



Coupled mutations involving diverse characters in Salmonella Blegdam
by Leonard Raymond Bullas

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY in Genetics

Montana State University

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Abstract:

Three independent derivatives of Salmonella blegdam, strain SB2, have exhibited the capacity to produce a temperate bacteriophage which lyses SB2. On seven occasions, lysogenic cells were isolated directly from these derivatives. The event leading to lysogeny or phage production occurs at mutational frequency. It was concluded, therefore, that SB2 carries at least most of the hereditary specifications of a temperate bacteriophage to which it is sensitive. Further mutational changes in phage related properties observed in SB2 derivatives included changes from sensitivity to immunity, changes from lysogeny back to sensitivity and changes from one level of lysogenic productivity and ultra-violet inducibility to another.

Most of the changes in phage related properties were accompanied by mutational changes affecting other unrelated bacterial properties which included nutritional requirements, motility, flagellar and somatic antigens, fermentation, colony morphology and antibiotic sensitivity. The prevalence of these coupled mutations argues that the changes in phage related properties are position effects rather than "point mutations".

Coupling between two or more mutations affecting non-phage related properties also occurs. Sometimes coupling is maintained for both forward and reverse mutation. This type of coupling usually involves a reciprocity, while one mutation restores a function, the coupled mutation abolishes another. In other situations, the coupling is lost once the mutations have occurred. Events occur which increase the probability of specific mutations. Some coupled mutations are "abortive" for they lead to functional genes which do not replicate. The cell has a "memory" for previous mutation so that reciprocal mutational changes occur between loci which were previously mutated.

Different auxotrophs showed different levels of UV inducibility. Examination of the degree of UV inducibility of particular auxotrophs led to the hypothesis that the site on the chromosome of a particular mutation was correlated with the location of prophage. Thus prophage is visualized as a movable element which can locate at many sites on the bacterial chromosome but is particularly 'attracted' to sites of mutation.

The hypothesis was advanced that the coupled mutations were controlled by an episomal element to which the symbol S was applied. This element can change position on the chromosome and suppress the activity and alter the mutability of adjacent genes. Prophage and S may occasionally become linked thus coupling bacterial mutations with phage property mutations.

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SALMONELLA BLEGDAM

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ABSTRACT

Three independent derivatives of Salmonella blegdam, strain SB2, have exhibited the capacity to produce a temperate bacteriophage which lyses SB2. On seven occasions, lysogenic cells were isolated directly from these derivatives. The event leading to lysogeny or phage production occurs at mutational frequency. It was concluded, therefore, that SB2 carries at least most of the hereditary specifications of a temperate bacteriophage to which it is sensitive. Further mutational changes in phage related properties observed in SB2 derivatives included changes from sensitivity to immunity, changes from lysogeny back to sensitivity and changes from one level of lysogenic productivity and ultra-violet inducibility to another.

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Different auxotrophs showed different levels of UV inducibility. Examination of the degree of UV inducibility of particular auxotrophs led to the hypothesis that the site on the chromosome of a particular mutation was correlated with the location of prophage. Thus prophage is visualized as a movable element which can locate at many sites on the bacterial chromosome but is particularly 'attracted' to sites of mutation.

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GENERAL INTRODUCTION

The phylogenetic relationship between viruses and the genes of their hosts is of considerable theoretical and practical interest. Various bacteriophage-bacterium systems have proven extremely useful in approaching the problem. Within these systems a diversity of situations has been described, and these situations can be assigned (with varying degrees of certainty) to stages in the phylogeny of virulent phage from the normal bacterial genes (or vice versa). These include such phenomena as "weakly virulent" phage, virulent phage capable of lysogenization with the help of related temperate phage, classic inducible temperate phage, phage occupying specific sites on the bacterial linkage map, non-inducible temperate phage, defective temperate phage, host induced modifications, etc.

Until recently, however, no system has been available in which the entire sequence from sensitive to lysogenic bacterium could be studied (without the intervention of extra-clonal phage). This appears now to be provided by the Escherichia coli B - P2 system. The work of Bertani (1957) and Cohen (1959) had led to the suspicion that E. coli B carried genes capable of functioning in the temperate phage P2, and Rutberg and Hédin (1960) succeeded in provoking P2 release by pressurized sphaeroplasts of this strain. The preliminary report does not characterize the released phage in detail, but its identification as P2 implies a capacity to lysogenize E. coli B. Surprisingly, the transition from P2-sensitive to P2-releaser (and thence to P2-lysogenic by feasible population

interactions) is abrupt, and does not involve a long series of sequential events of mutational frequency.

A second system involving the entire sensitive-to-lysogenic transition has been discovered in Salmonella blegdam and is the subject of this report. Important differences from the E. coli B - P2 system have been revealed, but the general interpretation invokes, as does that of Rutberg and Hédin, profound spatial rearrangements within the cell as the basic cause of the transition.

In the course of the investigation, several other intimately related but independently significant phenomena were uncovered and are considered here. These include "shift mutations" from one auxotrophic state to another; reversible shifts from a monophasic (H antigen) condition to a diphasic condition involving a total of three antigens (two previously undescribed for S. blegdam); synchronized mutations of two or more such diverse genes as those determining immunity, phage production, auxotrophy, flagellar antigens, somatic antigens, sugar fermentation, colony morphology, flagellar production, flagellar function and antibiotic sensitivity; mutational events resulting in altered frequency of mutation; and mutant genes which show linear inheritance.

MATERIALS AND METHODS

A. Bacterial and Bacteriophage Strains

All bacteria employed in the present work were derived from two Salmonella blegdam strains originally studied at the University of Adelaide, Australia (Atkinson and Bullas, 1957). The following details about the origin of these strains are pertinent.

