Spackle and Immunity Functions of Bacteriophage T4

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Cells of *Escherichia coli* B infected with the immunity-negative (*imm*2) mutant of bacteriophage T4 are able to develop a substantial level of immunity to superinfecting phage ghosts if the ghost challenge is made late in infection. This background immunity is not seen in infections with phage carrying the spackle (*s*) mutation in addition to the *imm*2 lesion. The level of immunity in *s*⁻ infections is intermediate between that of *imm*⁻ and wild-type infections under standard assay conditions. With respect to genetic exclusion of superinfecting phage, cells infected with *imm*⁻ phage are completely deficient, whereas infections with the *s*⁻ phage are only partially deficient compared to wild-type infections. Whereas *s*⁻-infected cells are unable to resist lysis from without by a high multiplicity of infection (MOI) of superinfecting phage, cells infected with *imm*⁻ phage show less than wild-type levels of resistance and the majority of cells remaining intact are unable to incorporate leucine or form infective centers. Under conditions of superinfection by low MOI of homologous phage, *imm*⁻-infected cells are lysis inhibited, whereas *s*⁻-infected cells do not show this property. Superinfecting phage inject their DNA into *imm*⁻-infected cells with the same efficiency as seen in wild-type infections, but this efficiency is reduced when the cells are first infected with *s*⁻ phage. The *s* function of T4 appears not only to affect the host cell wall as previously postulated by Emrich, but may also affect the junctures of cell wall and membrane with consequences similar to those of the *imm* function.

Infection of susceptible strains of *Escherichia coli* by bacteriophage T4 particles which lack DNA (phage ghosts) causes severe metabolic disruptions of the host cell (8, 14, 17) which lead to the death of most, but not all, infected cells (12, 13). When cells are first challenged with ghosts and then superinfected with intact phage, few infective centers are formed (14, 17). If, however, infection by intact phage precedes that of the ghosts by as little as 1 min, an appreciable number of infective centers develop (9, 28).

This ability of phage T4 to make its host "immune" to the normally disruptive effects of ghosts requires the expression of the immunity (*imm*) gene carried by the phage (26). This same gene, which is non-essential for phage production, also directs the genetic exclusion of superinfecting phage genomes (6, 19, 26). In promoting development of immunity to ghosts and genetic exclusion, the *imm* gene product is thought to act upon the host cell membrane in a stoichiometric fashion (27), since the degree of immunity and genetic exclusion developed was

found to be proportional to the amount of the *imm* product formed, but not the time this product was allowed to act.

In their initial report of the immunity gene of phage T4, Vallée and Cornett (26) noted that, if sufficient time were allowed after infection by the T4 *imm*⁻ mutant, the level of immunity to superinfecting ghosts could reach 60% of that developed in *imm*⁺-infected cells which attain complete immunity very quickly after infection under the same conditions (see Fig. 1 of reference 26). In the standard assay (ghost challenge at 10 min after phage infection at 30°C), the background level achieved by the *imm*⁻ mutant is about 10% of the *imm*⁺ level; at this time the *imm*⁺-infected cells have developed greater than 90% of their final level of immunity. This report describes experiments which indicate that the background level of immunity in *imm*⁻-infected cells results from the action of the spackle gene of phage T4 which was first isolated and characterized by Emrich (10).

MATERIALS AND METHODS

Bacteria. *E. coli* B/5 (*Su*⁻), S/6 (*Su*⁻), K112λh8 (*Su*⁻), CR63 (*Su*⁺UAG), and CR63λh8 (*Su*⁺UAG) were from this laboratory. *E. coli* BB (*Su*⁺UAG) was

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obtained from the California Institute of Technology, Pasadena, Calif. *E. coli* B (*Su*⁻) was obtained from the Pasteur Institute, France. Plating indicator strains were grown in H broth (25). Experimental hosts were generally grown to a cell density of 4×10^8 cells/ml in H broth or M9G medium (M9 salts and 0.4% glucose) as indicated in the legends to the figures and tables. M9 salts contained (per liter of water): Na_2HPO_4 , 5.8 g; KH_2PO_4 , 3.0 g; NaCl , 0.5 g; NH_4Cl , 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g; and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 2.7 mg.

Bacteriophage. The wild-type and mutant strains of T4 used in this report are from the collection of H. Bernstein. All phage are T4D, with the exception of the T4B *rII* mutants, *rED19* and *rA105*. The spackle (s) mutant of T4D was generously provided by J. Emrich Owen. The number in parentheses after the phage strain number refers to the gene bearing the mutation.

