LYSOGENY IN *RHIZOBIUM LEGUMINOSARUM* AND *RH. TRIFOLII**

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[*Manuscript received December* 17, 1962]

Summary

Seven lysogenic strains—three of *Rh. leguminosarum* and four of *Rh. trifolii*—were identified among a total of 54 strains from these two species and *Rh. phaseoli*. The *Rh. leguminosarum* strains may be multiply lysogenic; two of them also produce lethal agents resembling bacteriocins in their effect. Lysogeny was confirmed by standard criteria of ultraviolet light inducibility, self-immunity, and reductive ability. Lysogenic conversion involving symbiotic characteristics was not observed. A minimum of six different temperate phages and two clear-plaque-forming variant phages were identified. The host range includes strains of the above three rhizobial species, but not of *Rh. melilotii*. Variation in host specificity of some phages following host passage is under investigation.

I. INTRODUCTION

Current interest in the possibility of modifying the symbiotic capabilities of *Rhizobium* by various techniques of bacterial genetics, including transduction, has given added impetus to investigations on the occurrence of lysogeny in this genus and in related genera. Marshall (1956) first reported a lysogenic strain of *Rh. trifolii*. Davis (1958) described a temperate phage of *Rh. meliloti*. Takahashi and Quadling (1961), using Marshall's lysogenic strain (SU 298) and indicator strain (SU 297), recently confirmed and extended Marshall's earlier findings. They found SU 298 to be apparently defectively lysogenic, releasing two different functional phages only after exposure to phage-like inducing particles produced by SU 297. The most extensive investigation of lysogeny in *Rhizobium* appears to be that of Szende and Ördögh (1960) who screened 152 strains of *Rh. meliloti* and found 44 of them to be lysogenic on the basis of self-immunity to the liberated phage. Two widely sensitive indicator strains, lysogenized with 13 temperate phage strains, were used to further classify the phages and assign them to seven groups.

This paper summarizes results from a study of lysogeny in three apparently closely related cross-inoculation groups (species) of rhizobia—*Rh. leguminosarum*, *Rh. trifolii*, and *Rh. phaseoli*.

* This research was initiated at Brookhaven National Laboratory, Upton, N.Y., under the auspices of the U.S. Atomic Energy Commission, and continued at the Division of Plant Industry, C.S.I.R.O., Canberra.

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II. Materials and Methods

(a) *Cultures and Media*

A total of 54 strains of *Rhizobium* (20 of *Rh. leguminosarum*, 14 of *Rh. phaseoli*, and 20 of *Rh. trifolii*) were examined in the lysogeny screening experiments.

The glucose–salts–yeast extract (GSY) medium described in a previous report (Schwinghamer 1960) was used for all experiments. The agar double layer procedure (basal layer 20 ml of 1·5% agar; surface layer 2 ml of 0·8% agar; in 10-cm petri plates) was employed for plating of bacteria and phage.

(b) *Screening for Lysogeny*

Strains were cross-tested in all possible combinations for donor or indicator ability by the standard spot-test technique, in which a loopful of cell suspension from one strain was deposited on agar plates layered with *c.* 5×10^6 cells of other test strains. The plates were exposed to ultraviolet light, and observation on lysis was made after 2 days' growth at 27°C. The spotting procedure was expedited in large experiments by adding a layer of sterile agar (vol. = 3 ml) and using 2-ml hypodermic syringes to deposit small drops of a strain successively on a series of different indicator test strains.

(c) *Radiation Treatments*

X-ray treatments (Brookhaven Laboratory) were applied with a General Electric "Maxitron 250" therapy unit (250 kVp, 30 mA, 1·0 mm Al+0·25 mm Cu filter, HVL 0·9 mm Cu, dose rate *c.* 700 r/min). The ultraviolet light (2537 Å) source was a 15-W General Electric "Germicidal" lamp. Doses are given as exposure times at a distance of 16 in. Cells were suspended and agitated in GSY broth or minimal salts solution (glucose and yeast extract omitted) in a dish covered with sterile, uncoated "Cellophane" during irradiation.

(d) *Isolation and Classification of Temperate Phage Strains*

Stock strains of phage were obtained from filtrates (0·45 μ "Millipore" filter) of ultraviolet-induced suspensions of apparent lysogenic strains. Each phage was subcultured at least twice by single-plaque isolation procedures on indicator strains not found to be lysogenic. The stocks were then spot-tested on all strains of *Rhizobium* for purposes of grouping phage of similar host range and testing donor strains for self-immunity. Positive responses were examined further by plaque tests.