Through 1952 - 1957 a survey was conducted at the University of Adelaide to determine the incidence of lysogeny amongst different Salmonella groups. Many independent isolations from nature were studied, most of them from children and adults suffering from gastroenteritis. Among the strains studied were about fifty which were classified as Salmonella blegdam. That is, they possessed the somatic antigens 9 and 12 (thus belonging to Group D in the Kauffmann-White Schema) and were monophasic for the flagellar antigenic complex gmq.

The method used to determine lysogenicity was an adaptation of the cross-plating method developed by Fisk (1948) in which each culture is treated as both a potential lysogenic strain and as an indicator. By this method, only one S. blegdam strain (#43) could be shown to be lysogenic. With reference to the phage produced by #43, some of the remaining strains were resistant, some sensitive. One of the latter (#49) was selected as the standard propagating strain for future work. Since collections of most other Salmonella are predominately lysogenic (see Burnet 1932; Boyd 1950; Atkinson et al 1952), the demonstration of only one lysogenic S. blegdam was remarkable. The detection of lysogeny

is, of course, a function of the array of indicator strains available. Accordingly, it was suspected that the resistance of some of the S. blegdam strains to the phage produced by #43 might be due to cross-immunity conferred by an undetected prophage. In fact the unusual behaviour of #49 in serial broth culture suggested that it too might harbor a prophage or prophage-like element in a hitherto undescribed state, for such cultures rapidly and massively become resistant to the phage produced by #43. This suspicion is supported by the experiments reported here.

Strains #43 and #49 were selected for further study and here have been renamed SB1 and SB2 respectively. All other strains dealt with are derivatives of one of these two (namely SB2) and have been given designations such as SB3, SB4, SB5 etc. representing chronological order of isolation.

The phage produced by SB1 was originally called B43h/49 but has been renamed PSB1. PSB1 forms large turbid plaques on SB2 and is more completely described under Results. The phages produced by other bacterial strains are similarly named by inserting P before the name of the bacterial strain.

B. Culture Media, Diluents and Supplements

Except where indicated, all final concentrations given below are in grams per liter of distilled water. All culture media were sterilized in the autoclave at 15 lbs. pressure for 20 minutes.

Nutrient Agar (NA): Difco nutrient broth 8, NaCl 5, agar 15.

L-broth (LB): Bactotryptone 10, yeast extract 5, NaCl 5, glucose 1. The pH was adjusted to 7.2 with 1 N NaOH before sterilization. (Lennox, 1955)

L-agar (LA): L-broth ingredients 21, agar 10.

Top Layer Agar (TL): Difco nutrient broth 8, NaCl 5, agar 6.5.

Motility Medium (MM): Bacto Casitone 10, yeast extract 3, agar 4, Difco gelatin 80, NaCl 5. The pH was adjusted to 7.2 with 1 N NaOH after sterilization. (Edwards, 1942)

Eosin Methylene Blue Medium (EMB): Casein digest 10, yeast extract 1, NaCl 5, K_2HPO_4 2, Eosin Y 0.4, Methylene Blue 0.065, agar 15, sugar 10. (Lederberg, 1950)

Davis Minimal Liquid Medium (DMB): Glucose 1, K_2HPO_4 7, KH_2PO_4 2, crystalline sodium citrate 0.5, crystalline $MgSO_4$ 0.1, crystalline $(NH_4)_2SO_4$ 1; the sugar and the other salts were sterilized separately and added together only after sterilization. (Lederberg, 1950)

Nitrogen free DMB: Ingredients as for DMB with the omission of the $(NH_4)_2SO_4$.

Davis Minimal Agar (DMA): DMB ingredients 11.6, agar 15; the glucose and agar were added to a portion of the water, the other salts added to the remaining portion, each mixture sterilized separately and then added together.

Saline: NaCl 8.6.

Antibiotics: The following were added to media where appropriate to give the indicated final concentrations: Penicillin G, crystalline potassium (Eli Lilly and Co.) 300 µg/ml; Dihydro-streptomycin sulphate (Nutritional Biochemical Corp.) 250 µg/ml; Chloramphenicol (Pfizer Co.) 30 µg/ml.

Growth Factors: The following were added to minimal media where appropriate to give the indicated final concentrations, according to Lederberg (1950): Arginine .02 gm/ml, cytosine .02, glutamic acid .02, lysine .02, methionine .02, nicotinic acid .001, para-amino-benzoic acid .001, phenylalanine .02, tyrosine .02, tryptophan .02.

C. General Methods

1. Storage of Bacteria and Phage

Bacteria were stored on NA slants at room temperature, with routine subculture only at 12 monthly intervals. Strains in more general use were subcultured more frequently whenever it was deemed necessary.

Bacteriophage lysates were stored in the refrigerator at 4°C, after sterilization at 65°C - 70°C in a water bath, for 30 minutes.

2. Working Cultures

For most purposes, overnight (approx. 20 hrs.) L-broth cultures were employed. For certain purposes log phase cultures were obtained by inoculating 20 ml of L-broth in a Petri dish with 0.1 ml of an overnight culture and placing on a rotary shaker at 37°C for two hours.

3. Irradiation Procedures

Ultra-violet irradiation and induction experiments were carried out with a 2 bar ultra-violet lamp, model 2324, manufactured by the Dazor Manufacturing Corporation, St. Louis, Missouri, U.S.A. The lamp was placed at a distance of 50 cm from the center of a Petri dish containing 5 ml of the culture to be irradiated, on a rotating platform which rotated at a speed of approximately one revolution per second. Times of irradiation varied with the experiment but all irradiations were carried out with the same arrangement of lamp and culture.

4. Isolation of Mutants

The specific procedures used for the isolation of many mutants are detailed under the Results. The general methods employed for the isolation of auxotrophs were those of Lederberg (1950) and are given below.