Preparation of phage stocks. High-titer stocks of phage were routinely prepared from plate lysates plated from single plaque phage suspensions and harvested by alternate low- and high-speed centrifugation as previously described (26). The phage stocks were stored at 10^{11} PFU/ml in M9 salts plus gelatin (100 $\mu\text{g}/\text{ml}$). [³H]thymidine-containing phage were prepared by infecting *E. coli* B in M9G medium plus Casamino Acids (0.2%) at 4×10^8 cells/ml with wild-type T4D (multiplicity of infection [MOI] = 5). Five minutes after infection the medium was supplemented with deoxyadenosine (to 300 $\mu\text{g}/\text{ml}$) and [³H]thymidine (to 100 $\mu\text{Ci}/\text{ml}$, 73.5 $\mu\text{Ci}/\mu\text{g}$). After 90 min of incubation, gelatin was added to 100 $\mu\text{g}/\text{ml}$ and phage production was terminated by adding chloroform to the infected culture. The [³H]thymidine-containing phage were harvested by alternate low ($\times 3$)- and high ($\times 2$)-speed centrifugations and resuspended in M9 salts containing gelatin (100 $\mu\text{g}/\text{ml}$). Since the [³H]thymidine leads to inactivation of plaque-forming ability (4), the phage were titered as described for ghosts. The final preparation contained 1.6×10^{11} killing particles/ml (9×10^{-5} counts per min per particle) and contained less than 2% trichloroacetic acid-soluble radioactivity. [³H]leucine-containing phage were prepared on M9G-grown *E. coli* B in a manner similar to that described above. Eight minutes after infection, the culture was supplemented with [³H]leucine to 100 $\mu\text{Ci}/\text{ml}$ and unlabeled leucine to 10^{-5} M. The resulting phage preparation contained 1.5×10^{11} PFU/ml with 6×10^{-5} counts per min per PFU.

Bacteriophage ghosts. Ghosts of bacteriophage T4D *ame51(56)* were prepared by osmotic shocking by the procedure of Duckworth (8). The titer of the ghost preparation, which contained less than 0.001% plaque-forming contaminants, was calculated from the proportion of surviving broth-grown bacteria employing the zero term of the Poisson distribution. The term MOK (multiplicity of killing) is used for ghosts rather than MOI (multiplicity of infection) which is used for phage.

Phage crosses. A logarithmically growing culture of *E. coli* BB (*Su*⁺*UAG*) was concentrated by centrifugation to 4×10^8 cells/ml and simultaneously infected with both parental phage (MOI of each = 5) in H broth at 30 C in the presence of 2 mM KCN. After 10

min for adsorption, the phage-infected cells were diluted 4×10^4 into prewarmed H broth and aerated for 90 min before termination of growth with chloroform. The progeny phage were first plated on *E. coli* BB (*Su*⁺*UAG*) from which individual plaques were stabbed with sterile toothpicks and transferred to plates seeded with the appropriate *E. coli* indicator strains and incubated at 30 or 40 C to detect *am* and *ts* recombinants. The desired recombinant types were picked from the permissive condition plate and replated for single plaques from which a stock was prepared and retested for the appropriate character(s).

Turbidity measurements. The turbidity of superinfected cultures was measured with a Klett-Summerson colorimetric photometer with a 520- to 580-nm filter.

Immunity to superinfecting ghosts. The standard assay for immunity in T4-infected cells to the disruptive effects of superinfecting T4 ghosts was routinely carried out by measuring the amount of protein synthesis after superinfection by ghosts (26). Host cells were grown in M9G medium at 30 C to a cell density of 4×10^8 cells/ml from which duplicate 1.0-ml samples were infected with T4 phage (MOI = 5). After 10 min at 30 C, a time interval sufficient to develop greater than 90% immunity in *imm*⁺-infected cells, one sample received superinfecting ghosts (MOK = 7) followed by the addition of [³H]leucine (final concentration, 5×10^{-5} M, 0.5 $\mu\text{Ci}/\text{ml}$) to both samples 5 min later. The reaction was stopped after 5 min by adding 0.5 volume of ice-cold trichloroacetic acid (10% wt/vol), and the suspensions were chilled on ice for at least 30 min before filtering. The filters (type HA, 0.45- μm pore diameter, Millipore Corp., Bedford, Mass.) carrying the trichloroacetic acid-precipitated bacteria were washed three times with room-temperature 5% trichloroacetic acid and dried. The radioactivity associated with each filter was determined by liquid scintillation spectrometry in a toluene-based cocktail containing Spectrafluor (Amersham/Searle Corp., Arlington Heights, Ill). All values were corrected for nonspecific adsorption of [³H]leucine by subtraction of the amount of radioactivity adsorbed to an equal volume of trichloroacetic acid-killed bacteria under the same conditions. The values for immunity presented in the tables and figures represent the amount of [³H]leucine incorporated by the phage-infected cells which were superinfected with ghosts. These values are expressed as the percentage of the amount incorporated by the duplicate control which did not receive ghosts. Assays with T4B phage (and their T4D controls) were performed with cells grown in M9G medium supplemented with L-tryptophan (final concentration, 100 $\mu\text{g}/\text{ml}$) a few minutes before addition of the phage.

