GENERALIZED TRANSDUCTION OF A GALACTOSE MARKER IN SALMONELLA GALLINARUM-PULLORUM

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ABSTRACT

GENERALIZED TRANSDUCTION OF A GALACTOSE MARKER
IN SALMONELLA GALLINARUM-PULLORUM

by Richard Willis Vaughan

Phage mediated transfer of the gal^+ character in Salmonella gallinarum-pullorum was demonstrated and partially characterized. Transductions were performed with phage lysates produced by ultraviolet induction of strain 38 and with lysates of phage P38, propagated on strain 35 or 53. Galactose fermenting clones were distinguished from a background lawn of galactose nonfermenting cells by their appearance when overlaid with agar containing 0.05% 2,3,5-triphenyl-2H-tetrazolium chloride. Fermenting clones appeared red on a white nonfermenting background. Inter- and intrastrain transductions were obtained. Little difference was observed between the transducing efficiency of UV-induced P38 lysates and lysates of P38 propagated on strain 53. At a multiplicity of input of 1, 90-99% of the P38 phage particles were adsorbed within 25 min.

GENERALIZED TRANSDUCTION OF A GALACTOSE MARKER IN SALMONELLA GALLINARUM-PULLORUM

By

Richard Willis Vaughan

A THESIS

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Dedication

To my wife for her understanding and moral support.

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INTRODUCTION

Transduction, a form of infectious heredity, was first observed by Zinder and Lederberg (1952). Transduction mediated with bacteriophages involves: (1) infection of donor cells with phage particles, (2) multiplication of phage particles within donor bacteria (lytic cycle), (3) acquisition of a fragment of donor bacterial genetic complement, presumably at random, (4) infection of recipient bacteria with the matured donor phage, and (5) expression of the acquired genetic fragment in recipient bacteria (transductants). Direct evidence that the genetic complement involved in transduction is deoxyribonucleic acid (DNA) has been provided by Kaiser and Hogness (1960), who have transformed recipient bacteria with DNA isolated from a transducing phage preparation.

The experiments studied in this research were prompted by the report of transduction of galactose (gal) characters in *Salmonella gallinarum-pullorum* (Snyder and Schoenhard, 1960, 1961; Snyder, 1961). It seemed desirable to clarify and partially characterize the general transducing system of *S. gallinarum-pullorum* using the gal marker.

Galactose negative mutants were obtained from two strains of *S. gallinarum-pullorum*, and successful transduction was performed. Transduction was demonstrated with lysates of phage 38 (P38) prepared by UV irradiation of strain 38 or with lysates of infected wild type strain 53 or 35.

The results reported are expected to serve a twofold purpose: (1) a foundation for further experiments to map the gal chromosomal region and (2) a model for elucidation of the arginine segment.

HISTORICAL

Since the original discovery of transduction, three processes have been shown to be possible: general, special, and abortive transduction.

Generalized transduction has been observed with phage PLT22 in *Salmonella typhimurium* (Zinder and Lederberg, 1952), phage P1 in *Escherichia coli* (Lennox, 1955; Jacob, 1955), staphylococcal typing phage 53 in *Staphylococcus aureus* (Morse, 1959), and bacteriophage SP-10 in *Bacillus subtilis* (Thorne, 1961). The phenomenon has also been shown to occur in *Vibrio cholerae* (Bhaskaran, 1958), *Pseudomonas aeruginosa* (Loutit, 1958; Holloway and Monk, 1960) and *Proteus mirabilis* (Coetzee and Sacks, 1960a). Any of the genetic characters of a donor bacterium can be transferred by phage particles to a recipient bacterium, singly or in pairs (Lennox, 1955) and the transductants may or may not be lysogenic for the transducing phage (Adams and Luria, 1958; Coetzee and Sacks, 1960b). The frequency of genetic transfer in generalized transduction is quite low, 1×10^{5} - 1×10^{8} per infecting phage particle, for any one trait (Adams and Luria, 1958).

Specialized transduction involving phage λ of *E. coli* (Morse *et al*., 1956a,b) results when λ is reduced to a defective state. Luria, Adams, and Ting (1960) reported an analogous mechanism utilizing phage P1 to transduce from *E. coli* lactose fermenting (lac^+) donors to *Shigella dysenteriae* Sh lac^- recipients.

Phage λ becomes defective when the bacterial genome galactose (gal) region substitutes for a segment of the λ genome (Arber *et al.*, 1957). Defective lambda (λ dg) is genetically blocked in its ability to enter the lytic cycle, however, ultraviolet (UV) irradiation of bacteria carrying λ or λdg induces λ or λdg to enter the lytic cycle (Weigel and Delbruck, 1951; Morse *et al.*, 1956a). The induced lambda lysate will transduce only characters of the gal region, 70% of which are unstable. Upon repeated isolation unstable transductants (heterogenotes) segregate gal^- progeny. UV induction of heterogenotes produces lysates in which transducing particles approximate the number of λ particles (Morse *et al.*, 1956a, b).

Abortive transduction was discovered by Stocker, Zinder, and Lederberg (1953). In abortive transduction there is only one bacterium present in the transductant clone, at any given time, which contains the donor genetic fragment (Stocker, 1956; Lederberg, 1956). General transduction involves integration into the replicating genome, resulting in true clonal inheritance of the transduced trait. Abortive transduction occurs when the genetic fragment is not integrated and does not replicate, being passed unilinearly from the recipient cell to only one of the daughter cells at each cellular division (Hartman and Goodgal, smear).

One major problem in genetic research is the development of a suitable technique to study a desired trait. Since mutants deficient in their capacity to ferment galactose were the object of this investigation, a suitable indicator medium which would permit colony classification was required. Eosin-methylene blue (EMB) agar (Lederberg, 1950) is an extensively used indicator medium for this purpose. Although EMB is satisfactory for transduction analysis of fermentation mutants of *E. coli* (Morse *et al.*, 1956a) and *S. typhimurium* (Zinder and Lederberg, 1952), it does not work well with *S. gallinarum-pullorum*. Lederberg (1948) reports the use of triphenyltetrazolium chloride where fermenting clones remain white and nonfermenting clones stain red with formazan. Ogur *et al.* (1957) report the use of a triphenyltetrazolium chloride agar overlay technique to detect respiration-deficient mutants in yeast, the deficient mutants remaining white and the respiring colonies turning red. Jordan *et al.* (1962) report the use of triphenyltetrazolium chloride incorporated into the plate agar to detect fermentation clones, the nonfermenters remaining white and the fermenters staining red.

MATERIALS AND METHODS

Bacterial Strains

Four strains of *S. gallinarum-pullorum* were employed: strain SP25 (Massachusetts 1), strain SP35 (South Carolina C), strain SP38 (South Carolina F), and strain SP53 (Kentucky CDC3522). Mutants of SP35 and SP53 are listed in Table 1.

TABLE 1.--Bacterial mutants

Designation	Production
SP-35 gal^{-}_{1}	UV-induced
SP-35 gal^{-}_{6}	HNO_2 induced*
SP-35 gal^{-}_{7}	HNO_2 induced*
SP-35 gal^{-}_{8}	HNO_2 induced*
SP-53 gal^{-}_{1}	UV-induced**
SP-53 gal^{-}_{2}	Spontaneous
SP-53 gal^{-}_{2R}	Spontaneous
SP-53 gal^{-}_{3}	UV-induced
SP-53 S^{r}	Spontaneous***

*Obtained from D. E. Schoenhard.
**Obtained from J. J. Robinson.
***Obtained from R. W. Snyder

Strain 38 carries the bacteriophage P38. Strains 25, 35, and 53 are sensitive to and lysogenized by P38. All bacterial stocks were maintained in duplicate 0.7% Brain Heart Infusion (BHI) agar stab cultures kept at 4 C and at room temperature. Working stocks were maintained at room temperature on BHI agar slants in screw cap vials.

Phage

Phage P38 is a temperate phage isolated from mixtures of bacterial strains 38-35 or 38-53. Initial isolation was done on BHI agar plates according to the standard soft agar overlay method of Adams (1959). The phage particles were suspended in T_2 buffer (Hershey and Chase, 1952) and stored at 4 C in screw cap vials over chloroform.