(e) *Inducibility of Lysogenic Strains*

Cells of strains tested for ultraviolet light inducibility were washed three times by centrifugation to reduce the free phage count to a relatively low level. Filtered aliquots were used for assay of residual free phage. The washed cells were plated (*c.* 200 cells per plate) in two series—one set of plates with an indicator strain, the other without indicator (for estimation of survival after ultraviolet light irradiation). The plates were then exposed to ultraviolet light for 0 and 50 sec and plaque counts

taken for comparison of the percentage of *plated* cells lysed. Irradiation of cells after plating simplified dilution procedure when a number of doses were applied, but excessive killing of indicator cells prevented use of high doses (above LD_{80} level).

(*f*) *Lysogenization*

Strains to be lysogenized were marked with antibiotic resistance (streptomycin resistance as one marker and resistance to chloramphenicol or viomycin as the second) to aid in differentiation of recipient strains from donor and indicator strains. Tests of reductive ability were made for at least one phage from each of the apparently lysogenic strains, in the sequence described below.

(i) *Exposure to Temperate Phage.*—Cell-free filtrates (chloroform added as a further precaution) of stock phage were spotted on plates layered with streptomycin-resistant cells of the recipient strains. Cells harvested from the ensuing turbid spot were plated again for repetition of the spot test, until no lysis was detectable in the spot. The latter condition, suggestive of a high level of lysogenization, was usually attained after only two exposures to the phage.

(ii) *Isolation of Phage-liberating Clones.*—Cells from (i) were washed twice and plated to give *c.* 30–60 colonies per plate, then covered with a layer of soft agar (vol. = 3 ml). After 3 days of incubation (colonies 1–2 mm in diameter), the plates were exposed to ultraviolet light and layered with indicator cells of the corresponding streptomycin-sensitive recipient strain. Two colonies showing definite zones of lysis (Plate 1, Fig. 3) were "excised" and the suspended cells washed once before plating in streptomycin agar to avoid carry-over of streptomycin-sensitive, non-lysogenic indicator cells. In later experiments the detection of lysogenic clones was expedited by plating washed cells from (i) directly into streptomycin agar and picking about five colonies at random for a phage-release spot test. Omission of step (ii) was warranted by the high frequency of apparent lysogenization (proportion of zone-forming colonies usually exceeding 50% after two successive exposures to phage) noted in earlier experiments.

(iii) *Inducibility.*—Washed-cell suspensions from two clones showing lytic ability were used for demonstration of induction by ultraviolet light. Unlike the induction procedure already described for the original lysogenic strains, the cells were irradiated *before* plating, and inducibility was expressed as the ratio of plaque counts for irradiated versus non-irradiated suspensions. With some strains the cells were plated before lysis; with other strains (see third footnote, Table 3), where plaque formation appeared to be obscured by ultraviolet-delayed lysis, cells were plated following lysis.

(iv) *Phage–Host Specificity.*—Phages were re-isolated from single plaques and spotted on appropriate indicators for comparison of host range with that of original temperate phage stocks.

(v) *Nodulation Test.*—Lysogenized clones were given a host plant nodulation test to fully establish donor versus recipient strain identity and to note possible effects of lysogenization on symbiotic capability.

III. Results and Discussion

(a) *Detection of Lysogeny in Spot Tests*

Strains L1 and L5 (L4 as a common indicator)* were identified as probable lysogenic strains in preliminary experiments involving spot tests of X-irradiated cell suspensions, each comprising a composite of cells from approximately 10 strains. With the L1–L4 donor–indicator combination as a test system, several variables limiting detection of lysogeny in spot tests were examined briefly before screening individual strains by the method (ultraviolet irradiation of already-spotted plates)