Genetic exclusion of superinfecting phage. *E. coli* B/5 (*Su*⁻), grown in H broth (30 C) to a cell density of about 4×10^8 cells/ml, was concentrated by centrifugation to a density of 2×10^8 cells/ml. To duplicate 1.0-ml portions of bacteria were added either 100 μl of M9 salts followed in 15 s by 50 μl of the primary phage (MOI = 5), and superinfected with 50 μl of wild-type phage (MOI = 5) 8 min later, or 100 μl of chloramphenicol (final

concentration, 100 $\mu\text{g/ml}$) followed in 15 s by 100 μliters of a mixture of primary phage and wild type (MOI of each = 5). Measurement of infective centers was taken 9.5 min after simultaneous infection, or 5 min after superinfection, by diluting the phage-bacteria mixture 20-fold into H broth containing T4 antiserum (inactivation rate $K \approx 4$) and incubating for 2 min (30 C) before diluting 100-fold into ice-cold broth and plating with *E. coli* S/6 (*Su*⁻) as indicator. Adsorption of the primary phage was >98% in all experiments, as determined by removing a sample just before superinfection and shaking with chloroform before plating with *E. coli* BB (*Su*⁺).

Injection of superinfecting phage DNA. The procedure for measuring injection and solubilization of superinfecting DNA was adapted from Anderson and Eigner (1). *E. coli* B was grown in M9G medium (30 C) to a density of 4×10^8 cells/ml and harvested by centrifugation at room temperature. The cells were resuspended in the original volume of M9G medium (30 C) and infected with the primary phage (MOI = 5) while in a gyratory shaker water bath. After 10 min, the culture was superinfected with [³H]thymidine-labeled phage (MOI = 8), and incubation continued for 5 min before diluting the culture into 14 volumes of ice-cold water containing 333 μg of bovine serum albumin per ml (total volume, 75 ml). The diluted culture was subjected to shearing forces by blending at top speed (~16,000 rpm) for 10 min in a 100-ml cup of a Lourdes Instrument Corp. multi-mixer immersed in an ice-water bath. Samples were assayed for total counts per minute and acid-soluble counts per minute before and after blending. Such assays were made both for the complete culture and a supernatant fluid fraction (obtained by centrifuging 2.0 ml of sample for 5 min at 7,000 $\times g$, 4 C). Acid-soluble material was measured by adding 0.5 ml of sample to 0.5 ml of ice-cold 10% trichloroacetic acid. After 30 min in an ice-water bath, the precipitate was pelleted, and 0.5 ml of the supernatant was removed for liquid scintillation counting.

The radioactivity of the variously treated samples was measured by counting 0.5 ml of sample in 5 ml of scintillation fluid [2 volumes of toluene and 1 volume of Triton-X containing per liter of solution: 2,5-diphenyloxazole, 7.5 g; *p*-bis-2-(5-phenyloxazolyl)-benzene, 95 mg. This protocol yielded about 10,000 counts per min per 0.5 ml of the total culture sample. Control experiments with [³H]leucine-labeled phage indicated that 74 to 76% of the adsorbed phage were sheared from the cell surface by this blending regimen while lysing 4 to 6% of the cells as measured by the release of β -galactosidase.

The ability of the superinfected cultures to form infective centers was measured before and after blending by diluting a portion of the diluted culture into ice-cold M9G medium containing T4 antiserum ($K \approx 4$) for 7.5 min before plating with *E. coli* S/6 indicator.

Chemicals and other procedures. [Methyl-³H]thymidine and L-[4,5-³H]leucine were purchased from New England Nuclear Corp., Boston, Mass. The release of β -galactosidase from blended superinfected cells was measured by the procedure of Revel et al. (22).

RESULTS

Background level of immunity. Immunity development by infecting phage was measured by the level of continued protein synthesis after challenging the infected cells with superinfecting ghosts (see Materials and Methods). In a wild-type (*imm*⁺)-infected cell population, protein synthesis after the ghost challenge achieves about 90% of the level observed in *imm*⁺-infected cells not challenged by superinfecting ghosts. Under the same conditions, *imm2*-infected cell populations exhibit about 10% levels of protein synthesis after ghost challenge. However, if the ghost challenge is delayed, the *imm2*-infected cell population will eventually reach a maximum level of 60% immunity (26). This observation suggests two possible explanations: (i) the *imm2* mutant makes a partially functional *imm* protein and as a result *imm2*-infected cells were able to develop immunity but at a slower rate than *imm*⁺ phage; (ii) another gene(s) in addition to the *imm* gene also governs immunity development.

Does the *imm2* mutant make a partially functional protein? If the *imm2* mutation gives rise to an amino acid substitution in the wild-type protein, this mutation would be of the missense class and could be leaky due to residual activity of the missense protein. This possibility could not be tested directly, since the *imm* protein has not been isolated.