Media

Difco BHI agar, BHI broth, nutrient agar, and nutrient broth were used where indicated.

BHI soft agar contained 7 g Bacto-Difco agar, 37 g dehydrated BHI broth, and 1000 ml distilled water. The sterilized agar was dispensed 2.5 ml per sterile 13 mm x 100 mm test tube.

Soft agar (7 g Bacto-Difco agar/1000 ml distilled water) was dispensed 2.5 ml per 13 mm x 100 mm test tube.

M-9 broth (Dr. Myron Levine, personal communication) was employed for production of phage lysates.

KH_2PO_4	1.67 g

Na_2HPO_4	3.33 g
NH_4Cl	0.56 g
Casamino acids (Difco)	7.50 g
NaCl	0.50 g
Glucose	2.00 g
$MgSO_4$ 1M	2.50 ml
Distilled water	1000.00 ml

A casein hydrolysate broth was used for producing galactose negative mutants.

NH_4NO_3	1.0 g
Na_2SO_4	2.0 g
K_2HPO_4	7.0 g
KH_2PO_4	3.0 g
NH_4Cl	2.5 g
$MgSO_4 \cdot 7H_2O$	0.1 g
Casein hydrolysate (Nutritional Biochemical Co.)	3.0 g
Glycerol	15.0 ml
Distilled water	1000.0 ml

Casein hydrolysate and glycerol were added to 925 ml of distilled water and autoclaved. Separate 10X solutions of $MgSO_4 \cdot 7H_2O$ and NH_4Cl were prepared and autoclaved. One ml of 10X $MgSO_4 \cdot 7H_2O$ and 10 ml of 10X NH_4Cl were added to the casein hydrolysate-glycerol solution. A solution containing 10X by weight of the above amounts of NH_4NO_3, Na_2SO_4, K_2HPO_4, and KH_2PO_4 in 500 ml of distilled water was prepared. The solution was autoclaved and 50 ml added to the casein hydrolysate-glycerol solution.

Casein hydrolysate agar was prepared by addition of 15 g agar to the casein hydrolysate-glycerol solution before autoclaving.

Casein hydrolysate-galactose broth was made by supplementing casein hydrolysate broth with 10 ml of a 20% galactose solution.

Eosin-methylene blue agar (Lederberg, 1950) supplemented with 1.0% galactose (Difco) (EMBGal) was used for isolation of gal$^-$ mutants and purification of gal$^+$ transductants.

Casein hydrolysate (acid hydrolyzed, Nutritional Biochemical Co.)	8.0 g
Yeast Extract (Difco)	1.0 g
NaCl	5.0 g
K_2HPO_4	2.0 g
Eosin Y	0.4 g
Methylene Blue	0.065 g
Galactose (Difco)	10.0 g
Agar	15.0 g
Distilled water	1000.0 ml

All transductions were performed on a modification (LGal agar) of Lederberg's (1950) EMB agar.

Casein hydrolysate (acid hydrolyzed, Nutritional Biochemical Co.)	8.0 g
Yeast Extract (Difco)	1.0 g

NaCl	5.0 g
K_2HPO_4 (anhydrous)	2.0 g
Bacto agar (Difco)	15.0 g
Galactose (Pfanstiehl)	10.0 g
Distilled water	1000.0 ml

Five hundred ml of LGal agar were prepared by the following method. Seven and one-half g of agar were added to 360 ml of distilled water in a 500 ml flask, heated to boiling, and autoclaved. Twenty g of casein hydrolysate were dissolved in 500 ml of distilled water, dispensed 100 ml per 6 oz prescription bottle, and autoclaved. One hundred ml were poured into melted agar kept at 50 C. One hundred ml solutions of the following were dispensed into 6 oz prescription bottles and autoclaved: 25% NaCl, 10% yeast extract, 20% K_2HPO_4, and 25% galactose. Five ml of NaCl, 2.5 ml of yeast extract, 2.5 ml of K_2HPO_4, and 10 ml galactose were added to the melted agar. The final solution was shaken and 20-25 ml poured into 100 mm x 15 mm plastic Petri dishes. All agar plates were dried overnight at 37 C before use.

After suitable incubation of the transduction plates, 15 ml of 2, 3, 5-triphenyl-2H-tetrazolium chloride agar (Ogur *et al*., 1957; Nagai *et al*., 1961) were overlayed on them.

Agar (Bacto Difco)	15.0 g
Galactose (Pfanstiehl)	10.0 g

2,3,5-triphenyl-2H-tetrazolium chloride (TTC, Eastman)	0.5 g
KH_2PO_4	0.067 M
Distilled water	1000.0 ml

Solutions of 0.402 M KH_2PO_4 buffer adjusted to pH 7.0 with 10N NaOH, 0.15% TTC, and 6.0% galactose were separately dispensed in the amount of 250 ml per 16 oz bottle. Five ml of 4.5% agar were placed in 20 mm x 150 mm test tubes. Before use, the tubes of agar were melted and allowed to cool to 50 C. Two and one-half ml of buffer, 2.5 ml of galactose and 5.0 ml of TTC were added. The TTC agar tubes were allowed to stand at 50 C for 30 minutes before overlaying.

All dilutions were made in 0.5% saline or T_2 buffer (Hershey and Chase, 1952).

Gelatin	0.01 g (1 ml of a 1% solution)
$Na_2HPO_4 \cdot 12\ H_2O$	7.4 g (or Na_2HPO_4, 3g)
KH_2PO_4	1.5 g
NaCl	4.0 g
K_2SO_4	5.0 g
$MgSO_4$ 0.1 M	10.0 ml
$CaCl_2$ 0.01 M	10.0 ml
Distilled water	1000.0 ml

Solutions of gelatin, $MgSO_4$, and $CaCl_2$ were prepared separately and added after autoclaving. The final buffer had a pH of 7.0.

Saline contained 5 g NaCl per 1000 ml distilled water.

All autoclaving was done at 15 pounds pressure for 20 min.

Incubation

Incubation was carried out at 37 C unless otherwise stated.

Ultraviolet Source

A 30 watt, 35 inch long General Electric Germicidal Lamp was employed for inducing mutation and determination of a UV survival curve. Irradiation was done at a distance of 41 cm using a Superior Electric Company type 116-11056 powerstat set at 80 to control line voltage. The lamp was calibrated by measuring the impinging radiation with a General Electric light meter recording in foot candles. Foot candles were converted to ergs/mm^2 by the following method:

$$\frac{x \text{ foot candles}}{10{,}000} \times 1320 \text{ watts/m}^2 \times 10^7 = \text{ergs/m}^2$$

$$\frac{\text{ergs/m}^2}{10^6} = \text{ergs/mm}^2$$

UV Survival Curve

Strain 53 was inoculated into 10 ml of nutrient broth and incubated 12 hours. One ml of culture was centrifuged, washed twice with 0.5% saline and resuspended in 10 ml of saline. One-tenth ml samples of suitable dilutions were spread on duplicate nutrient agar plates to determine the number of viable organisms. Seven ml of saline culture were irradiated in a plastic Petri dish. One-tenth ml samples were withdrawn at 10 sec intervals. Suitable dilutions were spread on duplicate nutrient agar plates.

UV Induction of Mutants

Strain 35 or 53 was inoculated from a BHI slant culture into 10 ml of casein hydrolysate-galactose broth. The culture was incubated with aeration for 20 hours (stationary phase cell concentration = 1-2 x 10^9/ml). Seven ml of a 1:500 dilution were irradiated 15 seconds (approximately 10% survival) in a plastic Petri dish. The irradiated sample was centrifuged (6000 x G) for 15 minutes. The pellet was resuspended in 7 ml of casein hydrolysate broth and incubated 2-3 hours. One-tenth ml samples of 1:10 and 1:100 dilutions were spread on casein hydrolysate agar plates or EMBGal. After 48 hours incubation, EMBGal plates were observed for white gal^- colonies. After 24 hours incubation, casein hydrolysate agar plates were replicated (Lederberg, 1952) with velveteen to EMBGal agar. Replicated EMBGal plates were incubated 48 additional hours before observation. All mutants were carried through a minimum of 3 subcultures before tests were made to classify them. Gram stains, fermentation patterns, morphological comparisons, and P38 phage sensitivity were used to confirm that the mutants were strains of *S. gallinarum-pullorum*.

