# HOST-CONTROLLED MODIFICATION OF *RHIZOBIUM* BACTERIOPHAGE

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*Summary*

Host-controlled phenotypic variation of host specificity was observed with two rhizobiophage strains, ØL1 and ØL5, following growth on six strains of *Rhizobium leguminosarum* and *R. trifolii*. The six hosts could be assigned to four groups, each group representing a different pattern of host specificity. Initial adaptation of ØL5 to hosts L2, L7, and L25 appeared to involve mutation, although replication in cells of these hosts generally involved additional phenotypic restriction. Restricted and unrestricted forms of a phage did not differ significantly in their ability to adsorb to several hosts. One-step analysis of the L25-specific form of ØL5 grown in L25 cells indicated a low average burst size of approximately one unrestricted plaque-forming unit in a small proportion of cells which were able to produce infective centres on L4. One-cycle analysis of L4-specific ØL5 modified by growth in L25 confirmed the phenotypic nature of phage variation in this phage-host system, and indicated that specificity for L4 was not replicated in L25.

## I. INTRODUCTION

Host-controlled phenotypic modification or variation is known to occur in phage active on a number of genera of bacteria, including the *Escherichia–Shigella–Salmonella* group (see review by Luria 1953), *Staphylococcus* (Ralston and Krueger 1952), *Streptococcus* (Collins 1956), and *Pseudomonas* (Holloway and Rolfe 1964), but has not previously been reported for phage of *Rhizobium*. Aside from the basic interest attached to the incompletely understood mechanisms of phenotypic variation, this type of variation is of practical interest in various areas of microbial research, including routine maintenance or identification of phage strains.

This paper describes the occurrence of phenotypic modification and several instances of genotypic change of host specificity in two phages isolated from lysogenic strains of *R. leguminosarum*. Results of studies concerning the nature of these modifications will be presented in a later paper.

## II. MATERIALS AND METHODS

### (*a*) *Bacteria and Phage*

Strains of *Rhizobium*, original phage designations, and sources of cultures are given in Table 1.

Host-modified forms of phage are indicated by the commonly used phage–host notation; thus, ØL5·L25 represents ØL5 grown on L25 and carrying the host

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specificity imparted by L25. The terminology used for describing the various aspects of host-controlled modification is mainly that of Luria (1953) (see also summary of terminology by Paigen and Weinfeld 1963).

Background information on the bacterial or phage strains, linking the current work with previously reported observations (Schwinghamer and Reinhardt 1963), is given in Section III where relevant.

### (*b*) *Media and Plating*

The glucose–salts–yeast extract (GSY) medium (Schwinghamer 1960) was used for culture of bacteria and phage. The procedures for phage culture and assay were essentially those of Adams (1959).

TABLE 1

RHIZOBIUM STRAINS, PHAGE DESIGNATIONS, AND SOURCES OF CULTURES

| Rhizobium Strain* | Temperate Phage† | Bacterial Culture No. | Source |
|---|---|---|---|
| L1 (ØL1) | ØL1 | | |
| L4 | | 128C45 | Nitragin Co., Milwaukee, Wisconsin |
| L5 (ØL5) | ØL5 | 128C2 | Nitragin Co., Milwaukee, Wisconsin |
| L7 | | 128C10 | Nitragin Co., Milwaukee, Wisconsin |
| L2 | | ATCC-10314 | American Type Culture Collection, Washington, D.C. |
| L25 | | 3A2 | Agricultural Laboratories Inc., Columbus, Ohio |
| T23 | | 3D1k2 | Dr. L. W. Erdman, Department of Agriculture, Beltsville, Maryland |

* The prefixed letters L and T represent the *Rhizobium* species *R. leguminosarum* and *R. trifolii* respectively. Prophage of these two lysogenic strains is represented within parenthesis.

† The symbol "Ø" denotes "phage".

### (*c*) *Determinations of Host Specificity and Efficiency of Plating*

Efficiency of plating determinations of phages grown sequentially in different hosts were made with chloroform-treated single-plaque suspensions. Use of the single-plaque procedure instead of one-cycle growth analysis for most of the host-specificity tests was dictated by the extensive nature of the tests, involving sequences of modification of two phages by six hosts. The validity of the results as obtained by single-plaque assays is considered in Section IV.

