication*) in which the 0.1-ml sample of diluted phage was placed in each plate on the base agar surface and 3.1 ml of lawn was added from a warm pipette. Zsigray's method was advantageous when many plates were to be layered in rapid succession and produced the same titers as the method by which lawns containing phage were poured from a tube. The plates were held at room temperature for 30 min before being inverted carefully and incubated at 30 C, which was better for plaque development than 26 or 37 C. When more moisture was needed, a pan of water was placed in the incubator. Plastic lids replaced the clay after overnight incubation. The same procedures revealed infective centers when appropriate dilutions of cells infected with SP-15 were plated. The plaques from infected cells were visible more clearly, and developed sooner, than plaques from suspensions of free phage particles or carrier spores. All titers were calculated from counts made after 24 hr of incubation, although the number of plaques from infected cells did not increase after the first 16 hr. Plaques were counted under a dissecting microscope. Both of these techniques, which entailed mixing phage with lawn before solidification, gave titers for lysates that were lower by 10 to 25% than titers obtained when the same lysates were assayed by the spotting technique (9), but under any given set of conditions the titers were reproducible. It is not possible to estimate how many infected spores go undetected by any procedure, but no procedure detects more than the spotting technique.

**Phage propagation.** Although SP-15-infected spores of *B. subtilis* W-23, or of several of its derived strains, and of *B. licheniformis* 9945A usually gave rise to lysates that transduced well and had good PFU titers (up to  $2 \times 10^{10}$  PFU/ml), certain variables were difficult to control after infected spores had been stored for several years. Reliable methods for the propagation of phage by mixing uninfected bacteria with SP-15 lysates can now be reported.

For propagation on 9945A, a modification of the soft agar layering procedure of Thorne (11) was developed. PA base agar (25 ml) was pipetted into the required number of heavy-bottomed glass petri dishes and allowed time only to solidify. For each 10 plates to be layered, the following mixture was warmed to 37 C:  $10^9$  spores in 2 ml of water, 2 ml of PA broth-diluted SP-15, (approximately  $2 \times 10^9$  PFU), 2,000  $\mu$ g of FeCl<sub>3</sub>·6H<sub>2</sub>O, and 50  $\mu$ g of ZnSO<sub>4</sub>·7H<sub>2</sub>O in 0.5 ml of water. Then 15 ml of PA base agar and 35 ml of PA broth (both at 46 C) were added, and 5.4 ml of the combined mixtures was pipetted quickly over each plate of base agar. These plates were incubated at 37 C for 18 hr. For harvesting, each soft layer was flooded with 5 ml of PA<sup>++</sup> broth and macerated with a glass spreading rod. The macerated material was pipetted into tubes for centrifugation for 15 min at approximately  $1,000 \times g$ , which was then increased to  $5,900 \times g$  for an additional 15 min. The supernatant liquid was sterilized by filtration through membranes (AA or DA, or both, followed by HA; Millipore Corp.). The average yield from each plate was 5 ml of filtrate with a titer of approximately  $10^{11}$  PFU per ml.

Propagation of SP-15 on W-23 strains was satisfactory in broth. Exploratory experiments with different infected spore stocks as inocula for broth cultures demonstrated that preparation of SP-15 lysates with good transducing efficiency depended upon several variables, including the number of CFU used to inoculate PA<sup>++</sup> broth cultures and the ratio between uninfected and infected spores in the inoculum. An infected culture was constructed as follows. A 50-ml amount of PA<sup>++</sup> broth containing  $1.1 \times 10^9$  spores was shaken in a 250-ml flask at 37 C for 3.5 hr. Then  $1.1 \times 10^6$  PFU of SP-15 were added and incubation was continued for another 22 hr. A microscopical examination of the 3.5-hr culture revealed good germination with many cells already motile. One lysate that was prepared by this protocol, with spores of W-23-S<sup>r</sup>/1 mg, had a titer of  $1.3 \times 10^{10}$  PFU/ml and transduced Ile<sup>-</sup> to Ile<sup>+</sup> with an efficiency of 2.3 transductants per  $10^6$  PFU.