Table 1

LYSOGENIC STRAINS OF RH. LEGUMINOSARUM AND RH. TRIFOLII

Strains	Source	Induction by Ultraviolet Light: Percentage of Cells Forming Plaques*		Self-immunity Test†	Phage Produced‡	Other Characteristics
		No Ultraviolet Light	Ultraviolet Light			
L1	Nitragin Co.	1	32	0	ØL1; others (?)	Indicator for ØL5, ØL18; bacteriocinogenic (?)
L5	Nitragin Co. (No. 128C2)	2	27	0	ØL5; others (?)	Indicator for ØL1, ØL18; bacteriocinogenic (?)
L18	L. Erdman (No. 3HoC3)	7	29	0	ØL18; others (?)	
T3	Nitragin Co. (No. 162P28)	<1	12	0	ØT3	
T10	Nitragin Co. (No. 162P30)	<1	19	0	ØT10	
T5	Clover nodule; Upton, N.Y.	—	—	0	ØT5	Sensitive to antagonism by many other strains
T26	L. Erdman (No. 3D1q27)	<1	20	0	ØT26	

* Cells irradiated 50 sec immediately after surface plating in agar.

† Zero denotes immune response.

‡ Prefixed symbol "Ø" denotes "phage".

described earlier. The effect of ultraviolet dose and donor cell concentration is illustrated in Plate 1, Figure 1. Maximum expression of lysis in the spot area was obtained with the near-maximum dose (40–50 sec, *c.* LD_{60}; indicator cells at 5×10^6 per plate) allowing growth of a uniform indicator lawn, and a donor cell concentration of *c.* 10^6 per millilitre. Donor cell levels exceeding 10^7 per millilitre favoured manifestation of certain "antagonism" reactions but obscured lysis by phage because of spot area overgrowth by these typically gum-forming bacteria.

* See footnote to Table 2 for definition of symbols.

Some of the positive spot-test responses did not appear to involve phage. Cells of several strains formed large, clear zones (Plate 1, Fig. 2) on many indicators. Phages have not been isolated from the zones or from donor filtrates, and the nature of the zones suggests production of a readily diffusible antibiotic agent. Another type of weak spot response, more indicative of bacteriocin action, was observed on indicators spot-tested with filtered lysates of lysogenic strains L1 and L5. These antagonism interactions are being investigated further and will be more fully described in a later report.

(*b*) *Lysogenic Strains and Temperate Phages*

The seven strains of *Rh. leguminosarum* and *Rh. trifolii* found to be lysogenic are listed in Table 1. Lysogeny was not observed in the smaller number of *Rh. phaseoli* strains tested although it is reasonable to assume that the phenomenon occurs also in this group of rhizobia.

Strains T3 and T10 release phages which are essentially identical in their limited host range and in plaque morphology. These two strains were observed to differ only in their antibiotic response, T3 being less sensitive to chloramphenicol.

L1, L5, and L18 may contain more than one prophage. At least two apparently distinct phage types have been isolated from the lysates of each plated on certain hosts, but it has not been established with certainty whether or not the less prevalent phage in each case occurred as a variant subsequent to release by the lysogenic donor. In the case of L1 and L5, lysates consistently formed a much higher number (ranging from 30 to 100 times) of plaques on L4, or reciprocally on each other, than on T23. Several single-plaque isolates from T23 were able to lyse T23 as readily as the other two hosts. Host range spectrum experiments now in progress, using single-plaque isolates grown alternately on L4, T23, or other indicators, should clarify whether L1 and L5 produce two distinct phages, a single phage with a marked difference in efficiency of plating, or a single phage which is altered by mutation or by transient, host-induced modification.

L18 lysates produced a striking range of plaque types (Plate 1, Fig. 4), notably on indicators L25 and L26. Plaques varied greatly in size and marginal appearance, including both diffuse-edge and abrupt-edge types. The former type appeared to be particularly unstable, subcultures of it giving rise to a similar range of variation. An isolate from an abrupt-edge plaque reproduced as a stable form and was used in lysogenization experiments. The occasional appearance of such plaques in the periphery of the numerically predominant diffuse-edge plaques points to their probable mutant derivation from the latter type.

Phage strains forming clear plaques (Levine 1957) have been encountered in normally turbid forms of temperate phage from both L5 and L18. Such clear plaques appeared at a low frequency following maintenance of temperate strains on several different indicators, presumably as mutants with little or no reductive capacity. The "clear" strains resembled the turbid parent forms in host range and in plaque morphology (Plate 1, Fig. 5), excepting the clear-plaque character. An interesting mosaic pattern of plaques results when a mixture of turbid and clear types is plated at titres giving plaque confluence (Plate 1, Fig. 6).