MnCl₂ - Penicillin Method

SB2 was grown in L-broth at 37°C for 24 hours. The culture was then centrifuged and the sediment resuspended in 2 ml of 0.3 M NaCl and placed in a water bath at 37°C for one hour. The culture was again centrifuged, washed once in distilled water and resuspended in 2 ml of 0.04 M MnCl₂ solution for two hours at 37°C. After another centrifugation 1 loopful of the deposited cells, emulsified in the small amount of liquid that remained in the tube after decantation, was inoculated into 5 ml of L-broth and incubated overnight at 37°C.

The overnight culture was washed twice in sterile, distilled water and resuspended in 5 ml of nitrogen free DMB and incubated at 37°C for six hours. The culture was again centrifuged and resuspended in 5 ml of DMB with the addition of 300 units of penicillin-G. After overnight incubation, the culture was diluted out in saline and 0.1 ml of appropriate dilutions (10^{-4} , 10^{-5} , 10^{-6}) were plated out on NA to obtain isolated colonies. Plates showing suitable colony density were replicated to DMA (Lederberg and Lederberg, 1951) and incubated for 24 hours. The replicated DMA plates were then compared with the original NA plates and those colonies which appeared on the NA plates but not on the DMA plates were picked off and streaked on NA. A single colony was rechecked to test its auxotrophy, and its growth requirement identified.

Ultra-Violet Irradiation

NA plates spread with 0.1 ml of an undiluted overnight L-broth culture of SB2 were irradiated with UV for about 30 seconds and incubated overnight. The surviving colonies were then replicated to DMA and auxotrophic colonies identified in the same manner as used in the $MnCl_2$ - penicillin method.

5. Identification of Growth Factor Requirements

The method followed closely that described by Lederberg (1950). DMA plates were spread with 0.1 ml of appropriate growth factors and 1 loopful of the overnight L-broth culture, washed twice in saline, was streaked on the dried plate. The nature of the growth requirement was first identified by testing for growth with yeast extract and vitamin, amino acid and purine-pyrimidine pools. The individual components of the appropriate

pool which elicited growth were then tested singly and the specific growth factor identified.

6. Lysogenicity Tests

The method was adapted from that described by Fisk (1942) and is described here as the Mixed Growth Test. The potential lysogenic strain was grown in mixed culture on an L plate with SB2. After overnight incubation, the mixed growth was removed from the plate, emulsified in 1 ml. of L-broth and centrifuged to clarify. The clear supernatant fluid was then tested for lysis of SB2 on L-agar.

At the same time the culture was tested for sensitivity to lysis by phage PSB1 by the method of Craigie and Yen (1938) and described here as the Patch Test Method. This combination of two tests defines three types of culture:

- (1) Cultures lysed by phage PSB1 but producing no phage in mixed culture with SB2 - called 'non-lysogenic';
- (2) Cultures not lysed by phage PSB1 but producing no phage in mixed culture with SB2 called 'immune';
- (3) Cultures not lysed by phage PSB1 and producing lysis of SB2 after growth in mixed culture - called 'lysogenic'.

Similar Mixed Growth Tests were performed using bacteria other than SB2 as potential propagating and indicator strain as described under Results.

7. Assays of Cells and Phage

Cells were assayed by diluting in saline and plating on NA.

Phage was assayed by the agar layer method originally introduced by

Gratia (1936), quoted by Adams (1959) and in general use by most phage workers. A two ml sample of melted TL medium at 45°C was inoculated with 0.2 ml of an overnight culture of indicator cells (SB2 unless otherwise stated); 0.1 ml of appropriate dilutions of phage in L-broth was added to each tube and the mixture poured onto the surface of a dried L-plate. After solidification, plates were incubated at 37°C overnight and the plaques counted.

Where desirable viable cells were destroyed by heating the lysate before assay at 65-70°C for 30 minutes.

8. Single Step Growth Curves of Phage

Single step growth curves of phages on SB2 were obtained following closely the methods outlined by Adams (1959). Three kinds of preliminary information were necessary: (1) The growth rate of SB2, (2) the rate of neutralization of the given phage by antiserum prepared against PSB1 and (3) the rate of adsorption of the given phage to SB2.

The growth rate of SB2 was determined as follows: Twenty ml of L-broth, inoculated with 0.1 ml of an overnight L-broth culture of SB2 was placed in a Petri dish on a rotary shaker at 37°C and the viable cells estimated by taking hourly samples. From the growth curve it was found that 1×10^8 viable bacteria/ml were obtained after about two hours incubation. Bacteria for the determination of neutralization rate and adsorption rate and the single step growth curve itself were grown under the same conditions as for the cell growth curve.

The rate of neutralization of phage by anti-PSB1 serum was determined

as follows: Antiserum was diluted 1/10 and 1/100 in L-broth. SB2, grown under standard conditions to approximately 1×10^8 /ml was assayed. Phage was also diluted to approximately 1×10^8 /ml and assayed. To 0.9 ml of the dilutions of antiserum was added 0.1 ml of the phage and the mixture placed at 37°C. At five and 10 minutes after mixing 0.1 ml samples were removed, added to 9.9 ml of chilled broth, and assayed for phage. The percentage of phage inactivated was calculated and the K value determined by the method of Adams (1959). The dilution of antiserum required to neutralize 99% of phage in five minutes was then calculated.

The rate of adsorption of a phage to SB2 was determined as follows: SB2 was grown under standard conditions to 1×10^8 /ml and assayed. Phage was diluted to 1×10^8 /ml and assayed. To 4.5 ml of culture at 37°C was added 0.5 ml of phage. At 10 and 15 minute intervals after mixing, 0.1 ml samples were removed and added to 9.9 ml of chilled L-broth. Volumes of 1 ml were centrifuged and assayed for phage. The percentage of phage adsorbed at 10 and 15 minutes was then calculated.