Preparation of Transducing Lysates

Spontaneous.--*Salmonella gallinarum-pullorum* bacteriophage P38 was prepared according to a modified method of Z. Hartman (1956). One-tenth ml of an overnight M-9 broth culture of bacteria was transferred into 10 ml of M-9 broth. The culture was aerated for 3 hours. Phage particles were added to a final concentration of 1-2 x 10^6 per ml, and the culture aerated 6-8 hours. The culture was centrifuged at 6000 x G for 10 minutes followed by filtration of the supernatant fluid. Filtration was done through a glass micro-millipore filter using a 0.45 μ HA membrane without a supporting pad. Phage suspensions were assayed and stored over 0.5 ml chloroform at 4 C.

UV induction of P38 (UVI P38).--One ml of an overnight strain 38 M-9 broth culture was transferred to 10 ml of M-9 broth. The culture was incubated 11 hours with aeration (1-2 x 10^9 bacteria/ml), centrifuged and resuspended in 10 ml of saline. The saline suspension was irradiated, centrifuged and resuspended in 10 ml of M-9 broth. Further incubation (2-3 hours) with aeration was done in the dark. The culture was centrifuged and the supernatant fluid filtered through a millipore filter. Lysates were assayed and stored over chloroform. UV induced lysates usually contained 1-4 x 10^8 plaque forming units per ml (pfu/ml).

Method for Phage Assays (after Adams, 1959)

Two-tenths ml of an overnight BHI broth culture of indicator strain 25 were transferred into melted 0.7% soft agar tubes kept at 45 C. The phage to be assayed were serially diluted in T_2 buffer. One-tenth ml portions of a suitable phage dilution were transferred into duplicate bacteria-soft agar tubes. The tubes were poured onto BHI agar plates. Poured plates were rocked gently with a circular motion to spread the soft agar over the entire surface of the plates. The assay plates were incubated face-up for 10-12 hours. Plaques were counted on plates showing 30-300 plaques per plate. Phage titers (pfu/ml) were calculated from the average number of plaques on the duplicate plates multiplied by the dilution factor for the assay.

Preparation of P38 Antisera

Bacteriophage suspensions to be injected were prepared as follows: (1) Strain 53 was grown overnight in 10 ml BHI broth, (2) one ml was inoculated into 100 ml of BHI broth contained in a fluted 500 ml shaker flask, (3) the culture was shaken on a rotary shaker for 3 hours, (4) phage P38 was added to give 1-2 x 10^6 phage particles per ml final concentration, (5) the culture was shaken an additional 8 hours, heated at 60 C for 30 minutes and placed in a refrigerator overnight, (6) the culture was centrifuged at 6000 x G for 15 minutes (25 ml per 50 ml centrifuge tube), (7) the supernatant fluid was centrifuged at 25,000 x G for 2 hours, (8) resuspension of the pellet was done in 5 ml of a solution of 0.001% gelatin and 0.5% NaCl by letting it stand in the cold for 2 hours with occasional gentle agitation, (9) phage particle suspensions were combined and centrifuged at 6000 x G for 15 minutes, (10) the supernatant fluid was decanted and Deoxyribonuclease (DNAse) (Nutritional Biochemical Co.)added to a final concentration of 5 μ g/ml, (11) the solution was incubated 1 hour at 37 C, filtered through a millipore filter and centrifuged at 25,000 x G for 2 hours, (12) the phage pellet was resuspended as per step 8, and (13) the phage suspension was sterilized by filtration through a millipore filter. All centrifuge procedures were carried out in a Serval SS-1 Angle head centrifuge in a 4 C cold room. Phage titers usually were between 5 x 10^{10} and 5 x 10^{11}pfu/ml.

Five ml of phage suspension diluted to 1 x 10^{10} pfu/ml in saline were emulsified with 5 ml of incomplete Freund's Adjuvant (Difco). Once a week, for 3 weeks, 2.5 ml were injected intramuscularly (IM) into both rear legs of 2 two-month-old rabbits. The rabbits were rested for 2 weeks and injected (booster injection) with 2.5 ml per rear leg with 1 x 10^{10} pfu/ml in 0.85% saline. After two weeks the rabbits were bled via cardiac puncture. The booster injection and bleeding cycle were repeated twice at 10-day intervals.

The serum of each bleeding was ooled, centrifuged, decanted, sterilized by filtration through a millipore membrane, and frozen. All serum was heat-inactivated at 56 C before use. Assay of phage neutralizing titer was performed according to Adams (1959) with the exception that the initial phage dilution was 1 x 10^8 instead of 1 x 10^7 pfu/ml.

Phage Adsorption Determination (Adams, 1959)

In order to perform a satisfactory transduction, it was desirable to obtain a knowledge of the approximate time required for adsorption of 90-99% of input phage. Strain 53 was inoculated from a stock BHI slant into 10 ml of LC broth (Luria et al., 1960) and incubated with aeration. A direct estimation of cell concentration was made with a Petroff-Hausser counter. The culture was diluted to 2 x 10^7 bacteria/ml in LC broth. Phage from a stock of P38 propagated on strain 53 (P38/53) was diluted in LC broth to 2 x 10^8 and 2 x 10^7 pfu/ml. Bacteria and diluted phage were equilibrated to and kept at 37 C before further use. Phage P38/53 (2 x 10^8 pfu/ml) was diluted in LC broth and assayed to serve as a stock titer control. One ml of 2 x 10^7 bacteria was transferred to each of two 13 mm x 100 mm test tubes. One ml of 2 x 10^7 pfu/ml was added to one tube to give a multiplicity of input (MI) of 1. One ml of 2 x 10^8 pfu/ml was added 90 sec later to the second tube to give a MI of 10. Immediately after addition of phage 0.1 ml samples were withdrawn from each tube (0 time) and diluted 1:100 in T_2 buffer containing chloroform to stop phage adsorption. One-tenth ml samples were withdrawn and diluted at 3 minute intervals for a period of 21 minutes. Diluted phage suspensions (free phage) were freed from chloroform by 15-30 minutes of aeration at 37 C. Free phage particles were diluted appropriately and assayed using LC soft agar and LC agar (Luria et al., 1960).

Transduction Procedure

Overnight 10 ml BHI broth cultures (recipient cells) were centrifuged, supernatant fluid decanted, the pellet rinsed with 2 ml of T_2 buffer and resuspended in an equal volume of T_2 buffer. They were recentrifuged, rinsed, and resuspended in 3 ml of the same buffer. The organisms of the suspension were then counted in a Petroff-Hausser counter and diluted with T_2 buffer to 2×10^9 or 2×10^8 bacteria/ml. Five-tenths ml of suitably diluted phage particles, depending upon the desired MI, were added to 0.5 ml of culture. The mixture was allowed to adsorb for 25 minutes at 37 C in a water bath. One-tenth ml samples of the mixture were spread on 5 LGal agar plates. Incubation was at room temperature for 2 hours (Luria et al., 1960) to increase lysogenization before incubating an additional 70 hours at 37 C. Bertani (1958) has shown that decrease in temperature of incubation increases the frequency of lysogenization. Each plate was carefully overlaid with 15 ml of TTC agar. Overlaid plates were incubated 24 hours before scoring.

Recipient cells were diluted to 1×10^8 or 1×10^9 and 0.1 ml samples were spread on 5 LGal plates to serve as a control to detect back mutants. Treatment of these plates was identical to that of the plates for transduction. One-tenth ml of T_2 buffer and phage stocks were spotted on BHI agar plates to serve as a control to determine the purity of the phage stocks.

Frequency of transduction was calculated by subtracting the average number of gal^+ colonies appearing on reversion plates from the average number of gal^+ colonies on the transduction plates, multiplying by the plating factor and dividing by the total pfu.

Transductants were checked for galactose fermentation by streaking with a needle on EMBGal agar plates. Plates were scored for fermentation after 48 hours incubation.