TABLE 2

HOST RANGE OF TEMPERATE PHAGES AND CLEAR-PLAQUE DERIVATIVES

Symbols T, PT, and C represent turbid, partly turbid, and clear plaque or spot responses. Reactions in parentheses refer to relatively low plaque-forming efficiency. Blanks denote negative responses, i.e. no detectable plaque formation

Phage	Indicator Strains*																	
	Rh. leguminosarum									*Rh. phaseoli*				*Rh. trifolii*					
	L1	L2	L3	L4	L5	L6	L19	L20	L25	P4	P6	P15	P19	T1	T8	T20	T23	T27	T29
ØL1				PT	PT												(PT)		
ØL1 (T23-type)				(PT)	PT												PT		
ØL5	T			T													(T)		
ØL5 (T23-type)	T			(T)													T		
ØL5 (clear)	C			C													C		
ØL18	T	T	T	T	T	T	T	T	T	T	T	T	T	T		T	T	T	
ØL18 (clear)	C	C	C	C	C	C	C	C	C	C	C	C	C	C		C	C	C	
ØT3														T					T
ØT10														T					T
ØT5															T	T			
ØT26							T												

* Letter prefixes L,P,T denote the species (*leguminosarum, phaseoli, trifolii*) identity of the strains.

(c) *Host Range Specificity*

The host range pattern of stock strains of phage, including two variant clear types, is summarized in Table 2. The reactions for *Rh. leguminosarum* and *Rh. trifolii* indicator strains are based on tests conducted at Brookhaven National Laboratory, N.Y., and at the Division of Plant Industry, C.S.I.R.O., Canberra, with two different sets of phage stocks; *Rh. phaseoli* indicators were not tested at Canberra.

Phage from the three *Rh. leguminosarum* lysogenic strains, especially L18, showed the widest host range, whereas the range for phage from *Rh. trifolii* lysogenic strains was comparatively limited and more intraspecific. Three strains of *Rh. meliloti*, a species generally regarded as not being closely related to the other three groups mentioned, were not lysed by these phages or by four virulent phages isolated from pea or clover nodules.

(d) *Lysogenization*

As a final criterion of lysogeny, one phage strain from each lysogenic donor was tested for reductive ability (Table 3). The procedure for each phage was extended by isolation of a lysogenized clone from two sister strains bearing a different second marker, or by isolation of two clones from the same recipient strain.

Nodulation tests served the dual purpose of further confirming donor–recipient identity where the two strains represented different species, and of checking for possible modification of the symbiotic characters via "lysogenic conversion" (cf. review by Bertani 1958). Evidence of such conversion was not observed. With the exception of the two T8V^{r}S^r strains,* recipient *Rh. leguminosarum* strains formed nodules only on peas and *Rh. trifolii* strains nodulated only clover—a pattern of host specificity identical to that of the corresponding non-lysogenized marked strains. Pending further host tests, the *Rhizobium* identity of T8 and its derivatives rests largely on sensitivity to rhizobiophage. Concerning loss of "effectiveness" (ability to fix nitrogen during symbiosis) in several strains (Table 3), this loss was not attributable to lysogenization, but was instead found to be correlated with the presence of the viomycin-resistance marker (retention of effectiveness in L4S^{r}V^r an exception). Confirmatory studies of this apparent association are being made.

The inducibility data of Tables 2 and 3 do not represent the maxima for each strain, but merely illustrate significant enhancement of phage release by ultraviolet light under a given set of conditions. The values do, however, reflect the approximate order of inducibility of the phage as noted in other experiments. Phage release by the *Rh. trifolii* lysogenic strains and by strains carrying corresponding prophages was somewhat erratic. On the other hand, relatively high spontaneous or induced rates of lysis could be consistently demonstrated for prophages of the *Rh. leguminosarum* donors, notably for the "leaky" L18.

Inoculation of each lysogenized strain with all available temperate phages revealed only one definite case of cross-immunity, namely between T1 strains carrying ØT3 and ØT10 prophages. In view of their identical host specificity, these phages may

* See first footnote, Table 3, for definition of symbols.