With the three preceding kinds of information single step growth curves were constructed as follows: SB2 was grown under standard conditions to 1×10^8 /ml and assayed. Phage was diluted to 1×10^8 /ml and also assayed. The phage antiserum was diluted out to the dilution calculated to neutralize 99% of phage in five minutes (1/4). The protocol of the single step growth curve was:

Time	Tube	Procedure
0 mins	1. adsorption	1. 0.1 ml phage added to 0.9 ml SB2.
5 mins	2. neutralization	2. 0.1 ml of 1. to 0.9 ml antiserum.
10 mins	3. dilution	3. 0.1 ml of 2. to 0.9 ml broth.
11 mins	4. F.G.T.	4. 0.1 ml of 3. to 9.9 ml broth.
12 mins	5. S.G.T.	5. 1.0 ml of 4. to 9.0 ml broth.

F.G.T. = First Growth Tube.

S.G.T. = Second Growth Tube.

All tubes were placed in a water bath at 37°C. At five minute intervals 0.1 ml samples were removed from the F.G.T. and S.G.T. and assayed for phage. From the results, the latent period and the average burst size were calculated.

9. Serological Methods

a. Antisera

Antisera against the Salmonella H antigens a, b, c, d, i and 1, 2, 3, 5, 6, 7 were obtained from Lederle. Other antisera were specifically prepared according to the following procedures.

(1) Vaccines

Methods in general use and described by Edwards and Ewing (1951) were applied. The H suspension consisted of an overnight L-broth culture of SB2 which had been inoculated from the edge of a swarm on MM. To the culture was added an equal volume of 0.6% formol-saline and the whole heated at 56° for 30 minutes.

O suspensions were prepared by removing the growth of SB2 from several

NA plates with saline, washing once in saline and placing the resulting suspension in a current of steam at 100°C for 2 1/2 hours. For injection, the suspension was diluted to an opacity equivalent to 10^9 organisms/ml.

Antiserum against phage PSB1 was made using a heat sterilized preparation of PSB1 with a titer of 5×10^8 /ml.

(2) Production of Antiserum

Antisera were prepared in rabbits. Animals were bled from the marginal ear vein prior to the immunization and the resulting sera were first used as controls in agglutination or neutralization tests to detect any non-specific reaction.

Rabbits were injected intravenously into the marginal ear vein with 0.25 ml, 0.5 ml and thereafter 1.0 ml volumes of vaccine at intervals of three or four days. Injections were continued until a total of at least 10 ml of vaccine had been administered. Animals were bled on the fourth day after the last injection and if the antiserum so obtained was shown to have a high titer, the rabbits were bled again on the following second and fourth days. If more antisera were needed a second course of injections was commenced four to six months after the first course and the animals bled as before.

Antisera were stored in the freezing compartment of the refrigerator.

b. Agglutination Procedures

(1) H Agglutinations

Bacteria to be tested for H antigens were grown in L-broth overnight and an equal volume of 0.6% formol-saline added. The

suspensions were then heated at 56°C for 30 minutes to sterilize. Antisera were diluted in saline to appropriate concentrations. The Lederle antisera were used at the recommended 1/1,000 dilution. The dilutions found to be most useful for the SB2 H antiserum were 1/20, 1/1,000 and 1/10,000. Each agglutination tube contained 0.5 ml of diluted antiserum to which was added an equal volume of the antigen suspension. Tubes were placed in a water bath at 56°C for two hours and read for agglutination.

(2) O Agglutinations

Bacteria to be tested for O agglutination were grown on NA overnight. A thick suspension of the culture was made in 1 drop of saline on a slide. The O antiserum was diluted 1/5 in saline. One drop of the antiserum was mixed well with the bacterial suspension on the slide and the slide rotated to detect agglutination.

c. Phage Neutralization Tests

Antiserum was diluted 1/100 in L-broth. To 1.0 ml of diluted antiserum was added an equal volume of phage with a titer of about 10^7 phage particles/ml. The phage-antiserum mixtures were placed in a water bath at 45°C for 30 minutes and then assayed for unneutralized phage. The control consisted of a mixture of phage and L-broth which was similarly assayed. The percentage of phage neutralized under the given conditions was then estimated.

10. Cytological Methods

Bacteria examined to detect flagella were stained by the tannic

acid - basic fuchsin method of Leifson (1951). Bacteria emulsified carefully in distilled water after overnight growth on L-agar were found to give best results.

RESULTS

A. The Mutational Origin of Lysogeny

1. Capacity of SB2 to Give Rise to Lysogenic Cells

a. The Origin of SB5

With the object of isolating auxotrophic mutants, strain SB2 of Salmonella blegdam was subjected to $MnCl_2$ - penicillin screening. In one experiment, two different auxotrophs were obtained - SB4, a pyrimidine requiring strain satisfied by either cytosine or uracil, and SB5 which required lysine.

Immediately after isolation each strain was tested for sensitivity to phage PSB1. Since the parental strain SB2 is sensitive to this phage, the test constituted a convenient check against contamination. SB4 was lysed by PSB1 as expected but SB5 was not lysed. Since this result raised the possibility that SB5 was a contaminant, it was neglected for about three months. During this time it was stored on a NA slant at room temperature.

After three months of storage SB5 was tested to see if possibly it was lysogenic for a phage which, like PSB1, would lyse SB2. Using the sensitive Mixed Growth Technique (see Materials and Methods section) SB5 was grown with SB2 and the supernatant of the centrifuged mixed culture spotted on SB5 and SB2 in isolation. Lysis of SB2 but not SB5 was observed. Serial dilution of the supernatant confirmed that the lytic agent was a bacteriophage which formed hazy plaques indistinguishable from those of PSB1.