In some experiments, 0.1 ml of transduction mixture was diluted 1:100, 2 ml centrifuged and the supernatant fluid assayed for free phage content to determine the per cent of phage adsorption.

Test for Lysogeny of Transductants (Levine, 1957)

Transductants were carried through 5 subcultures on EMBGal agar. An isolated colony from subculture 5 was suspended in 0.5 ml of P38 antisera diluted 1:100 with 0.85% saline. The mixture was incubated at 37 C for 30 minutes in a water bath. A loopful was streaked on EMBGal plates containing P38 antisera at a dilution of 1:250. After 48 hours incubation an isolated colony was suspended in 0.5 ml of saline. This procedure frees bacteria from passively carried phage. A BHI plate was overlaid with soft agar containing indicator strain 25 to form a bacterial lawn and spotted with a loopful of cell suspension. The seeded BHI plates were observed for plaque formation after 10-12 hours incubation. If plaques appeared in the area spotted with the test organism, then it was classified as lysogenic.

RESULTS

Production of Mutants

Induction of mutation with ultraviolet light has been reported to be a complex process affected by many factors (Doudney and Haas, 1958, 1959; Haas and Doudney, 1957a, 1957b, 1959; and Witkin, 1956). One factor was the amount of irradiation employed. An ultraviolet survival curve (Figure 1) was determined to serve as a basis for obtaining a mutagenic dose.

Strain 53 was subjected to the following dosage: 15, 25, 40, 43, 45, 55, and 60 seconds. The number of plates employed per dose varied from 30-100 while the number of colonies per plate ranged from 1-150. In some cases induction was repeated 2-3 times for a single UV dose. One gal^- mutant (53 gal^-_3) was isolated from strain 53. Four gal^- mutants (35 gal^-_1, 35 gal^-_2, 35 gal^-_3, and 35 gal^-_4) were isolated from strain 35 of which 35 gal^-_2 and 35 gal^-_4 are sensitive to galactose. All mutants isolated were induced with 15 sec (10% survival) of irradiation. It is of interest that the one induction experiment performed with strain 35 utilized 20 plates and resulted in 4 gal^- mutants.

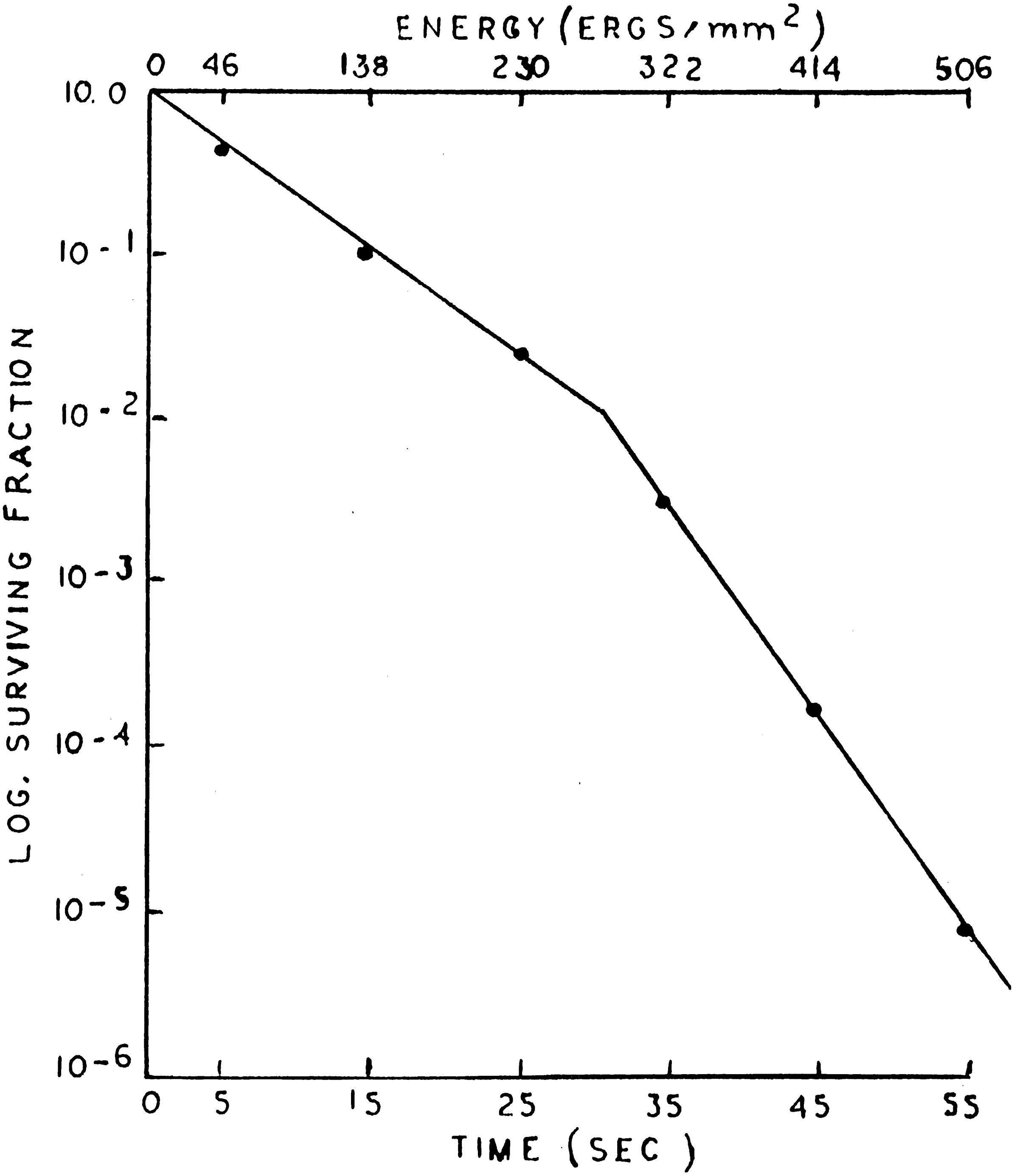

FIG. I UV INACTIVATION OF STRAIN S3

Adsorption of P38 to Strain 53

In studying the principle of transduction it was desirable to know the percentage of phage adsorbed to recipient cells as a function of time. The results of such an experiment were recorded in Figure 2. The velocity constants were calculated from the formula

$$K = \frac{2.3}{Bt} \quad x \quad \log \frac{p_o}{p} \text{ (Adams, 1959)}$$

where, p_o = phage assay at zero time, p = phage not adsorbed at time t min, B = bacteria/ml, and K = velocity constant ml/min. The P38-strain 53 system had a K = 1.59×10^{-8} ml/min for a MI of 1 and a K = 1.2×10^{-8} ml/min for a MI of 10.

Since 90-99% phage adsorption was assumed to be adequate for transducing procedures, the adsorption time was calculated by solving the above equation for t. At a MI of 1, 99% adsorption required 28.6 minutes. Hence, 25 minutes seemed sufficient to obtain 90-99% phage adsorption.