To achieve some indication as to which of the above explanations may be correct, a second *imm*⁻ mutant of T4 (*imm1*) independently isolated by Childs (6) was tested for a background immunity level. As shown in Table 1, a significant amount of background immunity was developed in the *imm1* mutant (15.9%),

TABLE 1. Immunity to superinfecting ghosts developed in wild-type-, *imm1*-, and *imm2*-infected *E. coli* B/5^a

Phage	Percent immunity ^b
None	0.23
Wild type	72.6
<i>imm1</i>	15.9
<i>imm2</i>	5.4
Wild type + <i>imm1</i>	73.8
Wild type + <i>imm2</i>	58.7
<i>imm1</i> + <i>imm2</i>	10.8

^a *E. coli* B/5, grown in M9G medium (30 C) to a cell density of 4×10^8 cells/ml, was infected with the indicated phage strains (MOI = 5) and superinfected with ghosts (MOK = 7) 9 min later.

^b Immunity was measured as described in Materials and Methods.

compared to the level (5.4%) obtained with *imm2* in these assays. The data further indicate that the *imm1* and *imm2* mutants fail to complement and thus are allelic. Had the *imm1* mutant shown no background immunity, the possibility of a second gene with *imm* function could have been eliminated. However, the results obtained did not distinguish between the two explanations.

Mutations in most early function genes, including all of those which have no known function, had been screened for the immunity defect without success (28). In addition, the isolation procedure of Childs (6), specific for exclusion-negative mutants, had shown only one region to contain the exclusion function, and the exclusion-negative mutant *imm1*, which is allelic to *imm2*, was located in this region. This suggests that, if other gene functions are involved in the development of immunity, mutants defective in such genes might be undetectable in the presence of the *imm⁺* gene. It seemed possible that such gene functions contributing to immunity development could be detected, however, in the absence of *imm* gene function. Thus, mutants of two early T4 genes, *rII* and spackle (*s*), which are thought to affect the host cell envelope, were assayed for immunity development singly and when coupled with the *imm2* mutation.

Phage-induced immunity and the *rII* gene. Numerous studies on the physiological defects occurring in abortive *rII⁻* infections have indicated that *rII* mutants are defective in a membrane function (5, 15). The response of *rII* mutant-infected cells to divalent and monovalent cations in the infection medium (16, 24) and the reported detection of the *rII* proteins associated with the membrane of phage-infected cells (11, 21, 29) have been the principal observations suggesting a membrane function for the product of the *rII* genes. Since the *rII* region is transcribed early in infection (23), it seemed possible that it might influence immunity to superinfecting ghosts when coupled with the *imm2* mutation, even though *rII⁻* mutants alone have no effect on immunity development (28).

The *imm2* and *rII⁻* mutations were combined by crossing *rED19* × *am(42)-imm2* and *rA105* × *am(43)-imm2*, from which progeny were selected to contain the *rII⁻* and amber markers. It was expected that most of these recombinants will also carry the unselected *imm2* mutation. These *rII-imm⁻* phage were assayed for their immunity character in a host permissive for *rII* mutants (Table 2). These results show that the presence of the *rII⁻* mutation does not reduce the background level of immunity seen with the

imm2 mutation alone. Also, the fact that the immunity development in the complete absence of the *rII* products (the *rA105* mutation deletes both the *A* and *B* cistrons) appears no different from the missense mutant (*rED19*) implies that the *rII* proteins are probably not interacting with the *imm* protein.

Phage-induced immunity and the spackle (*s*) gene. The spackle (*s*) mutant of T4 originated spontaneously from a lysozyme-deficient strain of T4 and was detected by its ability to lyse the infected cell even in the absence of the phage lysozyme (10). This phenotype implicated the *s⁻* phage as defective in a function which normally strengthens or maintains the integrity of the cell envelope after infection. This view appeared justified from the unique inability of *s⁻*-infected cells to develop resistance to lysis from without (LFW) (10). The *s* gene, then, most likely specifies a protein which

TABLE 2. Immunity to superinfecting ghosts in T4-infected *E. coli* B/5

Phage	Percent immunity ^a
None	0.18
Wild type	91.8
<i>imm2</i>	8.3
<i>am(42)-imm2</i>	9.2
<i>am(43)-imm2</i>	8.9
<i>rA105</i>	89.2
<i>am(42)-imm2-rED19</i>	9.3
<i>am(43)-imm2-rA105</i>	9.7
<i>s⁻</i>	60.2
<i>s⁻-am(42)-imm2</i> isolate 1 ^b	1.8
<i>am⁺</i> revertant	1.0
<i>s⁻</i> backcross recombinant	70.1
<i>ts(40)-am(42)-imm2</i> backcross recombinant	9.1
<i>s⁻-am(42)-imm2</i> isolate 2 ^b	3.4
<i>am⁺</i> revertant	1.4
<i>s⁻</i> backcross recombinant	62.8
<i>ts(40)-am(42)-imm2</i> backcross recombinant	11.5

^a Immunity to superinfecting ghosts was determined as described in Materials and Methods, except that L-tryptophan (final concentration, 100 µg/ml) was added to the host cell culture several minutes before the assay for experiments using *rED19*- or *rA105*-containing phage. The values of immunity for *imm2* and *s⁻* shown here are the averages of seven determinations.