TABLE 2. Effect of  $Mg^{2+}$  on transduction of *Bacillus subtilis* by SP-15<sup>a</sup>

Composition of transduction mixture					Yield of transductants		
Phage lysate		No. of recipient cells/ml	Ratio of PFU to cells	Final molar concn $Mg^{2+}$ <sup>b</sup>	Per ml	Per PFU	
Prepn	Age (days)						
854D	20	$1.2 \times 10^9$	0.70	0	257	$3.1 \times 10^{-7}$	
				0.0005	297	$3.6 \times 10^{-7}$	
				0.005	583	$7.1 \times 10^{-7}$	
				0.0125	1190	$1.5 \times 10^{-6}$	
				0.025	2137	$2.6 \times 10^{-6}$	
	25	$1.6 \times 10^9$	0.46	0	320	$4.2 \times 10^{-7}$	
				0.005	1695	$2.2 \times 10^{-6}$	
				0.01	3330	$4.4 \times 10^{-6}$	
				0.02	3400	$4.5 \times 10^{-6}$	
				0.04	3405	$4.5 \times 10^{-6}$	
				0.08	2455	$3.2 \times 10^{-6}$	
				0.16	2520	$3.3 \times 10^{-6}$	
				0.32	1200	$1.6 \times 10^{-6}$	
				0.23	0	135	$3.6 \times 10^{-7}$
					0.005	1050	$2.8 \times 10^{-6}$
0.01	1590	$4.2 \times 10^{-6}$					
0.02	2115	$5.6 \times 10^{-6}$					
0.04	2075	$5.5 \times 10^{-6}$					
0.08	1500	$4.0 \times 10^{-6}$					
	1295	$3.4 \times 10^{-6}$					
	680	$1.8 \times 10^{-6}$					
	844C	59	$1.2 \times 10^9$	0.83	0.005	1355	$1.4 \times 10^{-6}$
					0.032	2345	$2.4 \times 10^{-6}$
851M	36	$1.2 \times 10^9$	0.31	0.005	515	$1.4 \times 10^{-6}$	
				0.032	860	$2.4 \times 10^{-6}$	

<sup>a</sup> Recipient cells were strain W-23-S<sup>r</sup> M26.

<sup>b</sup> This value does not include residual  $Mg^{2+}$  in the cell culture.

**Effect of  $Mg^{2+}$  on transduction.** A linear relationship between the concentration of  $Mg^{2+}$  and the yield of transductants was observed when the variable in transduction experiments was  $Mg^{2+}$  concentration (Table 2). Linearity extended from below 0.005 M to 0.02 to 0.04 M  $MgSO_4$ , indicating that 0.03 M was a suitable concentration for  $Mg^{2+}$  in transduction mixtures of SP-15 with W-23 strains.

Of more than practical interest is the observation that  $Mg^{2+}$  concentration is limiting for the number of transducing particles that can effect transduction in an otherwise optimal mixture of phage and recipient cells. During the years when the approximate concentration of  $MgSO_4$  in transduction mixtures was 0.005 M, we had often observed that increases in MOI to above 1 were accompanied by decreased yields, per PFU, of transductants. When transduction experiments were executed in the presence of 0.03 M  $MgSO_4$ , the yield of transductants per PFU was not

affected by MOI, remaining nearly constant if given an adequate concentration of motile cells. In this connection, it should be noted that adsorption of SP-15 is limited to flagellated cells (J. Mele and C. B. Thorne, *personal communication*). Experiments done with 0.03 M  $MgSO_4$  showed that the cotransduction frequency of *lys* and *tyr* in W-23 TD-G8 was 80.3%, in good agreement with the value reported for cotransduction of the same pair of markers in W-23 M10-M62 (12) when 0.005 M  $MgSO_4$  was used for transduction mixtures. We do not know the step(s) in transduction for which  $Mg^{2+}$  is limiting, whether the phenomenon affects the SP-15-*B. licheniformis* transducing system, or whether other divalent metal ions might be equally or more influential.