Assay of P38 Antiserum

Phage antiserum was essential for the proof of lysogeny in transductants. The results recorded in Figure 3 demonstrate the ability of the serum to inactivate P38. The velocity constant was calculated from the formula

$$K = 2.3 \ \frac{D}{t} \ x \log \ \frac{p_o}{p} \ \text{(Adams, 1959)}$$

where, p_o = phage assay at zero time, p = phage not

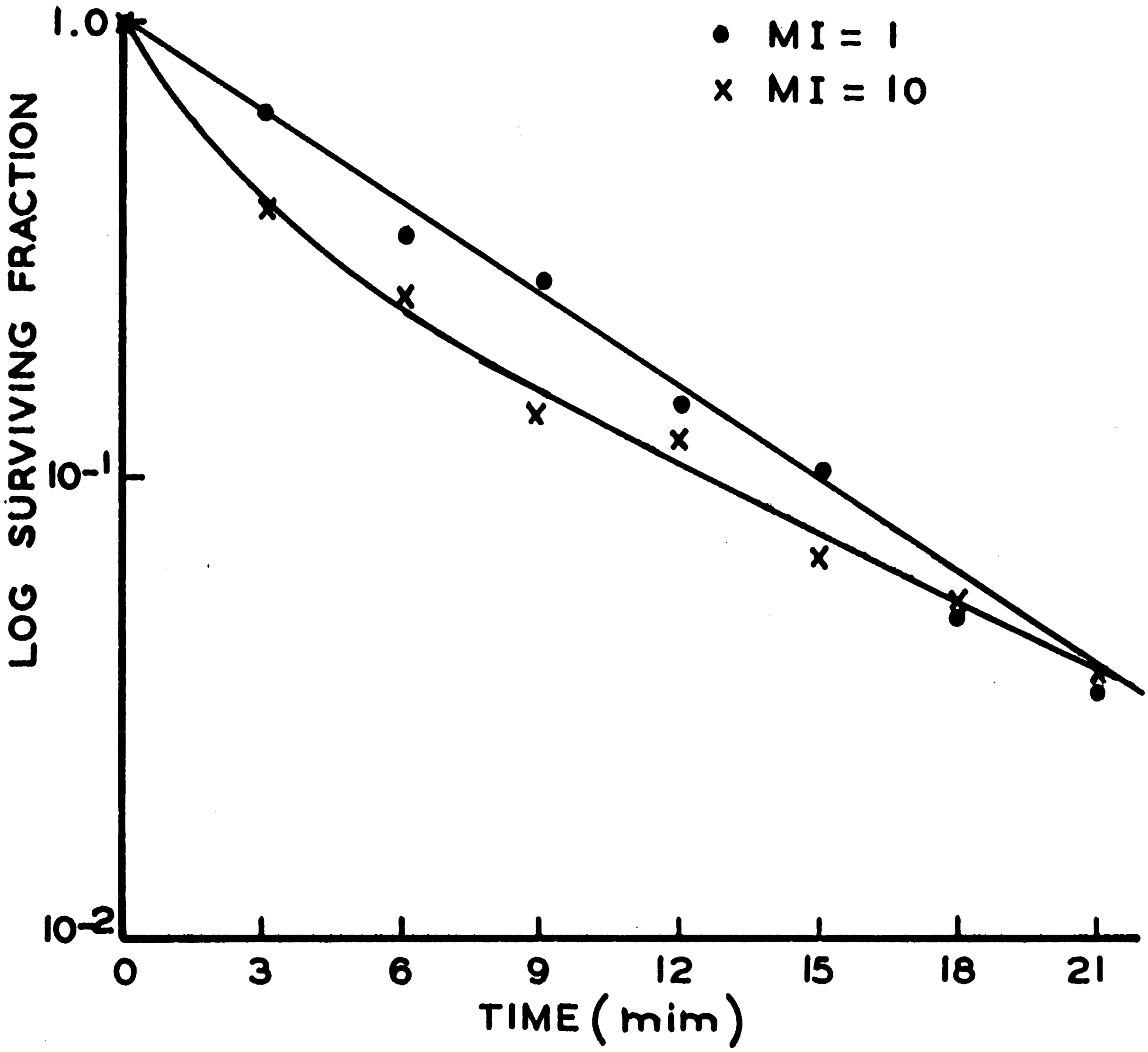

FIG. 2. ADSORPTION OF P38 TO STRAIN 53

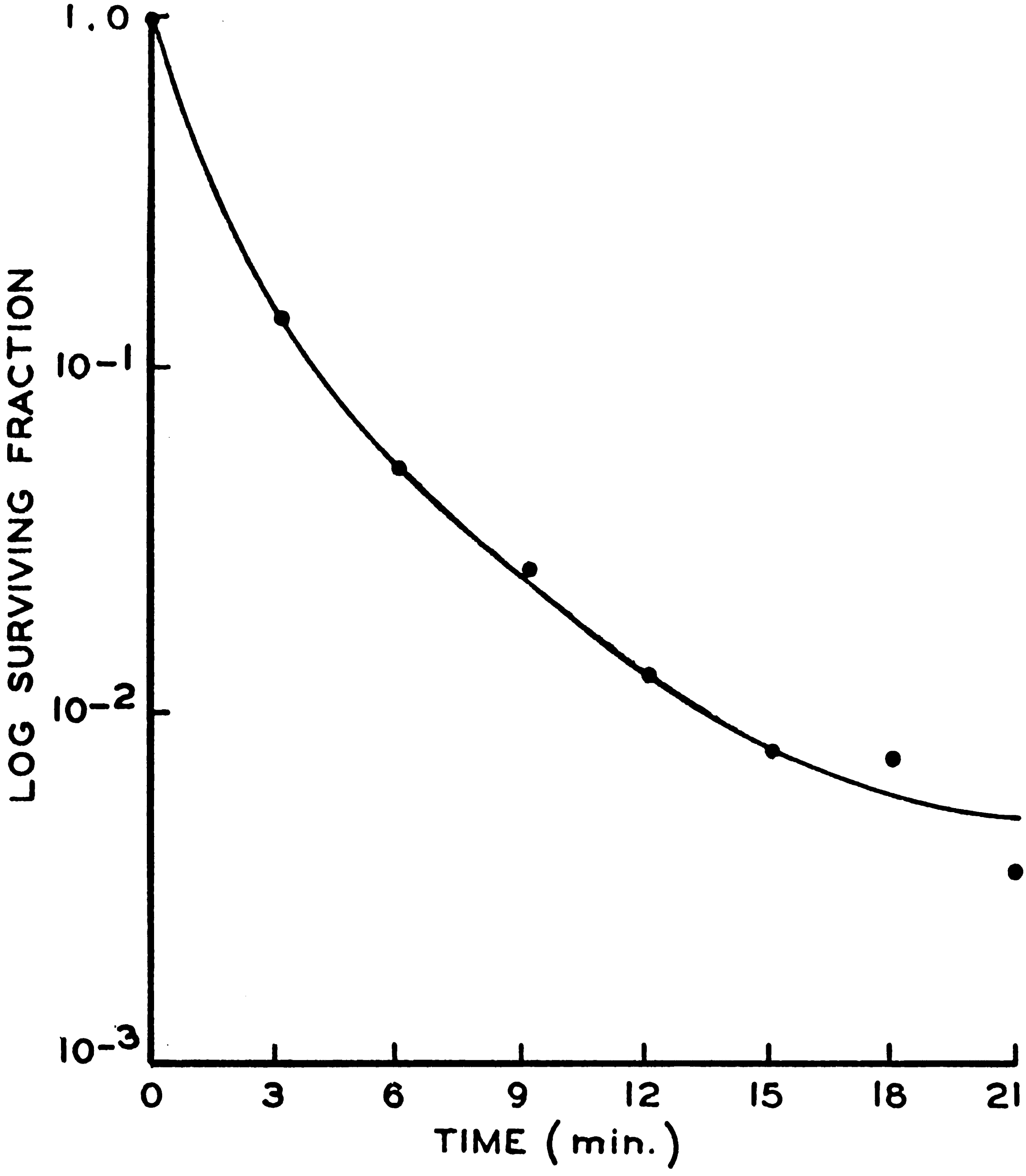

FIG. 3. NEUTRALIZATION OF P38 WITH A 1:1000 DILUTION OF ANTI-P38 SERUM.

inactivated at time t min, D = final dilution of serum in the phage serum mixture, and K = velocity constant. Figure 3 indicated first order kinetics were followed over the limited range 93-99.3% inactivation. A velocity constant $K = 217.2 \text{ min}^{-1}$ was determined for this region. Adams (1959) stated the equation describes the course of the reaction only over a limited range of inactivation, usually between 90-99%. Figure 3 indicated a serum diluted 1:1000 should inactivate 99% within 13 min. Hence, a 1:100 and a 1:250 serum dilution was considered sufficient to validate the test for lysogeny.

Detection and Specificity of Gal$^+$ Clones

Early attempts to transduce the gal character met with little success. One of the problems was a suitable medium to detect the transductants. It was known that transduction occurred in a liquid medium, therefore, the solid medium being used probably was not satisfactory.