In one-step and one-cycle growth experiments, phage previously grown on L25 or L4 was adsorbed for 40 min on log-phase L25 cells, which were then washed twice by centrifugation. Infected cells were grown at 28–29 °C for various time intervals before assaying for titres of infective centers and progeny phage on L4 and L25 indicator plates.

### (*d*) *Phage Adsorption*

Adsorption of restricted phage was compared on permissive and non-permissive hosts. Standard adsorption procedures (Adams 1959) were used but the following experimental conditions are noted: temperature = 28°C; multiplicity of infection = 0·1 or less; cell concentration *c.* 1–4 $\times 10^8$/ml; adsorption stopped by chilling and dilution; adsorption values based on unadsorbed phage in chloroform-treated samples.

## III. Results

### (*a*) *Fully Reversible Modification of ØL1 and ØL5 by L4 and T23*

Previous work (Schwinghamer and Reinhardt 1963) with lysogenic strains L1(ØL1) and L5(ØL5) had indicated anomalous host-specificity characteristics for phage derived from these strains. Lysates of L1(ØL1) formed semi-turbid plaques with full efficiency on L5(ØL5) and on L4 but with lower efficiency on T23. Similarly L5(ØL5) lysates formed turbid plaques on L1(ØL1) and L4 at a higher frequency than on T23. With both phages, however, lysates of T23 produced more plaques on T23 than on L4. Subsequent experiments, involving assay of single plaques harvested alternately from L4 and T23 in successive plating experiments, have shown this phage variation in host specificity to be due to host-controlled modification, rather than to phage mutation or multiple lysogeny.

A partial list of efficiency of plating values for L1(ØL1) and L5(ØL5) lysates and for single-plaque isolates taken from L4 or T23 indicator plates is given in Table 2. Plaques of both ØL1 and of ØL5 were consistently smaller on T23 (1 mm or less) than on the other hosts (1–2 mm), although plaque size or morphology on any host was largely unaffected by the host on which phage was last grown. As with other known systems of host-induced modification, the efficiency of plating pattern depended simply on the most recent host on which the phage was grown, and successive growth cycles (e.g. ØL1·L4·L4) on a given host did not measurably alter the efficiency of plating. The L4–T23 modification system differed from the L2–L7–L25 system described in the next section in that the efficiency of plating in the former system was fully reversible, at a level of approximately $10^{-2}$.

### (*b*) *Incompletely Reversible Modification of ØL5*

In addition to forming plaques on the hosts already mentioned, induced lysates (chloroform-treated to kill residual cells) of L1(ØL1) and L5(ØL5) produced a partial clearing of indicator cells when spotted on L2, L7, and L25. Progressive dilution of the lysates resulted in gradual loss of spot-test response without evidence of plaque formation. When the lysates were plated on these three hosts the indicator response ranged from partial clearing, at a phage titre (assayed on L4) of *c.* $10^8$–$10^9$ per plate, to various degrees of "pitting" (resembling very small plaques) at *c.* $10^7$–$10^8$ per plate. When ØL5 from either L5(ØL5) or L4 lysates was plated on L2, L7, or L25, the few definite plaques found contained phage fully adapted to the new host. The uncertainty of distinguishing plaques under the condition of irregular indicator lethality was such that the efficiency of plating value of $10^{-6}$ given in Table 2 and Figure 1 represents only a crude estimate derived from a number of experiments.