Serial streakings of SB5 on NA were then initiated. After 15 successive streakings, each originating from a well isolated colony on the preceding

plate, twenty colonies were picked and each subcultured to 5 ml L-broth. After overnight incubation the saturated cultures were tested for lysogenicity by the Mixed Growth Technique using SB2 as the second strain. All were positive. In addition, each culture was centrifuged and the supernatant subjected to a temperature of 65°-70°C to kill residual cells. The heated supernatants were then assayed for free phage by plating appropriate dilutions on SB2. Each contained free phage in concentrations varying from 160 to 4,000 per ml, with an average of 880.

Thus SB5 appeared to be a classic lysogenic strain in which the capacity to produce phage is an inherited property of all (or the vast majority) of the cells, and in which phage is released with a frequency which is low but still sufficiently high that free phage can be routinely detected in the medium in which it has been grown.

b. The Phage Released by SB5

The phages released by SB5 and SB1 were further studied. The plaque morphology of the former (hereafter called PSB5) was indistinguishable from that of PSB1. Both phages form plaques containing a central disk of resistant bacterial growth (sometimes centered with a small spot of lysis) surrounded by a clear ring of lysis. These plaques therefore resemble the plaque type of the temperate Type-A phages of S. typhimurium, photographs of which were published by Boyd (1950, 1954). Plaque morphology was the same regardless of whether the phages were plated on SB2 directly from the supernatant of the lysogenic or had been previously propagated on SB2. Lysates of both PSB1 and PSB5 regularly contained a small number of clear plaque

mutants. These phages formed plaques lacking the central disk of bacterial growth, but covered instead with a large number of resistant microcolonies that gave the plaques a "peppery" appearance when examined with a 10x lens.

Both PSB1 and PSB5 lyse SB2 but do not lyse either SB1 or SB5. Thus, by these limited criteria, their host ranges are identical.

Simultaneous phage neutralization tests employing antiserum prepared against PSB1 showed 92% neutralization of PSB1 and 90% neutralization of PSB5. Both phage preparations had been propagated on SB2. Thus PSB5 is serologically very similar to PSB1.

Atkinson and Bullas (1957) demonstrated that PSB1 (= phage B43h/49) was resistant to 80°C for 30 minutes. Simultaneous tests of PSB1 and PSB5, both propagated on SB2, showed identical responses. Thirty minute exposures to various temperatures showed no decrease in lytic activity up to 75°C, reduced lytic activity at 80°C and complete destruction of lytic activity at 85°C. Thus, in gross response of lysates to heating, the two phages were indistinguishable.

Six independent single step growth curves of PSB1 on SB2 showed latent periods of 30-35 minutes and burst sizes of 15 to 48 with an average of 31. Five independent single step growth curves of PSB5 on SB2 showed latent periods of 30 to 35 minutes and burst sizes of 31 to 61 with an average of 49. The single step growth curves for phage PSB1 and PSB5 are shown in figure 1. Insofar as these lytic cycle parameters are concerned, therefore, phages PSB1 and PSB5 are very similar. The difference in burst size may well be significant but the variation in values for each phage was too great