Reconstruction experiments were performed by mixing approximately 10^3 gal$^+$ cells with 2×10^8 53 gal$^-_1$ or 53 gal$^-_2$ cells and plating 0.1 ml on EMBGal agar. Upon incubation, papillae developed on the white gal$^-$ bacterial lawn but failed to give the characteristic color of fermenting clones. Mutant 53 gal$^-_1$ is leaky and results were not decisive after 48 hours. When 53 gal$^-_2$ cells and gal$^+$ cells of strain 53 (W53) were streaked next to each other on EMBGal agar, it was observed that a substance diffused from the gal$^-$ cells. This material obscured the characteristic color of fermenting clones. Neutral red agar (Morse and Alaire, 1958), another acid indicating medium, was tried but abandoned.

A medium (LGal agar) employing TTC, an oxidation-reduction indicator, as an agar overlay was successful. One hundred and sixteen dark red papillae were observed on a lawn of 53 gal$^-_2$ cells as compared to an expected 108. Thirty papillae were picked of which 1 was gal$^-$, 20 were gal$^+$, and 9 failed to grow. A 48 hour incubation period was determined to be insufficient as papillae were too small to be picked with consistency. It was noted that isolated 53 gal$^-_2$ clones gave a pink-light red color when overlaid with TTC agar, making it difficult to distinguish between fermenting and nonfermenting clones. However, a lawn of gal$^-$ cells remained white.

Donor phage UV-induced P38 were mixed with 53 gal$^-_2$ recipient cells and plated on LGal agar with and without galactose. This was done to decide whether galactose was an essential ingredient for papillae development. No papillae developed on the medium without galactose while an average of 21 papillae per plate was observed on the galactose containing medium. Recipient cells were plated on the same medium which allowed a transduction frequency of 1.11×10^{-6} to be calculated.

Further evidence that the red papillae are gal$^+$ clones was obtained by picking the papillae from 53 gal$^-_2$ transduction plates (Table 2).

TABLE 2.--Specificity of red papillae

Lysate of	Total Number Picked	Gal+	Gal-
UV-induced P38*	48	48	0
UV-induced P38*	27	23	4
P38/W53*	16	15	1
P38/W53*	12	12	0
P38/W53*	42	41	0

*Different experiments.

Transduction

The preliminary evidence for transduction reported in Table 2 was further substantiated by more complete experiments (Table 3).

Transduction frequencies of the homologous systems (recipient cells infected with phage propagated on the recipient) were considered to be insignificant. The average difference between the phenotypic reversions and transductants varied from -1.0 to 0.93. Results of the homologous infection experiments eliminate the phenomenon of phage conversion.

The transformation of gal^- to gal^+ by P38 is illustrated in Figures 4, 6, and 8. Control plates of mutants without phage are shown in Figures 5 and 7. Each dark red papilla is a clone of glactose fermenting cells.

TABLE 3.--Transduction of the **gal**$^+$ character to **gal**$^-$ recipients

Lysate	Recipient Culture	MI	Average Number of Gal Papillae/Plate: Control (no lysate)*	Average Number of Gal Papillae/Plate: Transductants	Transduction Frequency	% Phage Adsorption
UVIP38	53 gal$^-_2$	0.984	0.80	20.6	1.11×10^{-6}	
		0.984	0.80	21.8	1.23×10^{-6}	
		0.400	0.75	14.2	7.47×10^{-6}	92.4
		0.400	1.20	7.2	8.34×10^{-6}	92.7
UVIP38/53 gal$^-_2$	53 gal$^-_2$	10.00	0.75	0.6	0.00	94.0
P38/W53	53 gal$^-_2$	0.995	2.4	8.0	3.41×10^{-7}	
		0.99	1.2	19.4	1.23×10^{-6}	
		1.00	0.75	20.0	4.01×10^{-7}	93.8
		1.00	1.20	42.0	4.08×10^{-7}	97.5
P38W53/53† gal$^-_2$	53 gal$^-_2$	1.00	0.50	0.80	1.35×10^{-9}	99.5
		1.00	1.20	1.40	2.00×10^{-9}	98.6
		1.00	1.40	0.40	0.00	
		10.00	2.25	1.00	0.00	
		10.00	0.40	2.33	1.05×10^{-9}	

TABLE 3--<u>Continued</u>

Lysate	Recipient Culture	MI	Average Number of Gal+ Papillae/Plate: Control (no lysate)*	Average Number of Gal+ Papillae/Plate: Transductants	Transduction Frequency	% Phage Adsorption
P38/W53	35 $gal^{-}6$	1.00	2.00	39.40	$3.74x10^{-7}$	
P38/W35	35 $gal^{-}6$	1.00	2.00	13.80	$1.18x10^{-7}$	
P38W35/35 $gal^{-}6$		1.00	2.20	2.00	0.00	
P38/W53	35 $gal^{-}7$	1.00	1.20	39.40	$3.82x10^{-7}$	
P38/W35	35 $gal^{-}7$	1.00	1.20	8.40	$7.20x10^{-8}$	
P38W35/35 $gal^{-}7$		1.00	2.20	1.00	0.00	
P38/W53	35 $gal^{-}8$	1.00	0.80	42.75	$4.2x10^{-7}$	
P38/W35	35 $gal^{-}8$	1.00	0.80	15.50	$1.47x10^{-7}$	
P38W35/35 $gal^{-}8$		1.00	1.40	2.00	$6.00x10^{-8}$	
P38/W35 DNase**	35 $gal^{-}6$	1.00	2.40	62.2	$5.98x10^{-7}$	
P38/53S^r DNase**	53 $gal^{-}2$	1.00	4.00	64.0	$6.00x10^{-7}$	

*The gal^{+} papillae on the control plates are spontaneous reversion.

†P38W53/53 $gal^{-}2$ = P38/W53 propagated on 53 $gal^{-}2$.