TABLE 2

HOST RANGE AND EFFICIENCY OF PLATING FOR HOST-MODIFIED FORMS OF ØL1 AND ØL5

| Phage Strain | Normal Hosts* | Form of the Phage† | Efficiency of Plating‡ on Hosts: | | | | | Host Range Extended to:§ | Probable Type of Adaptation |
|---|---|---|---|---|---|---|---|---|---|
| | | | L1 | L4 | T23 | L7 | L2, L25 | | |
| ØL1 | L5(ØL5); L4 | Lysate of L1(ØL1) | | 1 | $10^{-2}$ | | | | |
| | | ØL1·L4 | | 1 | $10^{-2}$ | | | | |
| | | ØL1·L4·T23 | | $10^{-2}$ | 1 | | | T23 | Phenotypic |
| | | ØL1·L4·T23·L4 | | 1 | $10^{-2}$ | | | | |
| ØL5 | L1(ØL1); L4 | Lysate of L5(ØL5) | 1 | 1 | $10^{-2}$ | $10^{-6}$(?) | $10^{-6}$(?) | | |
| | | ØL5·L4 | 1 | 1 | $10^{-2}$ | $10^{-6}$(?) | $10^{-6}$(?) | | |
| | | ØL5·L4·T23 | $10^{-2}$ | $10^{-2}$ | 1 | $10^{-6}$(?) | $10^{-6}$(?) | T23 | Phenotypic |
| | | ØL5·L4·T23·L4 | 1 | 1 | $10^{-2}$ | $10^{-6}$(?) | $10^{-6}$(?) | | |
| | | ØL5·L2 | $5\times10^{-4}$ | $5\times10^{-4}$ | $10^{-2}$ | $5\times10^{-4}$ | 1 | (L7), L2, L25 | Mutation |
| | | ØL5·L25 | $5\times10^{-4}$ | $5\times10^{-4}$ | $10^{-2}$ | $5\times10^{-4}$ | 1 | (L7), L2, L25 | Mutation |
| | | ØL5·L7 | 1 | 1 | $10^{-2}$ | 1 | $10^{-1}$ | L7, L2, L25 | Mutation |
| | | ØL5·L25·L4 | 1 | 1 | $10^{-2}$ | $3\times10^{-1}$ | $10^{-1}$ | L7 | Phenotypic |
| | | ØL5·L25·L4·L4 | 1 | 1 | $10^{-2}$ | $3\times10^{-1}$ | $10^{-1}$ | | |
| | | ØL5·L25·L4·T23 | $10^{-3}$ | $10^{-3}$ | $3\times10^{-1}$ | $10^{-3}$ | 1 | T23 | Phenotypic |
| | | ØL5·L25·L1 | 1 | 1 | $10^{-2}$ | $3\times10^{-1}$ | $10^{-1}$ | L7 | Phenotypic |
| | | ØL5·L25·L2 | $5\times10^{-4}$ | $5\times10^{-4}$ | $10^{-2}$ | $5\times10^{-4}$ | 1 | | |
| | | ØL5·L25·L7 | 1 | 1 | $10^{-2}$ | 1 | $10^{-1}$ | L7 | Phenotypic |
| | | ØL5·L7·L4 | 1 | 1 | $10^{-2}$ | $3\times10^{-1}$ | $10^{-1}$ | | |
| | | ØL5·L7·L4·T23 | $10^{-3}$ | $10^{-3}$ | $3\times10^{-1}$ | $10^{-3}$ | 1 | T23 | Phenotypic |

* "Normal hosts" are fully sensitive to phage from the lysogenic donors; no adaptation is needed.

† Single-plaque populations used, except where lysates are indicated. Plaques were taken successively from the hosts in the order listed. The specificity pattern is determined largely by the last-named host (see text for partial exceptions following adaptation to L2, L7, or L25).

‡ Efficiency of plating values represent averages for two or more experiments. Ratios (excepting the uncertain $10^{-6}$ estimate, see text) derived for different experiments usually fell within a twofold to threefold range of variation.

§ Refers to extension of host range effected by growth on the *last* host given in the phage form designation, but note that the manner of adaptation to L7 also depends on whether the phage was previously adapted to L2 or L25 (see text). Where L7 is shown in parentheses the mutational adaptation was partly masked by superimposed phenotypic restriction. Changes in efficiency of plating involving re-adaptation to original hosts L1 and L4 (i.e. no extension of host range) are all phenotypic.

Unlike ØL5, ØL1 produced fewer apparent plaques on these hosts and none of the few "plaques" tested appeared to contain reproducible adapted phage. In view of the otherwise similar responses noted on L2, L7, and L25 for both phages, however, it is possible that more exhaustive analysis would reveal adaptive modification on these hosts also for ØL1.

The main patterns of host specificity demonstrated for ØL5 following initial passage through hosts L2, L7, or L25 are given in Table 2. A diagrammatic summary of the "pathways" of modification and their associated efficiency of plating values is shown in Figure 1, to illustrate continuity and non-reciprocal efficiency of plating relationships. Similar results were obtained for both the normal turbid-plaque isolates and for clear-plaque mutant derivatives of ØL5.