^b Triple mutants *s⁻-am(42)-imm2* were constructed by the crosses diagrammed in Fig 1A; backcrosses to segregate the *imm2* and *s⁻* mutations are diagrammed in Fig 1B. The *imm* and *s* alleles of the backcross segregants were determined from their levels of immunity development relative to the single *imm2* and *s⁻* mutants.

alters the host cell envelope. The spackle gene has been shown to express an immediate early function of T4 (20), as is also true for *imm* (J. B. Cornett, Ph.D. thesis, Univ. of Arizona, Tucson, Ariz. 1973).

The s^- mutant was tested for its ability to confer immunity to superinfecting ghosts. Table 2 shows that the spackle mutant does not develop full immunity (approximately 60%) to superinfecting ghosts under standard conditions where the normal levels for wild type (approximately 90%) and *imm* $^-$ (approximately 10%) are seen. The partial defect in immunity to superinfecting ghosts seen in s^- infections indicated that the *s* gene may be responsible for the background immunity. Accordingly, the double mutant s^-imm^- was constructed to determine the immunity levels to superinfecting ghosts developed in cells infected with phage lacking both gene functions. Since both the spackle and immunity functions are non-essential, construction of s^-imm^- recombinants had to be performed without the advantage of selective plating conditions.

The s^- parent which forms "rII-like" plaques (sharp borders) on *E. coli* B at 37 C (10) was crossed to an *imm* $^-$ mutant carrying an amber (*am*) lesion near the *imm2* mutation in host *E. coli* BB (Su^+UAG) (see Fig. 1A). The progeny of this cross were replica plated on *E. coli* S/6 (Su^-) and *E. coli* BB (Su^+UAG), and plaques showing clear borders on BB (spackle-negative phenotype) and which failed to grow on S/6 (amber-negative phenotype) were selected as putative $s^-am^-imm^-$ phage. From seven such isolates, two were retained for further testing. That the s^- mutation was present in these putative $s^-am^-imm^-$ phage was suggested by their inability to become lysis inhibited by homologous superinfection (Fig. 2). The presence of the *imm2* allele was suggested by the essentially complete lack of immunity to superinfecting ghosts (Table 2). To insure that these $s^-am^-imm^-$ phage did contain the s^- and *imm* $^-$ alleles, each isolate was also backcrossed (see Fig. 1B) to recover the respective single mutants which were identified by their characteristic levels of immunity development (Table 2). The results of these assays (Fig. 2; Table 2) demonstrate that both isolates did contain the s^- and *imm* $^-$ alleles.

The levels of immunity developed by the $s^-am^-imm^-$ phage were much lower than that of the singly mutated *imm* $^-$ (s^+) phage (Table 2), which indicated that the background level of immunity seen in *imm2*-infected cells was due to the presence of the *s* gene product. This interpretation was confirmed by the experiment illustrated in Fig. 3, which clearly shows that

FIG. 1. Diagram of the recombinants occurring during construction and confirmation of the genotype of the $s^-am(42)-imm2$ double mutants. Mixed infections of *E. coli* BB (Su^+UAG) were carried out as described in Materials and Methods. The map position of the spackle gene was taken from Emrich (10), and the map position of *imm* was taken from Cornett and Vallée (7). A, The $s^-am(42)-imm2$ recombinants (-----) were selected by their ability to form large sharp-edged plaques when grown on *E. coli* BB (Su^+UAG) at 40 C (spackle phenotype) and by their inability to grow on *E. coli* S/6 (Su^-) [amber(42) phenotype]. B, The s^- recombinants (—) were selected as large sharp-edged plaques when grown on *E. coli* S/6 (Su^-) at 40 C. The $ts(40)-am(42)-imm2$ recombinants (-----) were selected as plaques which grew on *E. coli* BB (Su^+UAG) at 25 C but which failed to grow at 42 C or on *E. coli* S/6 (Su^-) at either temperature. All recombinant phage stocks were grown from single plaque isolates.

there is no background level of immunity developed in the double mutant *imm* $^-s^-$ infection. This particular experiment was performed at 37 C, rather than the standard temperature of 30 C, to be comparable to the original experiment showing the background levels of immunity (see Fig. 1 of reference 26). Under standard conditions (30 C) it takes 10 min for *imm* $^-$ to develop 10% immunity, whereas at 37 C it requires less than 2 min.

When immunity to varying MOK of superinfecting ghosts is measured by survival of infective centers as well as protein synthesis (Table 3), it is seen that the absence of *s* gene function is significant only with higher MOK of superinfecting ghosts or when the phage also lacks the *imm* function, whereas phage lacking only the *imm* function are sensitive at all MOK. The s^-imm^- double mutant used here and in the subsequent experiments was clonally derived from a spontaneous *am* $^+$ revertant of an $s^-am^-imm^-$ isolate. The majority of experiments presented thus far have utilized *E. coli* B/5 as the standard host to enable comparison with

FIG. 2. Lysis inhibition by homologous superinfection in T4-infected *E. coli* BB. A logarithmically growing culture of *E. coli* BB (Su^+UAG) in H broth (37 C) was concentrated to 2×10^8 cells/ml by centrifugation and infected with the indicated T4 strains (MOI = 5). Five minutes after the primary infection, the phage-bacteria mixtures were diluted with 4 volumes of prewarmed (37 C) H broth and superinfected with the same phage type 5 min later (MOI = 5). Lysis of the superinfected cultures was determined by the decrease in turbidity.

previous work (26). It was noted during the current study that strain B/5 did not form colonies efficiently on H broth plates after growth in M9G medium. Thus, *E. coli* strain B, which did not exhibit this peculiar behavior, was used in this and subsequent experiments where plating for colonies or infective centers from M9G medium was desired.