**The P38/W35 and P38/53S^r lysates were treated with DNase.

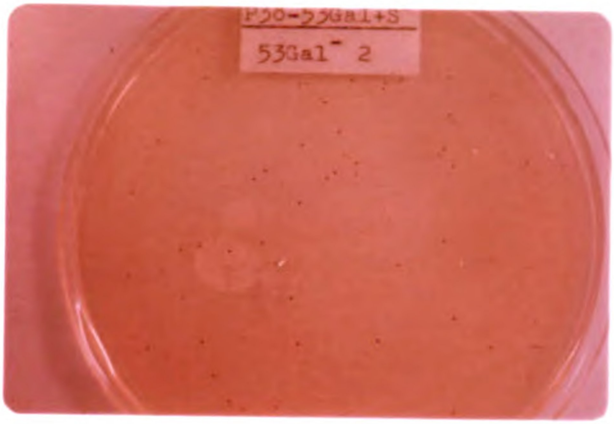

Figure 4. Transductants

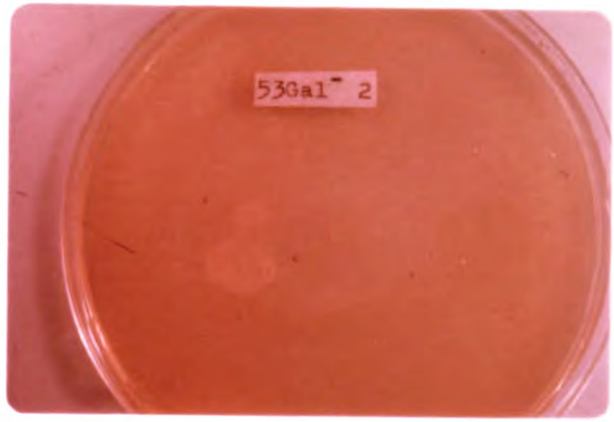

Figure 5. Control

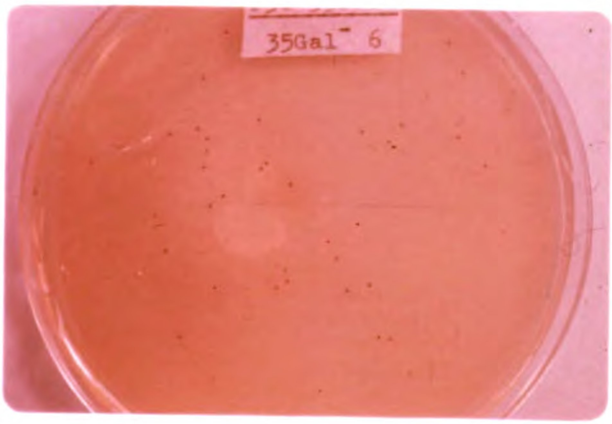

Figure 6. Transductants

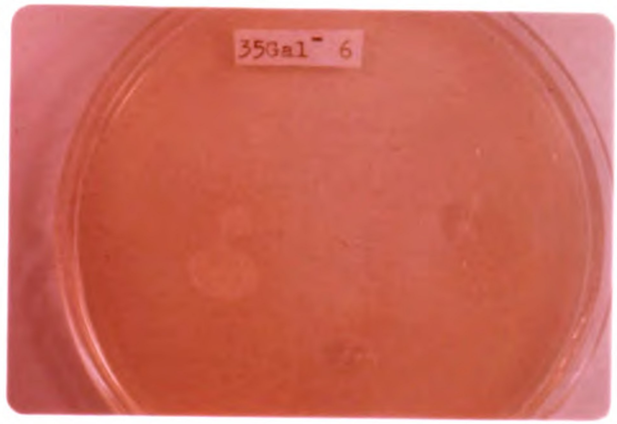

Figure 7. Control

Figure 8. Transductant papillae

The carbohydrate fermentation pattern (Appendix, Table 6) was determined for strain 35, strain 53 and their derived mutants. It was noted that 53 gal^-_2 differed from W53 in its ability to ferment rhamnose and galactose. One transductant derived from 53 gal^-_2 infected with P38/W53 was also tested. The transductant had regained its ability to ferment galactose but not rhamnose.

During the course of this research, a spontaneous rough form of 53 gal^-_2 (53 gal^-_{2R}) was isolated. Experiments were performed to determine its susceptibility to transduction (Table 4).

TABLE 4.--Infection of 53 gal^-_{2R} with transducing lysates

Lysate	Recipient Culture	MI	Average Number of Gal$^+$ Papillae/Plate: Control (no lysate)	Average Number of Gal$^+$ Papillae/Plate: Transductants	Transduction Frequency	% Phage Adsorption
UVIP38	53 gal^-_{2R}	0.4	1.0	2.0	$1.18x10^{-6}$	57.5
		0.4	0.8	1.4	$3.64x10^{-7}$	87.9
P38/W53	53 gal^-_{2R}	1.0	1.5	1.0	0.00	26.0
		1.0	1.4	2.2	$1.14x10^{-8}$	0.0

The transduction frequencies reported in Table 4 were not considered to be significant. There was an average difference of 1 gal^+ clone between the transduction and control plates. Concurrent transduction experiments employed the same lysates with 53 gal$^-_2$ as the recipients. These results, including the per cent phage adsorption, were recorded in Table 3. The inability of 53 gal$^-_{2R}$ to be transduced was believed to be caused by insufficient phage adsorption.

Transductions were performed with DNAse treated lysates to determine whether a transforming principle was operative. The results recorded in Table 3 indicated transformation did not occur. In fact, DNAse treatment of lysates seemed to enhance the transduction frequencies.

Ten P38/W53 to 53 gal$^-_2$ and 15 UV-induced P38 to 53 gal$^-_2$ transductants were tested for lysogeny and segregation of gal$^-$ clones. All were found to be stable gal$^+$ and lysogenic.

Transduction frequencies were determined as a function of the multiplicity of input (Table 5). Although the most efficient MI appeared to be 0.1, there was a difference of approximately 3 gal$^+$ clones between the control and transduction plates. A ratio of 1 phage per bacterium was chosen for most transduction procedures as it was felt the results would be more significant.

Galactose negative mutants 35 gal$^-_1$ and 53 gal$^-_3$ were not amenable to transduction. Mutant 35 gal$^-_1$ adsorbed and propagated phage but could not be transduced. A lawn of 53 gal$^-_3$ cells turned red when overlaid with TTC agar. Consequently, gal$^+$ transductants were not distinguishable from the background of mutants.

TABLE 5.--Efficiency of transduction as a function of MI

Lysate	Recipient Culture	Average Number of Gal^+ Papillae/Plate		Transductants	Transduction Frequency
		MI	Control		
P38/W53	53 gal^-_2	45.98	1.75	94.2	1.24×10^{-7}
		10.45	1.75	10.2	7.91×10^{-8}
		1.045	1.75	9.4	4.06×10^{-7}
		0.1045	1.75	4.6	1.56×10^{-6}
		0.0145	1.75	1.2	0.00
UV-induced P38	53 gal^-_2	1.04	2.6	7.6	3.80×10^{-6}
		0.52	1.4	17.2	3.04×10^{-6}
		0.052	1.4	4.2	5.40×10^{-6}

Mutant 53 gal^-_1 formed papillae but when overlaid with TTC agar, the papillae, appeared as star shaped craters. Sixteen craters were picked and streaked on EMBGal agar. No gal^+ colonies were isolated.

DISCUSSION

Production of Mutants

Transduction studies have as their first prerequisite, an adequate supply of mutants. Hence, much time was allotted to this problem.

Haas and Doudney (1957a,b) found in *E. coli* that pre-irradiation incubation in a minimal medium supplemented with yeast extract or ribonucleic acid precursors increased mutation frequencies. Witkin (1956) observed post-irradiation incubation in a medium containing an adequate supply of amino acids increased the frequency of UV-induced prototrophs with *E. coli* B/r and *Salmonella typhimurium* LT2. Therefore, *S. gallinarum-pullorum* was incubated in a medium containing casein hydrolysate as a source of amino acids and yeast extract.

Mutation induction is at a maximum when a synchronously dividing culture is irradiated 10-15 min before cellular division (Haas and Doudney, 1957b). The procedure of Haas and Doudney (1957b) was used with strain 53. However, no gal^- mutants were obtained. Employing a method of Abbo and Pardee (1960), 70% synchrony was obtained with strain 53. No mutants were isolated. Consequently, further use of synchronous cultures was abandoned.

A range of UV dosage was tried sufficient to induce mutation in *S. typhimurium* (Witkin, 1956) and *E. coli* (Haas and Doudney, 1957b). Induction with nitrous acid resulted in the isolation of 5 gal$^-$ mutants from strain 35 but none from strain 53 (J. M. Robinson, personal communication). Although the nitrous acid system was not thoroughly investigated, the results combined with data obtained from UV experiments, suggested strain 53 was refractory to mutation at the gal loci.

Mutants of *E. coli* have been described as being genetically blocked and unable to use galactose as a carbon source (Lederberg and Lederberg, 1953). Biochemical studies have shown these mutants to be deficient in the following enzymes of the Leloire pathway (Kurahaski, 1957; Kalckar *et al.*, 1959): (1) mutants which will grow in the presence of but will not metabolize galoctose lack galactokinase, (2) sensitive mutants are blocked in galactose-l-phosphate uridyl transferase or uridine diphosphogalactose-4-epimerase. Two galactose sensitive mutants have been isolated from strain 35 which may be of the type described for *E. coli*. Six gal$^-$ mutants of strain 35 and 3 gal$^-$ mutants of strain 53 may lack galactokinase.

Adsorption of Phage P38

Adams (1959) concluded that adsorption-rate constants in the range 10^{-8}-10^{-9} per min could be expected under optimal conditions. Thus, LC broth provided excellent conditions for adsorption of P38 to strain 53.

Since transduction experiments employed different conditions (e.g., T_2 buffer, strain 35, strain 35 gal$^-$ mutants and strain 53 gal$^-$ mutants) the per cent adsorption was determined after the 25 min incubation period. Adsorption was found to be in the 90-99% range.

Detection and Specificity of Gal$^+$ Clones

A sufficient number of mutants were obtained to allow partial characterization of the transducing system. However, a method was required to detect gal$^+$ clones among a majority of gal$^-$ cells.

Classical media used to indicate fermentation are dependent upon acid production with consequent change in color of a pH indicator. It was apparent from the reconstruction experiments that such media (EMBGal and Neutral Red) were not acceptable.