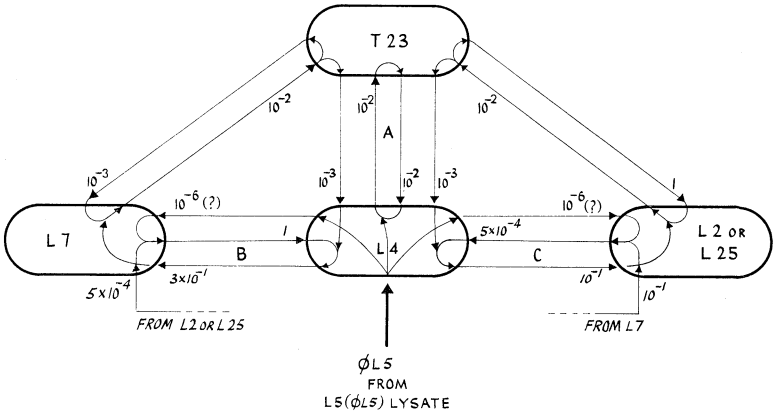


Fig. 1.—Diagrammatic illustration of fully reversible and partly reversible systems of modification of ØL5. Host strains L2, L4, L7, L25, and T23 are depicted by the heavily-lined bacterial cell outlines; growth of phage in cells of a strain is represented by the lighter lines passing through a cell outline. The efficiency of plating ratios refer to the phage titre determined on the host indicated at the head of a line, divided by the titre determined on the host indicated at the base end of the same line. Ratios are located near the head of each line concerned. All efficiency of plating values refer to progeny phage, not infective centres. Loops labelled *A*, *B*, and *C* represent three cycles of phage modification discussed in the text; e.g. cycle *A* is translated as: ØL5 initially grown on L4 produces approximately 100 times more plaques on L4 than on T23; subsequent growth of the phage on T23 reverses the efficiency of plating on the two hosts.

The following generalizations may be drawn from the ØL5 data for purposes of identifying apparently consistent patterns or types of modifications among the maze of host responses:

(1) Infectivity on L2, L7, and L25 can be "switched on" by initial passage through cells of any of these three strains. The only notable difference between the initial change induced by L7, as opposed to L2 or L25, is that the latter two hosts simultaneously impose a $5 \times 10^{-4}$ level of restriction for plating on L7, as well as on L1(ØL1) and L4. Thus growth of ØL5·L2 or ØL5·L25 on L4 (Table 2) removed the phenotypic restriction and exposed the basic adaptation, presumably of genotypic origin (see below), to L7.

(2) L1(ØL1) and L4 are similar as indicators or as modifers and may share a common mechanism of host control, whereas L2 and L25 appear to represent another type of control. L7 occupies a unique intermediate position between these two groups in that it resembles L2 and L25 during the first passage of phage, but resembles L1(ØL1) and L4 as host for phage which has already acquired specificity for L2, L7, or L25. T23 is distinct from the other strains in that phage multiplication in T23 cells depresses plating efficiency on L1(ØL1), L4, or L7, but not on L2 or L25. This depression was enhanced roughly tenfold for ØL5 previously modified by L25, then grown on T23. Perhaps of more significance is the relatively constant efficiency of plating (*c.* $10^{-2}$) on T23 for both ØL1 and ØL5 grown on any of the other hosts. Thus the six hosts used appear to represent four different types of host control.

(3) Fully reversible restriction is characteristic for ØL5 grown alternately on L4 and T23 (Fig. 1, cycle *A*), but incomplete reversibility of efficiency of plating predominates after a single passage of phage through L2, L7, or L25 cells. While phage grown previously only on L4 forms plaques on L2, L7, or L25 at an efficiency of *c.* $10^{-6}$ in its first exposure to these three hosts, the efficiency of plating for phage modified by these hosts remains high ($3 \times 10^{-1}$ on L7, $10^{-1}$ on L25) after another round of growth (Fig. 1, cycles *B* and *C*) on L4, instead of reverting to the original $10^{-6}$ level. Thus infectivity on L2, L7, and L25 cannot be reduced to the original low base level, regardless of which host the phage is grown on. This indicates that the original modification was a mutation, with phenotypic modification being superimposed during multiplication of phage in cells of L2, L7 (main restriction by L7 involves the efficiency of plating value of $10^{-2}$ on T23), or L25. Such superimposed phenotypic-genotypic changes are known to occur in Vi-phages of *Salmonella typhii* (Anderson and Felix 1953).

Phage derived from lytic infection or prophage activation gave similar results in the one case where comparison was made. Thus, phage from L25 lysogenized with ØL5·L25 gave efficiency of plating values similar to those obtained for ØL5·L25 grown in non-lysogenic L25 cells.