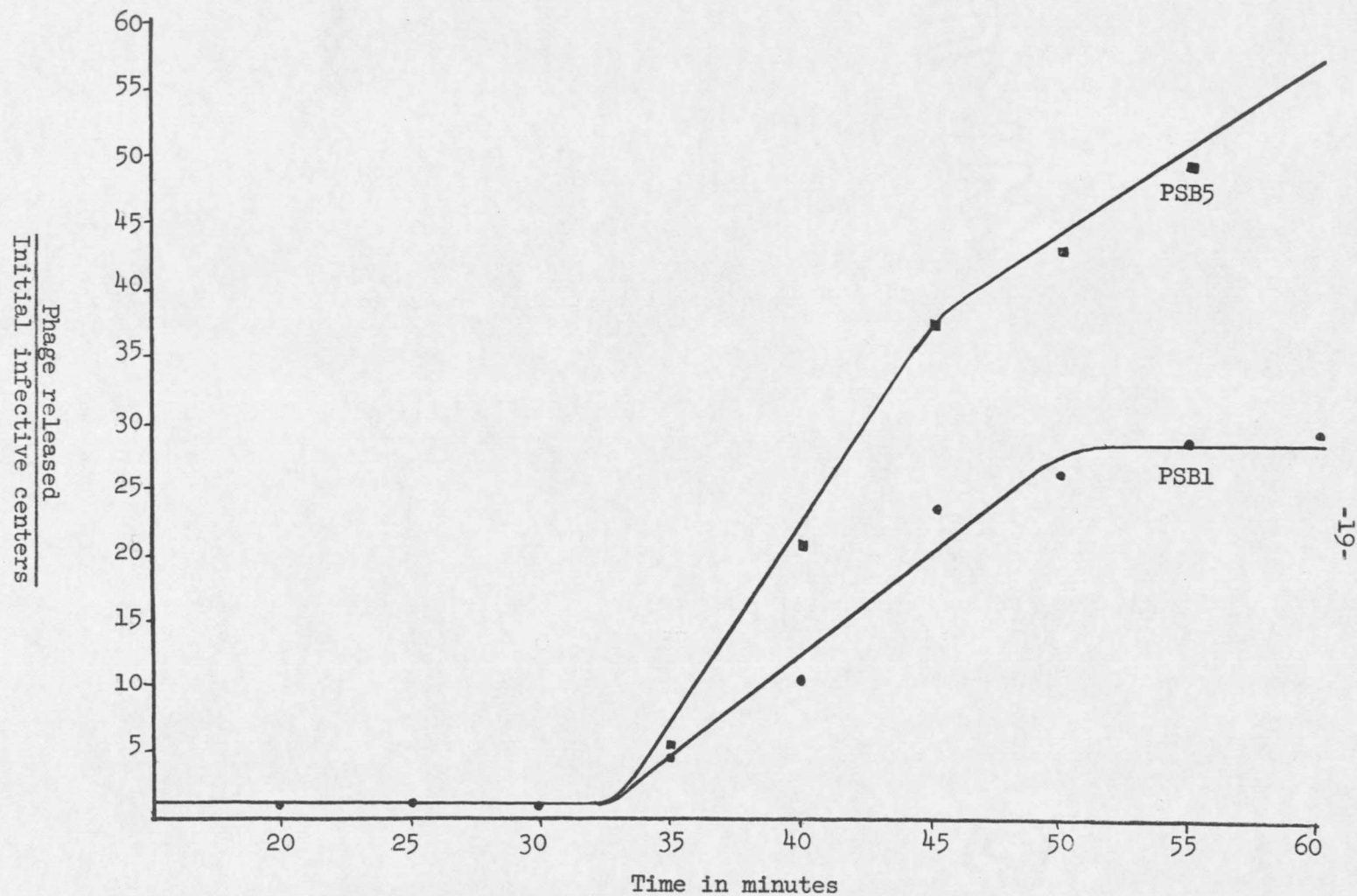


Figure 1. Single Step Growth Curves of Phages PSB1 and PSB5 on SB2. The points represent average values from six and five independent experiments respectively. The infecting phage had been propagated previously on SB2.

to allow a rigorous proof. This deserves further study.

The SB2 lysogenizing capacity of both PSB1 and PSB5 appears to be very low as illustrated by the following observations. The residual growth in an area of confluent lysis of PSB1 on SB2 was emulsified in saline, diluted and plated on NA. Thirty isolated colonies were picked at random into L-broth and the resultant cultures examined. Fifteen were still sensitive to lysis by PSB1 and 15 were immune to lysis by PSB1 but were not lysogenic by the Mixed Growth Test with SB2. On another occasion, 50 similar cultures were tested; on this second test, six were sensitive to PSB1 and 44 were immune but not lysogenic. On a third occasion, 30 similar cultures were tested, this time derived from the residual growth in an area of confluent lysis of PSB5 on SB2. Among these cultures, 28 were sensitive and two were immune but not lysogenic. As will be discussed later there is reason to suspect that the fluctuations in the frequency of immune bacteria is a function of the SB2 culture treated rather than the phage applied. The important point here is the low frequency of lysogenization under these conditions of undetermined, but probably rather high, multiplicity of infection. Lysogenic cells have been isolated following exposure of SB2 to PSB1, but only with considerable effort.

Thus in all tested criteria, phages PSB1 and PSB5 are similar or identical. Phage PSB1 has been shown capable of generalized transduction. For example, genes for motility and tryptophan can be transduced to appropriate strains (Bullas, unpublished). Other examples of PSB1 transduction are cited under Results. PSB5 has not been tested for this property.

c. The Question of Contamination

The discovery that SB5 was lysogenic for a phage indistinguishable from PSB1, together with the observation that it agglutinated to the same titer as SB2 when tested with H and O antisera prepared against the latter strain, confirms beyond reasonable doubt that it is a derivative of either SB1 or SB2, the only S. blegdam strains in the laboratory.

At the same time, two new and simple explanations for its origin suggested themselves. On the one hand, SB5 could be a cellular derivative of SB1 cells, either misidentified as SB2 or contaminating the SB2 culture. On the other hand, SB5 could be a cellular derivative of SB2 contaminated by PSB1 phage.

The first of these possibilities seems quite remote. The recovery of the sensitive SB4 from the same experiment argues that simple misidentification of the treated culture was not involved. And the fact that SB5 is, after all, a lysine requirer argues that if contamination were responsible for its origin this contamination must have been on a massive scale.

The second possibility is more difficult to exclude, since PSB1 was in fact in use at the time of the isolation of SB5. However, the following points argue against this possibility. At no time during several years of regular and simultaneous handling of SB2 and PSB1 had plaques of the latter appeared on the former, unless deliberate mixtures had been made. Furthermore, as discussed above, PSB1 is a very inefficient lysogenizer of SB2, even under conditions of massive lysis, which was not observed at any step in the isolation of SB5. In addition, the burst size data, although not

conclusive, suggest that PSB5, although similar, is not identical to PSB1.

These arguments were considered sufficiently convincing that the only remaining (and extremely interesting) explanation - that the lysogenic strain is a clonal derivative of the sensitive strain SB2 - might serve as a working hypothesis guiding future study.

2. The Conditions of Origin of Lysogenic Cells from SB2

a. The Role of the $MnCl_2$ Treatment

It is useful to distinguish between the two properties that distinguish lysogenic strains - the capacity to produce phage detectably (which we shall call the productivity) and immunity. Sensitive strains readily yield mutants which have acquired the second property. In some cases (for example, most mutants resistant to the virulent T phages and the Lp_2^r mutants resistant to lambda) immunity is attributable to a failure of the phage to adsorb. In other cases, immunity does not preclude adsorption; this is true of a few T phage resistant mutants and is generally true for immunity to temperate phages. The latter type of immunity is readily observable in SB2, as will be discussed in detail later.

Defective lysogenics are at least superficially similar to immune mutants arising from sensitives - both are immune but have low (or zero) productivity. The one has arisen by a gain of immunity, the other by a loss of productivity.

Since one of the two examined survivors of the $MnCl_2$ - penicillin treatment proved to be lysogenic, it was next appropriate to test the possibility that some part of this treatment massively converts sensitive cells into

lysogenic cells either directly or via prior provocation of massive phage production.

Strain SB2 was treated in exactly the same way as it had been treated in the experiment leading to the origin of SB5, up to and including the overnight incubation in penicillin. Then, 0.1 ml samples of the undiluted treated culture and of serial dilutions of the culture were plated on L-agar to which 0.1 ml of an overnight culture of untreated SB2 had also been added. At the same time, 10^{-3} , 10^{-4} and 10^{-5} dilutions were plated alone on NA to measure viable cells. In addition, the original undiluted culture was centrifuged, the clear supernatant tested for lysis on SB2 and the pellet resuspended in L-broth and tested for immunity to phage PSB1.