Genetic exclusion of superinfecting phage by the spackle mutant. The finding that s^- phage are partially deficient in developing immunity to superinfecting ghosts, a function primarily governed by the *imm* gene, prompted the following experiment to determine what role the spackle gene might play in a second *imm* gene function, the genetic exclusion of superinfecting phage. The results presented in Table 4 show that s^- phage-infected cells are able to

exclude only about 40% of the superinfecting phage under conditions where wild-type infections exclude 90% and *imm* $^-$ infections fail to exclude at all. As in the case of development of immunity to superinfecting ghosts, the *s* gene appears to play a lesser role in developing a function controlled mainly by the *imm* gene.

Insofar as the s^- phenotype parallels at a

FIG. 3. Development of immunity to superinfecting ghosts in T4-infected *E. coli* B/5. *E. coli* B/5 was grown in M9G medium (37 C) to a cell density of 4×10^8 cells/ml. Duplicate portions of cells were infected with the indicated T4 strains (MOI = 5). One portion was superinfected with ghosts (MOK = 7) at the times indicated to determine the level of immunity present, relative to a control portion not receiving ghosts. The measurement of immunity by incorporation of [3H]leucine is described in the Materials and Methods.

TABLE 3. Infective center formation and protein synthesis as measures of immunity in phage-infected *E. coli* B after superinfection with varying MOK of ghosts^a

Primary phage	Infective center formation (%) ^b			Protein synthesis (%) ^c		
	4 ^d	8	16	4	8	16
Wild type ...	88	81	82	98	97	74
s^-	92	89	66	95	77	51
<i>imm</i> 2	54	21	2.4	28	6.2	0.98
s^- - <i>imm</i> 2	2.6	4.2	0.28	5.5	0.71	0.38

^a The ghost challenge was at 10 min after phage infection (MOI = 5) of *E. coli* B (30 C). The measurements presented are those of the samples receiving superinfecting ghosts expressed as a percentage of the samples not receiving ghosts. The values represent the average of two determinations.

^b Portions of both primary and superinfected samples were diluted 20-fold into M9G medium containing T4 antiserum ($K \approx 4$) 13 min after the primary infection and incubated for 4.5 min (30 C) before subsequent dilution into ice-cold M9G medium and plating for infective centers.

^c Protein synthesis in both primary and superinfected samples was determined from a 5-min pulse of [3H]leucine (final concentration, 5×10^{-5} M, 0.5 μ Ci/ml) into ice-cold trichloroacetic acid-insoluble material from 15.5 to 20.5 min after the primary infection.

^d MOK of superinfecting ghosts.

TABLE 4. Exclusion of superinfecting wild-type T4 phage by *imm2*- and *s*⁻-infected *E. coli* B/5

Primary phage ^a	No. of infective centers/ml			
	Expt	Super-infection	Simultaneous infection	Exclusion (%) ^b
<i>am</i> (42)	1	3.0×10^7	2.9×10^8	90
<i>am</i> (42)	2	2.6×10^7	4.9×10^8	95
<i>am</i> (42)	3	3.7×10^7	3.4×10^8	90
<i>am</i> (42)- <i>imm2</i>	1	3.6×10^8	1.3×10^8	0
<i>am</i> (42)- <i>imm2</i>	2	4.1×10^8	3.4×10^8	0
<i>am</i> (42)- <i>imm2</i>	3	3.7×10^8	3.0×10^8	0
<i>am</i> (42)- <i>s</i> ⁻	1	2.0×10^8	3.3×10^8	39
<i>am</i> (42)- <i>s</i> ⁻	2	2.2×10^8	4.8×10^8	53
<i>am</i> (42)- <i>s</i> ⁻	3	2.5×10^8	3.7×10^8	33

^a The *am*(42) mutation is *am*NG205(42).

^b The percent exclusion is equal to: $100 - (N \times 100/N_0)$, where N_0 is the number of infective centers formed in the simultaneous infection and N is the number of infective centers formed after superinfection.

reduced level that of *imm*⁻, the *imm2* phage was then tested for properties characteristic of the spackle mutant: (i) the failure to exhibit lysis inhibition, and (ii) the failure to protect the infected cell from LFW resulting from high MOI of superinfecting phage.