**Stability during storage.** As reported earlier (12), SP-15 lysates prepared in PA<sup>++</sup> broth retained good transducing ability during storage at 4 C for several months. The data in Table 3

TABLE 3. *Stability of SP-15 particles during storage at 4 C*

Phage lysate			Transduction assay <sup>a</sup>		
Prepn	Age (days)	PFU/ml	Ratio of PFU to CFU	Yield of transductants	
				Per ml	Per PFU
844C	15	$1.4 \times 10^{10}$	0.77	1925	$1.4 \times 10^{-6}$
	59	$9.8 \times 10^9$	0.83	1355	$1.4 \times 10^{-6}$
854D	3	$1.0 \times 10^{10}$			
	20	$8.2 \times 10^9$	0.70	2137	$2.6 \times 10^{-6}$
	25	$7.6 \times 10^9$	0.46	3400	$4.5 \times 10^{-6}$
	60	$7.5 \times 10^9$	0.57	1850	$2.5 \times 10^{-6}$
	209	$3.0 \times 10^9$	0.18	1275	$4.3 \times 10^{-6}$
	223	$2.9 \times 10^9$	0.38	2463 <sup>b</sup>	$4.3 \times 10^{-6}$
	273	$3.2 \times 10^9$	0.14	1030	$3.3 \times 10^{-6}$
922GL	1	$1.3 \times 10^{10}$	0.61	3080	$2.3 \times 10^{-6}$
	190	$4.4 \times 10^9$	0.34	1906	$2.5 \times 10^{-6}$
922GL-a <sup>c</sup>	190	$2.2 \times 10^9$	0.34	1176 <sup>b</sup>	$2.7 \times 10^{-6}$

<sup>a</sup> Recipient cells ( $10^9$  to  $2 \times 10^9$  CFU/ml) were an isoleucine-requiring strain of *B. subtilis* W-23. The transduction assays on lysate 844C were with  $0.005$  M  $Mg^{2+}$ , and those on 854D and 922GL were with  $0.03$  M  $Mg^{2+}$ , above the residual  $Mg^{2+}$  of the cell culture.

<sup>b</sup> This represents the yield from 0.2 ml of lysate; all other yields were from 0.1 ml of lysate.

<sup>c</sup> This tube of lysate had been removed from 4 C to room temperature and shaken, repeatedly, for removal of samples.

show that SP-15 titers dropped gradually for a few months and leveled off at approximately 30 to 35% of the initial titer. Transducing efficiency did not appear to change significantly during extended storage. Tests with several lysates, including the single plaque-derived 922GL, confirmed this finding, suggesting that PFU and TP have the same degree of stability. In other words, the storage stability seems to be determined by the phage protein rather than by the DNA it contains.

**One-step growth experiments.** PA media were most satisfactory for SP-15 propagation on both *B. subtilis* and *B. licheniformis* and for PFU titration on *B. subtilis*. We should have preferred, therefore, to determine the latent period and burst size for SP-15 in PA broth, but one-step growth experiments in this medium were not satisfactory. An improbably long latent period preceded an unrealistically large burst size, making the phage growth curves reminiscent of Meynell's experience with phage  $\alpha$  on *B. anthracis* (5). We turned to TY broth, a medium in which the rate of cell growth became exponential without perceptible lag when overnight cultures from spores were subcultured. Figure 1 presents the results of a typical one-step growth experiment, on W-23-S<sup>r</sup>/1 mg, showing that the latent period