### (*c*) *Adsorption of Restricted and Unrestricted Forms of ØL5*

Differences in plaque-forming efficiency of ØL5 on various hosts were not attributable to adsorption, in agreement with results for host-controlled variation in other bacteria. Adsorption curves for phage previously grown on L4, L25, or T23 did not differ significantly, irrespective of which host was used to adsorb the phage. Adsorption of the different forms of ØL5 ranged from approximately 45 to 55% at 5 min and from 85 to 95% at 30 min.

Results of later experiments, involving infection of L4 cells by ØL5·L25 (Schwinghamer, unpublished data), indicate that the phage genomes are injected but are largely inactivated in a restricting host.

### (*d*) *One-step Growth of ØL5·L25 in L25 Cells*

Although the types of variation (excepting the changes observed for initial passage through L2, L7, or L25) described for ØL1 and ØL5 conform to commonly accepted criteria (Luria 1953) for host-controlled phenotypic changes rather than for mutation, some analyses were made of the distribution of unrestricted and restricted ØL5 forms in one-step L25 lysates. The frequency of unrestricted particles (i.e. able to form plaques on L4) in single-plaque phage populations from L25 indicators had been found to be *c.* $5 \times 10^{-4}$, whereas both forms produced plaques on L25. The ratio

TABLE 3

ONE-STEP GROWTH OF ØL5·L25 IN L25 CELLS

General procedure is described in Section II. Multiplicity of infection for experiments 1, 2, and 3 was 1·4, 0·8, and 2·1, respectively. Starting time (0 hr) for incubation corresponds to the end of the adsorption period, 40 min after addition of phage. Phage titre on L25 represents total phage (restricted plus unrestricted); titre on L4 represents unrestricted phage. Average burst size = phage titre at a given incubation period (other than 0 hr) divided by phage titre at 0 hr (i.e. infective centres). In experiments 1 and 2 the suspension of infected, washed cells was not diluted before incubation; in experiment 3 the suspension was diluted $10^{-2}$ to reduce loss of phage, notably unrestricted phage, by adsorption to non-lysed or non-infected L25 cells

| Expt. No. | Incubation Time of Infected Cells prior to Plating (hr) | Plaque Titre on: | | Ratio of Plaque Titre on L25 Relative to L4 | Average Burst Size of Cells which Produced Plaques on: | |
|---|---|---|---|---|---|---|
| | | L25 | L4 | | L25 | L4 |
| 1 | 0 | $5{\cdot}4 \times 10^{7}$ | $3{\cdot}9 \times 10^{5}$ | 138 | | |
| | 6·5 | $6{\cdot}8 \times 10^{8}$ | $4{\cdot}7 \times 10^{5}$ | 1447 | 12·6 | 1·2 |
| 2 | 0 | $6{\cdot}8 \times 10^{7}$ | $3{\cdot}1 \times 10^{6}$ | 22 | | |
| | 3 | $4{\cdot}6 \times 10^{8}$ | $2{\cdot}5 \times 10^{6}$ | 184 | 6·8 | 0·8 |
| | 4·5 | $1{\cdot}0 \times 10^{9}$ | $9{\cdot}0 \times 10^{5}$ | 1111 | 14·7 | 0·3 |
| | 6 | $7{\cdot}7 \times 10^{8}$ | $3{\cdot}3 \times 10^{5}$ | 2333 | 11·3 | 0·1 |
| 3 | 0 | $2{\cdot}1 \times 10^{7}$ | $5{\cdot}2 \times 10^{5}$ | 40 | | |
| | 2 | $2{\cdot}9 \times 10^{7}$ | $3{\cdot}9 \times 10^{5}$ | 74 | 1·4 | 0·8 |
| | 3·5 | $2{\cdot}3 \times 10^{8}$ | $4{\cdot}4 \times 10^{5}$ | 523 | 11·0 | 0·9 |
| | 5 | $4{\cdot}5 \times 10^{8}$ | $3{\cdot}7 \times 10^{5}$ | 1216 | 21·4 | 0·7 |

of total phage (predominantly restricted) to unrestricted phage invariably increased as lysis progressed (Table 3). Approximately 1/22 to 1/138 of the non-lysed cells (infective centers) produced plaques on L4, whereas fewer than 1/1000 of phage particles plated after lysis — i.e. progeny phage — carried specificity for growth on L4. The rate of lysis of ØL5-infected L25 cells was found to be relatively variable and lysis was not always complete even after 6 hr of growth, so that the 1/2000 ratio more commonly obtained for filtered or chloroform-treated plaque suspensions (Table 2) was not quite realized. The change in ratio of plaque-forming units before and after lysis results from the disparity in average burst size for the two forms of