The treated culture showed massive lysis by PSB1, showing that no appreciable fraction of the population had become immune as a result of its manipulation. No lysis by the supernatant was observed, indicating that no appreciable fraction of the treated cells had produced phage. While approximately 100 colonies appeared on the 10^{-5} NA assay plate, no plaques appeared on any of the mixed platings on L-agar. Since about 10^7 treated SB2 cells were plated on the most concentrated of these, the frequency of SB5-like cells in this treated culture was very low.

The results just cited by no means exclude the possibility that some aspect of the treatment provokes the conversion from sensitivity to lysogeny, but the rarity of such events in treated cultures lends significance to the fact that one out of two of the auxotrophs isolated in the original treatment was lysogenic. It is suggested that the treatment may have served basically

as a screening procedure, and that there is a fundamental relationship between the origin of lysogeny and the origin of auxotrophs, or of certain auxotrophs.

Since SB2 persists as a sensitive strain, it is clear that the spontaneous origin of lysogenic cells like SB5 must be relatively rare. In order to fix an upper limit to the frequency of such an event in an untreated culture, the following experiment was performed. Serial dilutions of an overnight L-broth culture of SB5 were mixedly plated on L-agar with various and much larger numbers of SB2 cells from similar overnight cultures. The diffuse plaques which appeared on these plates after 24 hours incubation always greatly exceeded the number of free phage expected at that dilution and represented SB5 clones which had released phage soon enough to produce visible plaques. The results (figure 2) show that about 1% of the plated SB5 cells produce detectable plaques, and that this percentage is essentially independent of the number of (majority) SB2 cells simultaneously plated. This means that as few as 100 SB5-like cells would be detected whenever 0.1 ml (ca. 10^8 cells) of an overnight culture of SB2 is plated on L-agar. One plate of SB2 deficient of plaques, therefore reveals that the frequency of SB5-like cells in that culture is less than 10^{-6} . Since plaques have never been observed on simple platings of SB2 alone on L-agar, the average frequency of such lysogenic cells is much less than 10^{-6} .

b. The Role of the Lysine Requirement

(1) The Physiological Independence of Lysogeny and the Lysine Requirement

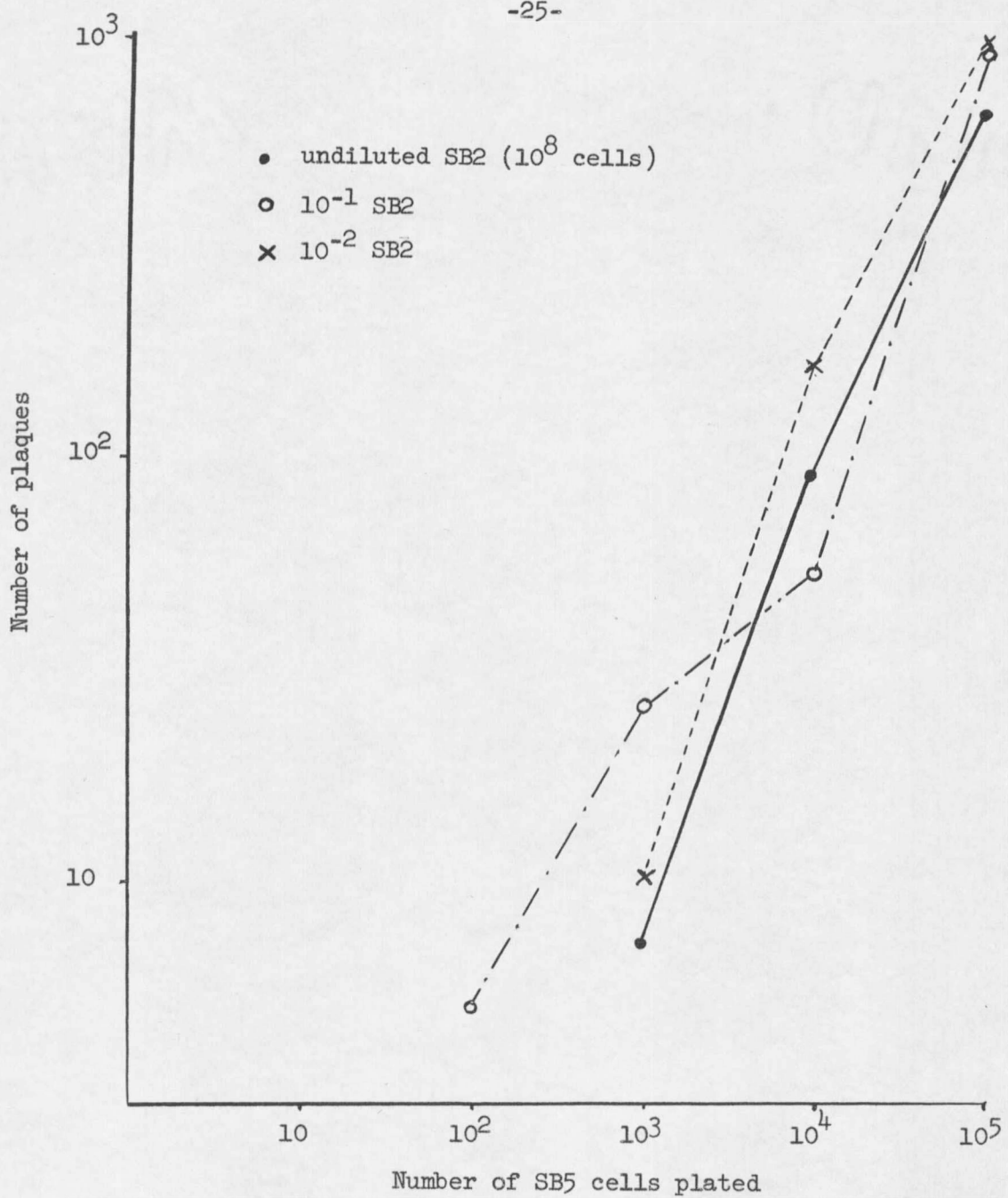


Figure 2. Plaque Forming Ability of SB5 Cells Plated out with SB2 Cells on LA.