was approximately 100 min, that the infected cells continued to burst for 1 hr, and that the average burst size was 25 PFU per infected cell. Our observations indicated that all infected cells, if plated during the first 30 min after infection, were represented by sharp, clear plaques after 16 hr of incubation at 30 C in a W-23-S<sup>r</sup>/1 mg lawn. Plaques of this phenotype were rare or missing on plates from samples taken after 100 min, when the numbers of very small plaques, typical for SP-15, began to increase rapidly. We indicated earlier that up to 25% of the PFU that can form plaques, when plated by the spotting technique, often fail to produce visible plaques when plated by other techniques. Thus, the average burst size for the experiment of Fig. 1 may have been nearer 30 than 25. When the data from a replication of this experiment were plotted, the latent and rise periods were superimposable on the curve of Fig. 1, but the average burst size was 34. These observations of long latent and rise periods and of small burst size, combined with the requirement of SP-15 for flagellated cells for adsorption, may explain why suitable conditions for phage adsorption and growth

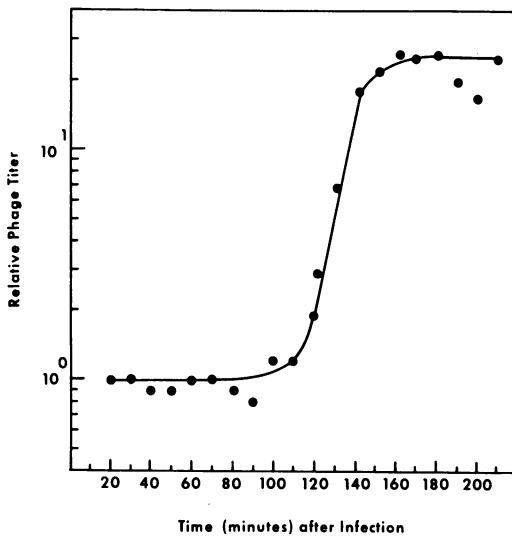


FIG. 1. Typical one-step growth experiment with SP-15 grown on *Bacillus subtilis*. Phage lysate was added to 30-min subcultured, exponentially growing cells of W-23-S<sup>r</sup>/1 mg in TY broth to achieve an actual multiplicity of infection of 0.11 during the 5 min allowed for adsorption. This mixture, having  $2.7 \times 10^8$  CFU/ml, was then diluted 1:100 in warm TY broth containing a 1:700 dilution of SP-15 antiserum, shaken for 5 min, and diluted in TY broth for final growth tube dilutions of  $10^{-5}$ ,  $2 \times 10^{-6}$ , and  $5 \times 10^{-7}$ . The points were plotted relative to the averaged number of infected cells that were found in the 20- and 30-min samples.

must be maintained in soft agar lawns throughout several cycles of phage replication to produce visible plaques. These same biological and physical properties may also explain why infection and lysis of several generations of host cells were necessary for the production of high-titer lysates.

**Protection of SP-15 during and after suspension in CsCl.** When we centrifuged SP-10 in CsCl at 4 C by the procedures of Okubo et al. (7), we observed that gradual addition of peptone diluent for the first 10-fold dilution of CsCl gradient fractions protected PFU and TP from severe damage. When SP-15 was held in 4 M CsCl overnight at room temperature and then diluted as for SP-10, only 0.01% of the PFU survived. This result was unexpected because we knew that lysates of SP-15 were remarkably stable, even at room temperature. With the idea that this loss in titer could be caused wholly by osmotic shock damage to SP-15, we tried various means others have suggested for protecting a variety of phages (2, 6). Loss in PFU titer after periods of 1 to 2 hr in CsCl at room temperature was prevented by making the  $10^{-1}$  dilution gradually with either 2 M  $MgSO_4$  or 3 M KCl, in Tris-Mg, before diluting further in peptone diluent. But after exposure of SP-15 for 24 hr at 26 C to CsCl, neither 2 M  $MgSO_4$  nor 3 M KCl for the  $10^{-1}$  dilution, followed by further 10-fold dilutions through 2-fold graded dilutions of  $MgSO_4$  or KCl, permitted recovery of more than 0.02 to 0.1% of the PFU recovered from Tris-Mg control suspensions diluted in the same manner. Protection was needed during at least 24 hr of exposure to CsCl when gradient centrifugation and follow-up assays were required. Table 4 shows that lowering the temperature to 4 C during 24 and 48 hr of exposure to CsCl pro-