phage. (Reference to more than one distinct "form" in a given phage population implies that the usual $5 \times 10^{-4}$ efficiency of plating value of ØL5·L25 on L4 could be ascribed to heterogeneity in the phage population as well as to heterogeneity in the restricting cell population.) On this basis it would appear that continuous growth of ØL5·L25 stock cultures in L25 cells would depress the frequency of unrestricted phage particles well below the $5 \times 10^{-4}$ level, unless stablized at this level by some process of "derestriction", possibly by recombination events as described for marker rescue (Lederberg 1957; Drexler and Christensen 1961; Arber and Dussoix 1962; Ihler and Meselson 1963; Uetake, Toyama, and Hagiwara 1964) in restricting hosts.

TABLE 4

ONE-CYCLE GROWTH OF ØL5·L25·L4 IN L25 CELLS

Experimental procedure as described for Table 2

| Expt. No. | Multiplicity of Infection | Incubation Time of Infected Cells Prior to Plating (hr) | Plaque Titre on: | | Ratio of Plaque Titre on L25 Relative to L4 | Average Burst Size of Cells which Produced Plaques on: | |
|---|---|---|---|---|---|---|---|
| | | | L25 | L4 | | L25 | L4 |
| 1 | 0·6 | 0 | $2{\cdot}1 \times 10^4$ | $8{\cdot}5 \times 10^2$ | 25 | — | — |
| | | 6·5 | $7{\cdot}6 \times 10^5$ | $6{\cdot}1 \times 10^2$ | 1246 | 36 | 0·7 |
| 2A | 2·4 | 0 | $2{\cdot}1 \times 10^4$ | $1{\cdot}2 \times 10^3$ | 18 | — | — |
| | | 6·0 | $2{\cdot}3 \times 10^5$ | $6{\cdot}8 \times 10^2$ | 338 | 11 | 0·6 |
| 2B | 12 | 0 | $2{\cdot}9 \times 10^5$ | $1{\cdot}1 \times 10^4$ | 26 | — | — |
| | | 6·0 | $5{\cdot}5 \times 10^6$ | $6{\cdot}0 \times 10^4$ | 92 | 19 | 5·5 |

The decline in apparent average burst size, to levels well below 1, probably results largely from some phenomenon associated with plating of non-lysed cells. Thus, when an infected L25 cell lyses after plating and liberates a cluster of 15–20 restricted phage particles, there is a reasonable likelihood of some indicator cells in the area of the bursts being infected with two or more restricted particles and releasing unrestricted phage capable of plaque formation. Such a multiplicity effect, described as "cooperative infection" by Paigen and Weinfeld (1963) and as "multiplicity activation" by Uetake, Toyama, and Hagiwara (1964), has in fact been encountered in separate studies with L25-modified ØL5 (Schwinghamer, unpublished data). Some support for this interpretation stems from the observation that plaques produced on L4 by infected L25 cells were variable in size and generally smaller than plaques produced by phage from L25 cells lysed before plating. Initiation of the smaller plaques would have been delayed by the 5–7-hr period required for L25 lysis and multiple infection of L4. Hence, where cooperative infection occurs in a phage system, it could conceivably distort efficiency of plating determinations for phage suspensions containing an appreciable carry-over of infected, non-lysed cells.

### (e) *One-cycle Growth of ØL5·L25·L4 in L25 Cells*

One-cycle growth experiments were performed with one of the ØL5 modification systems (see cycle *C*, Fig. 1) to support the previous evidence for phenotypic variation drawn from single-plaque analyses and one-step experiments. L25 cells infected with ØL5·L25·L4 were assayed for phage titre before and after lysis, on L4 and L25 indicators (Table 4). The procedure differed from that of Table 3 only in the use of ØL5·L25·L4 to follow the progress of a full cycle of modification, i.e. of L4-specific phage genomes passed through L25 and again assayed on the original L4 host. In experiment 1, where the multiplicity of infection was less than 1, the plating efficiency (1/1246) of progeny phage on L4 fell within the $5 \times 10^{-4}$ range previously noted for ØL5·L25. A single passage of the phage through L25 suffices to depress the L4 specificity to a degree similar to that found in single-plaque phage populations. The possibility of reversion by mutation and selection is thus reasonably excluded.