Application of a technique developed to detect respiration-deficient mutants in yeast (Ogur et al., 1957; Nagai et al., 1961) was successful. Mudd et al. (1951) have used TTC as an indicator of sites of dehydrogenases in bacteria and also gave reference to a concurrent oxidation-reduction interaction between diphosphropyridine nucleotide and flavoprotein, causing reduction of the colorless TTC to red formazan. Nagai et al. (1961) stated the exact nature of the enzymatic locus affecting the reduction of TTC into formazan is not yet identified, although succinic dehydrogenase, which catalyzes the succinate-to-fumarate conversion at a redox potential near that of TTC, and which is absent in the respiration deficient mutant, is assumed to be responsible.

The difference between TTC overlaid gal^- and gal^+ clones grown in the presence of galactose as a carbon source is probably a differential in the amount of dehydrogenases present.

The following information indicated the observed red papillae were gal^+ clones: (1) reconstruction experiments showed excellent agreement between the expected number of gal^+ papillae and the observed number of red clones, (2) twenty of the 30 subcultured red clones from the reconstruction experiment were gal^+, 1 was gal^- and 9 clones failed to grow, suggesting the one gal^- clone isolated could be attributed to a failure to pick the complete clone, (3) one hundred and thirty-four of the 139 subcultured red clones assumed to be transductants were gal^+ and 5 were gal^-, and (4) galactose was required for the development of the red papillae.

Transduction

Transformation, conjugation, and phage mediated transfer were three general phenomena of genetic exchange in bacteria which had to be considered.

Transformation (Avery _et al._, 1944) involves a transforming principle which is inactivated by DNAse. Experiments with DNAse treated phage preparations precluded the occurrence of transformation.

Conjugation (Lederberg and Tatum, 1946), which requires physical contact between two mating types, was considered negligible as bacteria-free phage lysates were used and the gal^+ clones on the control plates with no lysates could be attributed to phenotypic reversion.

Active plaque forming phage lysates were found to be essential for transfer of genetic information. The number of gal^+ clones which appeared on the LGal agar plates seemed to be dependent upon whether the mutant culture had been mixed with phage lysate. Mutant 53 gal^-_2 adsorbed P38/W53 (greater than 93%), and the gal^+ character was transferred. Mutant 53 gal^-_{2R}, spontaneously derived from 53 gal^-_2, adsorbed P38/W53 poorly (less than 27%), and the gal^+ character was not transferred suggesting phage were the vectors of genetic exchange.

Therefore, the genetic system was assumed to be a type of phage mediated exchange. Phage conversion, specialized and generalized transduction were considered as possibilities.

Phage conversion (Freeman and Morse, 1952; Groman, 1953) has been shown to be a property of the phage irrespective of the bacterial genome. Phage conversion did not occur in the P38 system since infection of gal^- mutants with homologous phage lysates resulted in none to an occasional gal^+ clone.

Specialized transduction (Morse _et_ _al_., 1956a,b; Luria _et_ _al_., 1960) is characterized by high frequencies (greater than 1×10^{-4}) and unstable heterogenotes. UV-induced P38 infected 53 gal^{-}_{2} cells transduced at a frequency of $1\text{-}8 \times 10^{-6}$ and were found to be stable. Strain 38 carries phage P38, but under the conditions employed, specialized transducing lysates were not produced. The P38/W53-53 gal^{-}_{2} system transduced at a frequency of 1×10^{-6} - 4×10^{-7}. Ten transductants from this system were stable and lysogenic. A specialized transducing system may be present in the P38/W53-53 gal^{-}_{2} system, but the gal^{+} transductants would have to be irradiated and the resultant lysates used for transduction. This was not done. Lysogeny of the 25 transductants tested suggested that transfer of the gal marker is intimately associated with P38.

The transduction frequency for the galactose marker was comparable to that found for general transduction in _E_. _coli_, 1×10^{-6} (Morse _et_ _al_., 1956a) and _S_. _typhimurium_, 1×10^{-6} (Hartman, 1957). Lysis, evident on the transduction plates, may have lowered the transduction frequency somewhat from a more optimal rate.

Intrastrain transduction with both strain 35 and 53 was demonstrated. Interstrain transduction from strain 53 to strain 35 suggested a close genetic homology between the two.

Comparison of the P38/W53 and UV-induced P38 transducing systems indicated little, if any, difference.

Behavior of the transductants was identical although they occurred at a higher frequency. It was felt, due to the low phage titer obtained, that further work with UV-induced lysates was not warranted.

Mutant 35 gal^{-}_{1} was not transducible, and other similar situations have been reported (Hartman and Goodgal, 1959).

SUMMARY

Spontaneous, nitrous acid-, and UV-induced nonfermenting galactose mutants were obtained on two strains (35 and 53) of S. gallinarum-pullorum.

A method for detecting respiration-deficient mutants of yeast was successfully adapted to distinguish gal^+ clones on a background lawn of gal^- cells. Reconstruction experiments demonstrated almost quantitative recovery of the introduced gal^+ cells.

The gal^- mutants, suspended in T_2 buffer, were observed to adsorb 90-99% of the P38 phage particles within 25 min. Wild type 53, using LC broth as a suspending medium, adsorbed 90% of the phage within 18 minutes.

Inter- and intrastrain generalized transduction was demonstrated using P38 as a vector. At a MI of 1 the gal^+ character was transduced to 53 $gal^-{}_2$ at a frequency of 1-8 x 10^{-6} by UV-induced P38 or at a frequency of 1 x 10^{-6} to 4 x 10^{-7} by P38 propagated on W53. A spontaneous rough form of 53 $gal^-{}_2$ was found not to be transducible and to adsorb considerably less phage than 53 $gal^-{}_2$. The gal^+ character was transferred to mutants 35 $gal^-{}_6$, 35 $gal^-{}_7$, and 35 $gal^-{}_8$ but not to 35 $gal^-{}_1$ by P38/35 phage particles. Interstrain transduction was accomplished by infecting 35

gal^-_6, 35 gal^-_7, and 35 gal^-_8 with lysates of P38/53. Mutant 35 gal^-_1 was also refractory to interstrain transduction. Twenty-five gal^+ transductants were tested and found to be actively lysogenic. The efficiency of transduction and type of transductants produced appear to be independent of method of preparing phage lysates.

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APPENDIX

TABLE 6.--Typical reactions of _Salmonella gallinarum-pullorum_ strains on differential tube media

Strains	Carbohydrates*										SIM medium†			Urea Broth†	Simmons-citrate agar†
	Xylose	Dextrose	Maltose	Lactose	Rhamnose	Mannitol	Dulcitol	Galactose	Salicin	Sucrose	H_2S	Motility	Indole		
W35	YG	YG	NC	NC	YG	YG	NC	YG	NC	NC	+	-	-	-	-
35 gal^-_1	YG	YG	NC	NC	YG	YG	NC	NC	NC	NC	+	-	-	-	-
35 gal^-_6	YG	YG	NC	NC	YG	YG	NC	NC	NC	NC	+	-	-	-	-
35 gal^-_7	YG	YG	NC	NC	YG	YG	NC	NC	NC	NC	+	-	-	-	-
35 gal^-_8	YG	YG	NC	NC	YG	YG	NC	NC	NC	NC	+	-	-	-	-
W53	YG	YG	NC	NC	YG	YG	NC	YG	NC	NC	+	-	-		-
53 gal^-_1	NC	YG	NC	NC	NC	NC	NC	NC	NC	NC	+	-	-	-	-
53 gal^-_2	YG	YG	NC	NC	YG	YG	NC	NC	NC	NC	+	-	-	-	-
53 gal^-_3	NC	YG	NC	NC	YG	YG	NC	NC	NC	NC	+	-	-	-	-
[53 gal^-_2‡ P38/W53+]	YG	YG	NC	NC	NC	YG	NC	YG	NC	NC	+	-	-	-	-

NC = No change or alkaline reaction. YG = Acid and gas formation. - = Negative.
+ = Positive.
* = Carbohydrates were in Difco Purple Broth Base. † = Difco medium.
‡ = 53 gal^-_2/P38W53$^+$ is a transductant from P38/W53 to 53 gal^-_2.