TABLE 3

LYSOGENIZATION EXPERIMENTS

Phage	Bacteria		Tests Confirming Lysogenization and Identity of Recipient Strains							
	Donor Strain	Recipient* Strain	Antibiotic-resistance Markers (μg/ml)†			Self-immunity Test	Induction by Ultraviolet Light: Ratio of Phage Titre relative to Control‡	Host Range Test of Isolated Phage§	Nodulation‖	
			Streptomycin	Chloramphenicol	Viomycin				On Peas	On Clover
ØL1	L1	$T23S^rC^r$ (*a*)	1500	1200		0	6·0	+	—	+, E
		$T23S^rC^r$ (*b*)	1500	1200		0	15·1	+	—	+, E
		$L5C^rS^r$ (*a*)	1500	2000		0	90·4	+	+, E	—
		$L5C^rS^r$ (*b*)	1500	2000		0	55·0	+	+, E	—
ØL5	L5	$L4S^rV^r$ (*a*)	1500		25	0	10·8	+	+, E	—
		$L4S^rV^r$ (*b*)	1500		25	0	—	+	+, E	—
ØL18	L18	$T23S^rC^r$	1500	1800		0	190	+	—	+, E
		$T23S^rV^r$	1500		7	0	900	+	—	+, I
ØT3	T3	$T1S^r$	1500			0	39·2	+	—	+, E
		$T1S^rV^r$	1500		8	0	54·1	+	—	+, I
ØT10	T10	$T1S^r$	1500			0	11·6	+	—	+, E
		$T1S^rV^r$	1500		8	0	—	+	—	+, I
ØT5	T5	$T8V^rS^r$ (*a*)	1300		8	0	7·1	+	—	—
		$T8V^rS^r$ (*b*)	1300		8	0	—	+	—	—
ØT26	T26	$L19S^rV^r$ (*a*)	1500		8	0	7·7	+	+, I	
		$L19S^rV^r$ (*b*)	1500		8	0	—	+	+, I	

* At least two strains used for each phage. Some [(*a*), (*b*)] are sister clones bearing the same markers; others differ in the second marker. Symbols S^r, C^r, and V^r denote resistance to streptomycin sulphate, chloramphenicol succinate, and viomycin sulphate respectively.

† Resistance of *donor* strains ranged from 5–30 μg/ml for streptomycin, 100–800 μg/ml for chloramphenicol, 1–3 μg/ml for viomycin.

‡ Lysogenized clones bearing prophages of ØL1, ØL18, and ØT1 tested for plaque number by plating irradiated cells *after* lysis; other clones tested by plating cells before lysis. Ultraviolet light exposure time 1·5–3 min for different experiments.

§ Plus (+) indicates agreement of host range with that of the input (donor) phage (Table 2).

‖ Plus or minus symbols denote presence or absence of nodulation: E, effective nodulation; I, ineffective nodulation (nodules small, more numerous, white; plant growth limited).