vided complete protection if followed by gradual dilution with 2 M  $MgSO_4$  or 3 M KCl but not by gradual dilution with peptone. To avoid loss from osmotic shock attributable to the protecting salts, the further dilution necessary for assay of PFU was made as indicated in the footnotes to Table 4. An explanation for the fourfold increase in titer after 24 hr in CsCl was not sought, but its reality was confirmed in a replicate experiment performed with another lysate. The 98% decrease in titer that was found upon diluting from cold CsCl into peptone probably reflects the effect of osmotic shock. The nature of a greater, and apparently irreversible, damage caused to SP-15 by more than a few hours of suspension in CsCl at room temperature was not investigated further. Optical grade CsCl was equally damaging. The procedure described earlier for handling samples of SP-15 in CsCl for transduction assays was based on the results reported above. This procedure, because compromises were necessary, provided slightly less protection for SP-15 particles than did the procedure recommended for PFU assay dilutions, but it made possible the dilution of 0.01-ml samples in a fluid that was compatible with transduction assay.

The buoyant densities in CsCl of SP-15 particles were not determined, but can be estimated by using the values of 1.762 g/cm<sup>3</sup> (12) and 1.703 g/cm<sup>3</sup> (6) for the buoyant densities in CsCl of the DNA of SP-15 and *B. subtilis*, respectively. When these values are substituted in the equation of Weigle, Meselson, and Paigen (14), with the assumption that DNA and protein each contributes half the mass of a phage particle, one finds that SP-15 particles filled with bacterial DNA should band at a density 0.0295 g/cm<sup>3</sup> lighter than those filled with phage DNA. This density difference would place SP-15 transducing particles and plaque-forming units 84 drops (1 drop = 0.01 ml) apart after centrifugation in a CsCl gradient. Okubo et al. (7) found the peaks of SP-10 PFU and TP to be only 17 drops apart; we obtained very similar results (*unpublished data*). Given the sharper separation that is predicted for the PFU and TP of SP-15 propagated on *B. subtilis* W-23, experiments can be designed to reveal whether TP are entirely free from phage DNA. Information on this point would contribute toward an understanding of the manner in which phage particles package bacterial DNA.

TABLE 4. Protection of SP-15 during and after suspension in CsCl

Suspending conditions			Diluting fluid	PFU/ml	Ratio of treated to controls × 100
Time (hr)	Temp (C)	Fluid			
24	26	Tris-Mg <sup>a</sup>	Graded Mg <sup>a</sup>	$1.0 \times 10^8$	100
24	26	CsCl <sup>b</sup>	Graded Mg	$5.0 \times 10^6$	0.05
24	4	CsCl	Graded Mg	$4.2 \times 10^8$	402
48	4	CsCl	Graded Mg	$1.2 \times 10^8$	120
48	4	CsCl	KCl <sup>c</sup>	$1.3 \times 10^8$	130
48	4	CsCl	Peptone	$2.5 \times 10^7$	2.5

<sup>a</sup> See Materials and Methods.

<sup>b</sup> CsCl of density 1.721 g/cm<sup>3</sup> in Tris-Mg was mixed with SP-15 lysate in Tris-Mg to give  $d = 1.5$  g/cm<sup>3</sup>.

<sup>c</sup> A  $10^{-1}$  dilution was made with 3 M KCl in Tris-Mg, and subsequent 10-fold dilutions were made in peptone diluent (1% Difco peptone, w/v).

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