Lysis inhibition. Cells infected with *s*⁻ phage phenotypically resemble *rII*⁻ infections in that these cells are not lysis inhibited by superinfecting homologous phage (10). The *rII*⁻ and *s*⁻ mutant types are distinguishable, however, by the inability of *rII*⁻ mutants to produce progeny in host cells carrying the prophage lambda, which does not restrict growth of *s*⁻ or *imm*⁻ phage. As shown in Fig. 2, the *imm*⁻ mutant, in contrast to the *s*⁻ strain, is lysis inhibited by superinfection. This property provides the one clear distinction between these two mutant types.

Resistance to lysis from without. A second phenotype of the *s*⁻ mutant which is unique to this strain is its inability to develop resistance to LFW (10). The ability of cells infected with *s*⁻ or *imm*⁻ mutants to resist LFW by superinfecting wild-type phage was determined in superinfected cultures by measuring the turbidity to assay the degree of lysis. The results (Fig. 4) show that, with respect to LFW, the *imm*⁻-infected cells are intermediate between wild type and *s*⁻. The turbidity measurements used here merely reflect the integrity of the superinfected cells but do not measure their viability (ability to form infective centers). In addition to cell integrity, the following experiments also measured the viability of the infected cells before and after superinfection with high MOI of phage by: (i) plating for infective centers; and

(ii) determining the amount of protein synthesis by incorporation of [³H]leucine in a 5-min pulse (Table 5). The proportion of viable superinfected cells in general reflects the proportion of physically intact cells for wild-type and *s*⁻ infections but was greatly reduced for the *imm*⁻ infections. The turbidity and viability measurements seen with *imm*⁻ superinfected cells indicate that superinfecting phage did not cause extensive LFW in these cells but appeared to "traumatize" the *imm*⁻-infected cells, thereby reducing their ability to form infective centers and to continue protein synthesis. The cause of the observed traumatization is not known and has not been studied further.

Injection of superinfecting phage DNA. It has been shown previously (1, 18) that, although superinfecting phage adsorb efficiently

FIG. 4. LFW by superinfecting wild-type T4 in T4-infected *E. coli* B. *E. coli* B, grown in H broth (37°C) to a cell density of 4×10^8 cells/ml, was primarily infected with the indicated phage strains (MOI = 5) and superinfected with wild-type T4 (MOI = 120) 7 min later. Lysis of the superinfected cultures was determined by the decrease in turbidity. The percent lysis, determined as the percent turbidity at 100 min relative to the turbidity at 4.5 min equal to 100%, was 90%, 42%, and 21% for the cultures primarily infected with wild type, *imm2*, and *s*⁻, respectively.

TABLE 5. Lysis from without, infective center formation, and protein synthesis in phage-infected *E. coli* B after superinfection with wild-type phage

Primary phage ^a	Turbidity (%) ^b			Infective center formation (%) ^c			Protein synthesis (%) ^d		
	50 ^e	70	87	50	70	87	50	70	87
Wild type	82	56	50	54	62		80	66	58
<i>s</i> ⁻	49	26	28	44	15	3.8	51	24	5.1
<i>imm</i> 2	66	47	40	0.4	0.15	0.16	1.3	2.1	2.9

^aMOI = 4 to 6.^bTurbidity measurements are expressed as percent turbidity at 90 min after superinfection relative to the turbidity 1 min before superinfection taken as 100%. The lowest possible value for turbidity is approximately 16%, which represents complete lysis from without under these conditions.^cAt 6 min after the primary infection and 3 min after superinfection, portions were diluted 20-fold into M9G medium containing T4 antiserum ($K \approx 4$) and incubated for 2.5 min before subsequent dilution in ice-cold M9G medium and plating for infective centers. Infective centers per milliliter (after superinfection) are expressed as percent of infective centers per milliliter without superinfection.^dProtein synthesis was measured during a 5-min pulse of [³H]leucine incorporation (final concentration, 5×10^{-5} M, 0.5 μ Ci/ml) into ice-cold trichloroacetic acid-insoluble material from 15.75 to 20.75 min after the primary infection. Protein synthesis in the superinfected cultures is expressed as percent of [³H]leucine incorporation in the control cultures not receiving the superinfecting phage.^eMOI of superinfecting phage added 10 min after the primary infection.

to bacteria, they are less efficient than primary infecting phage in injecting their DNA. This reduced ability to inject appears to result from a function expressed by the primary phage, since superinfecting phage inject with normal efficiency if the host cells are treated with chloramphenicol prior to the primary phage infection (1). A simple explanation for all the above results with *s*⁻ phage is to view the wild-type spackle gene product as hindering the injection (tail sheath contraction) of superinfecting phage and phage ghosts. This view assumes that the *s*⁻ mutation allows a greater efficiency of injection-contraction of superinfecting phage or ghosts and thus increases the probability of superinfecting phage to inject their DNA (escape exclusion) and superinfecting ghosts to kill (reduced immunity).

To test this hypothesis, *E. coli* B was infected with the mutant phage under study and superinfected with [³H]thymidine-labeled wild-type phage 10 min later. After 5 min, the superinfected cultures were subjected to shearing forces to remove adsorbed phage particles, and the percentage of adsorbed superinfecting phage which injected their DNA was determined as described in Materials and Methods. The results of this experiment (Table 6) indicate that, contrary to the hypothesized result, superinfecting phage are actually less efficient in injecting their DNA into *s*⁻-infected cells than *s*⁺-infected cells.