The estimates of average burst size on L4 (expts. 1 and 2A) approach unity, suggesting that L25 cells which form plaques on L4 may be "exceptional cells" which accept the phage but do not replicate the parental specificity (Arber and Dussoix 1962). This interpretation of non-replication is supported by the fact that the higher burst size on L4 in experiment 2*B* appears to be roughly proportional to the higher phage input (multiplicity of infection = 12). A multiplicity of infection effect of this type would be distinct from cooperative infection, which would give an increase in the number of L25 cells accepting L4-modified phage. The tenfold increase in plaque number on L25 by the 0-hr cells of experiment 2*B* (compared with expt. 2*A*) may, however, reflect some degree of cooperative infection of L25.

## IV. Discussion

The occurrence of phenotypic change in "re-adaptation" of ØL5·L25 to L4 was established in one-step and one-cycle growth experiments. Evidence for phenotypic variation in the other phage systems (Table 2; Fig. 1) was derived from single-plaque assay procedures. Here it is recognized that a change in efficiency of plating of the phage population from a plaque could conceivably have resulted from mutation plus selection during an estimated five to six generations of growth occurring in plaque formation on a modifying host. The following observations, however, minimize the likelihood of genotypic changes accounting for the results obtained (excepting the mutational adaptation to L2, L7, and L25):

(1) In almost every instance of adaptation to one host there was a simultaneous decrease in efficiency of plating on a previous permissive host. With adaptive host range mutants this may happen occasionally but not commonly.

(2) The frequency of "reversion" for specificity on the previous host, in most cases ranging from *c.* $10^{-2}$ to $10^{-4}$, is of a much higher order than is usually found for spontaneous mutation. Unadsorbed or desorbed phage from the plated phage population may be carried over from the excised plaque, but not at a frequency that would distort efficiency of plating values within this range.

(3) Repeated generations of growth of progeny phage on a particular host, following adaptation to this host, do not measurably alter the efficiency of plating ratios, thus providing no apparent evidence for selective increase of adaptive mutants in the population.

The interpretation of a mutational origin for the adaptation of ØL5 to L2, L7, or L25 is necessarily more tentative than the above interpretation of phenotypic variation; the former is based simply on the persistence of the adaptation, irrespective of the host on which the adapted phage was subsequently grown. More conclusive evidence, based on a clonal distribution of such variants, would be difficult to obtain in single-cell burst analyses because of the poorly defined plaques produced on L25 by the previously non-adapted phage.

Takahashi and Quadling (1961) briefly considered host-induced recombinational events as an alternative interpretation for the complex behaviour of phage liberated by lysogenic strains T9 and T10 of *R. trifolii*. The data presented by these authors supported their interpretation based on defective lysogeny, although the relative amounts and behaviour of the two phages in mixed bursts from T10 infected by phage 10 would appear to suggest an added component of phenotypic variation. In relation to this, it is recognized that although strains like L4, L7, and L25 have not been found to be lysogenic, they may well carry unknown prophages — functional or defective — which could influence the phenotype of the progeny from infecting phages (Anderson and Felix 1953; Lederberg 1957; Christensen 1961).

Host-induced modification has been shown to occur in two of the seven temperate phages isolated from *R. leguminosarum* and *R. trifolii*. The other five phage strains have not been closely examined for such variation but it is possible that a third phage (ØL18, cf. Schwinghamer and Reinhardt 1963), one which displays considerable plaque-type instability and gives a weak spot-test response on some hosts as described for ØL1 and ØL5, might also undergo phenotypic change of host specificity or plaque type. Likewise, in the case of ØL1 and ØL5 a weak spot-test reaction was also noted on some *Rhizobium* strains other than those described herein. On the basis of these experiments and observations it would appear that this type of variation may be of fairly common occurrence in rhizobiophage. From the standpoint of *Rhizobium* genetics research (of which these phage studies are a part) stock cultures of modified forms of phage are applicable to bacterial strain identification (Anderson and Felix 1953) and host range extension can be exploited in attempts to demonstrate transduction in *Rhizobium*. A combination of both phenotypic and genotypic change mechanisms provides such a phage with a high degree of host range flexibility which complicates strain identification and maintenance but could be an important factor in the survival ability of rhizobiophage in the legume plant rhizosphere.

## V. Acknowledgments

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