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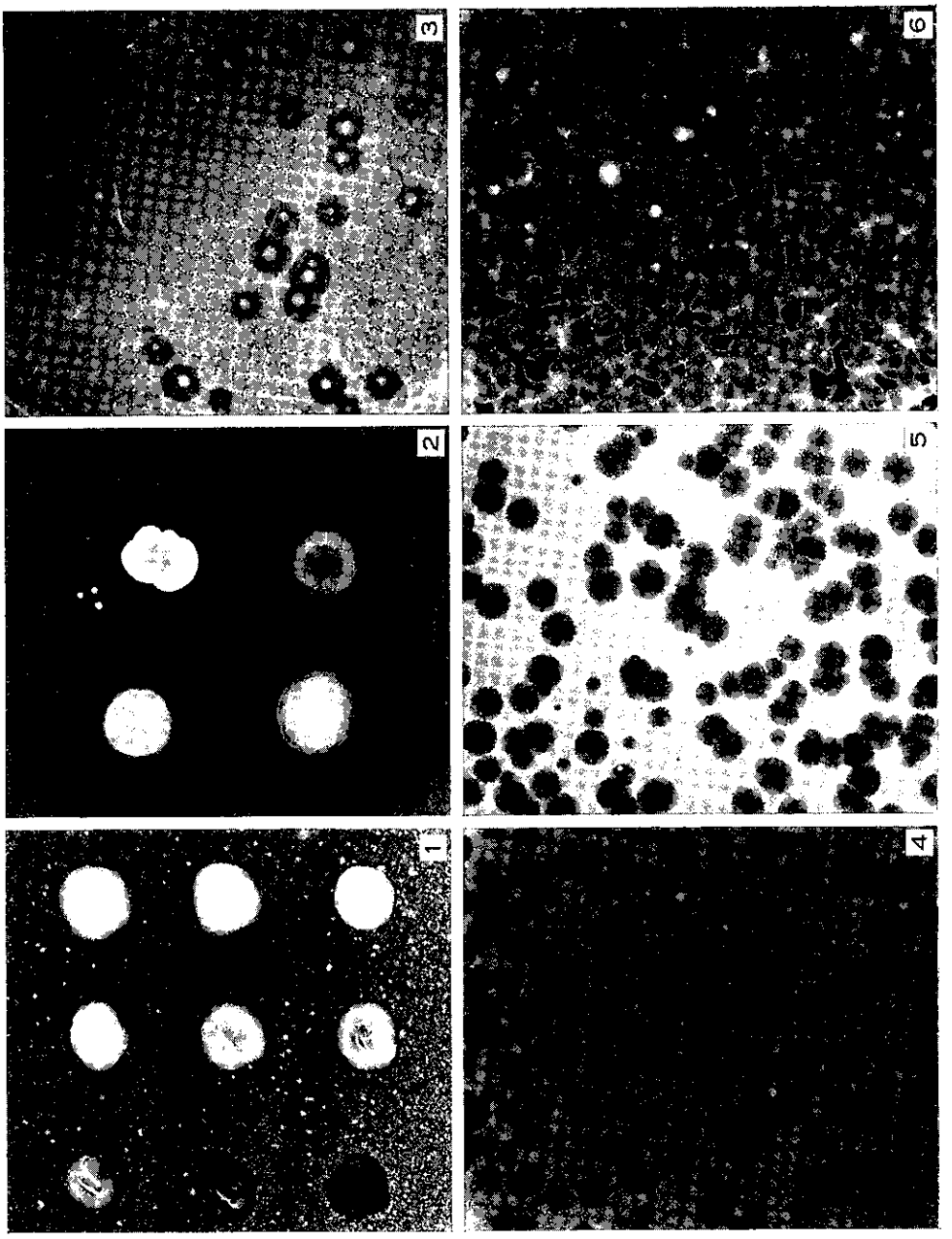

be regarded as a single entity. Another apparently cross-immune reaction between two distinctly different phages, i.e. "prophage interference" (review by Bertani (1958)) was observed for T23(ØL18) exposed to ØL5. The host range of ØL1 and ØL5 suggests a possible relationship, but prophage ØL1 was not excluded by L5 in the formation of the double lysogenic L5C^rS^r(ØL1, ØL5).

IV. Conclusion

The demonstrated reductive ability and interspecific host range of phages isolated from the seven lysogenic strains provides a basis for attempts at transduction (not demonstrated for *Rhizobium*) between *Rh. leguminosarum*, *Rh. phaseoli*, and *Rh. trifolii*.

V. Acknowledgments

The authors wish to acknowledge the technical assistance of Mrs. Helen Malecky. Thanks are also due to Dr. C. I. Davern for review of the manuscript and for helpful discussions during the course of the work.

Strains of *Rhizobium*, other than those listed in Table 2, were kindly provided by the following: Nitragin Company, Milwaukee, Wisconsin; Agricultural Laboratories, Inc., Columbus, Ohio; Dr. L. W. Erdman, U.S. Department of Agriculture, Beltsville, Maryland; Dr. O. N. Allen, University of Wisconsin, Madison, Wisconsin.

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Explanation of Plate 1

Fig. 1.—Effect of ultraviolet dose and donor cell concentration on extent of detectable lysis by phage in spot test. Donor strain L1; indicator strain L4. Horizontal rows, top to bottom: exposures of 0, 30, and 70 sec to ultraviolet light. Vertical rows, left to right: donor cell concentrations *c.* 5×10^6, 5×10^7, and 5×10^8 per millilitre.

Fig. 2.—Zones of "antagonism" produced on strain T5 by four different donor strains.

Fig. 3.—Colonies from lysogenized (concentric zone of lysis) $T1S^r$ cells plated beneath an indicator layer of T1 (non-lysogenized, streptomycin-sensitive) cells.

Fig. 4.—Range of plaque types produced on indicator L26 by phage from L18 lysate.

Fig. 5.—Turbid and clear plaques of L18 phage.

Fig. 6.—Mosaic appearance of confluent plaques from mixture of temperate and "clear" phages.