DISCUSSION

Vallée and Cornett (27) have previously presented the view that the *imm* gene product

TABLE 6. Injection of superinfecting phage DNA^a

Primary phage	Superinfecting phage	
	Adsorption (%)	Adsorbed phage which inject (%)
Wild type	85	30
<i>s</i> ⁻	88	8.6
<i>imm</i> 2	86	33
<i>imm</i> 2- <i>s</i> ⁻	83	46

^aInfective center production before and after blending, respectively, was ($\times 10^8$ PFU/ml): wild type, 1.4, 1.2; *imm*2, 0.7, 1.0; *s*⁻, 1.7, 1.8; *imm*2-*s*⁻, 0.36, 0.34.

stoichiometrically neutralizes the sites of potential ghost action on the cell membrane. This view assumes that: (i) phage T4 irreversibly adsorb to those areas of the cell wall which are contiguous with the cell membrane as observed in photomicrographs of plasmolyzed, T4 phage-infected *E. coli* (2); (ii) these cell wall-membrane adhesions serve as the sites of action of the *imm* protein in neutralizing the disruptive effects of ghosts and in initiating genetic exclusion of superinfecting phage DNA. This report is concerned with what role the spackle gene might play in these hypothesized events. The relevant phenotypes of mutants as determined in this report are summarized in Table 7. The *rII*⁻ mutant type is also included in Table 7 for comparison, since the *rII* products are also thought to interact with the host cell membrane. For simplicity, in the following discussion it is assumed that both *imm* and *s* gene products act at the same site.

TABLE 7. Comparison of the phenotypes of wild type, s^- , imm^- , s^-imm^- , and rII^- mutants of bacteriophage T4D after addition of superinfecting (SI) phage or phage ghosts

Phage	Immunity to SI ghosts (MOK = 7)	Genetic exclusion of SI phage	Lysis inhibition by low MOI of SI phage	Resistance to LFW by high MOI of SI phage
Wild type	>90%	>90%	+	+
s^-	~60%	~40%	-	-
imm^-	~10%	0	+	±
s^-imm^-	~1%	NT ^a	NT	-
rII^-	>90%	>90%	-	+

^a NT, not tested.

Previous work (10) with T4 spackle indicates that the spackle gene specifies a product which, when mutated, leaves the s^- -infected cell unable to resist LFW and not subject to lysis inhibition by superinfecting phage. In addition, this mutation allows the infected cell to lyse in the absence of e gene product (phage lysozyme). These observations led Emrich (10) to postulate that the spackle mutant fails to effect a normal strengthening of the cell wall after phage infection.

The imm function affects two properties of phage-infected cells: immunity to superinfecting ghosts and genetic exclusion of superinfecting phage. The spackle mutant also shows deficiencies in these same two properties. In contrast to imm^- , the immunity defect in s^- -infected cells becomes significant only at higher MOK of superinfecting ghosts (Table 3). This could be taken to indicate that s^- -infected cells exhibit reduced immunity (reduced protein synthesis after the ghost challenge) due to LFW occurring in a proportionate number of cells. This explanation for the spackle phenotype does not, however, account for spackle's partial deficiency in genetic exclusion (Table 4), since this assay requires the superinfected cell to remain intact. Since the s^- defect with regard to genetic exclusion does not reflect lysis of the superinfected cells, most probably the closely related property of immunity is not significantly affected either by LFW.

The possibility that the spackle mutant phenotype might result from the failure of superinfecting phage or ghosts to undergo tail sheath contraction was mentioned in the Results. It was thought that perhaps spackle acted stoichiometrically at the adsorption sites to control the extent of injection. This would leave the imm function to control the fate of the injected DNA or the killing effect of the contracted ghosts. The results presented in Table 6 proved contrary to this model for spackle action. Instead of observing an increase in injection of superinfecting phage DNA, it was in fact reduced in the

s^- -infected cells compared to wild-type infections. Injection of phage DNA most probably requires the proper interaction between phage and the cell wall (3). The low efficiency of injection by superinfecting phage into the s^- -infected cells indicates that spackle function most likely affects the host cell wall. These results are in accord with the conclusion previously reached by Emrich (10). However, other data in this report indicate that the spackle function may also affect the cell membrane.

The observation that 60% of the superinfecting phage DNA which is injected into an s^- -infected cell passes through the membrane to escape genetic exclusion, compared to 8% in an s^+ -infected cell (Table 4), suggests that the wild-type spackle function also alters the cellular membrane. This effect on the membrane may be a consequence of the action of the spackle gene product on the cell wall. Such a condition could be realized if the spackle gene product, like the product of the imm gene, acts on the cell wall-membrane adhesion points where superinfecting phage and ghosts adsorb to the cell. The spackle product may be needed to fully neutralize these sites against the potential action of superinfecting ghosts or phage.

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