The lysine requirement of SB5 is somewhat "leaky". Well washed suspensions inevitably show a faint lawn of growth on DMA. Nonetheless, prototrophic revertant colonies are readily obtained from the larger colonies which appear on such plates. A number of large colonies from a DMA plate were picked and streaked on NA to give isolated colonies. These were then checked for growth requirements and lysogeny. All were prototrophic and lysogenic. On one other occasion, to be discussed in a later section, prototrophs were isolated from an old slant of SB5 which were immune but not productive. Although the observations with the prototrophs isolated directly from the SB5 slant show that the properties of auxotrophy and productivity can be lost together, the prototrophic revertants of the lys⁻ auxotrophs show that the two properties are not invariably associated with one another.

(2) The Origin of Phage from other Auxotrophs

(a) Phage Production by other Auxotrophs
on Supplemented DMA

In addition to SB4 and SB5, four other auxotrophs have been derived from SB2. A tyrosine requirer (SB8), a glutamic acid requirer (SB9) and a slow growing strain of unidentified deficiency (SB12) were all isolated following a single UV treatment. A tryptophan requirer, (SB30) was isolated following MnCl₂ - penicillin treatment. Of these six independent auxotrophs, only SB5 was lysogenic; the rest were sensitive to lysis by phage PSB1. It is clear that the origin of auxotrophy is not always accompanied by the origin of lysogeny. On the other hand, the

suggestion of some relationship between the two events was strengthened by the observation of rare phage production by two of these additional auxotrophs.

In the course of testing the growth requirements of SB4 and SB9, washed suspensions of the newly isolated auxotrophs were plated on DMA and DMA plus appropriate growth factors. Four plaques appeared on SB4 plated on DMA-uracil; none on the platings of this strain on DMA, DMA-cytosine nor DMA-lysine. Many plaques appeared on the plate of SB9 on DMA-glutamic acid; none on the platings of this strain on DMA or DMA-lysine. No plaques appeared on simultaneous platings of SB2 on DMA and NA, and no plaques were observed when the same washed suspensions of SB4 and SB9 were plated on NA.

Some of the bacterial growth from the plates on which plaques were seen was emulsified in saline, centrifuged and tested for lysis of SB2. These extracts from SB4 on DMA-uracil and from SB9 on DMA-glutamic acid each lysed SB2, and on appropriate dilutions plaques were seen. The phages, henceforth called PSB4 and PSB9, were then propagated on SB2 and the resulting lysates compared with PSB1 and PSB5. All phages have identical host ranges, lysing SB2, SB4 and SB9 but not SB1 or SB5. All are indistinguishable in plaque morphology. Further, PSB9 is similar to PSB1 and PSB5 in serology, heat resistance and lytic cycle kinetics.

A second plating of the washed suspensions was then made: SB4 on DMA, DMA-uracil, DMA-glutamic acid and NA, and SB9 on DMA, DMA-glutamic acid, DMA-lysine and NA. Plaques were again observed on the plating of SB9 on DMA-glutamic acid but on no other plate. Extracts from these other plates

were also negative.

Stock cultures of SB⁴ and SB⁹ were subcultured to NA slants from the washed suspensions used above. Several months later, two further platings on DMA and supplemented DMA were made. No plaques were observed on any of the plates, nor was phage revealed by mixed growth of extracts from these plates with SB². Although these deliberate later attempts to observe phage production by SB⁴ and SB⁹ were unsuccessful, SB⁴ and a derivative of SB⁴ spontaneously manifested phage production on other occasions. These instances will be discussed shortly. For the moment we shall consider the significance of the phage production just described.

First, the possibility of phage contamination must again be considered, and here it is more difficult to exclude by a priori reasoning. Yet the pattern of appearance of plaques was not random. Specifically, they did not appear on SB² under any condition nor did they appear on NA for any strain and they appeared more profusely on SB⁹ on DMA-glutamic acid than on any other growth factor or on SB⁴ with any growth factors. Second, if the plaques were not due to contamination and their distribution not fortuitous, certain conditions associated with plating on DMA seem to induce phage production. Third, the rare and erratic occurrence of plaques under these conditions suggests that the populations plated were heterogeneous, that only a small fraction were capable of this induction. This would, of course, be the situation if SB⁴ and SB⁹ cultures contain a very small number of lysogenic cells. Regardless of the nature of the phage producing cells (excluding contamination as a possibility), the suspicion that auxotrophic

mutations and the origin of lysogeny are related, is therefore strengthened.

(b) The Origin of Lysogenic Strain SB23

The second instance of phage production by SB⁴ occurred in the course of routine tests of sugar fermentation on EMB media. The majority of SB⁴ colonies on EMB-xylose were dark with a green sheen, but two colonies were pale but not translucent. Both of these proved still to require cytosine and to be sensitive to phage PSB1. However, the growth on NA of one of these cultures became motley; when again tested this culture was immune to PSB1 and contained a phage which lysed SB2. The culture was streaked and several isolated colonies tested for lysogeny by mixed growth with SB2; all were lysogenic and still required cytosine. One of these was slanted as SB23. The phage it produces (PSB23) is like PSB1 in host range, plaque morphology, serology, heat resistance and lytic cycle kinetics. Once again the question of contamination arises. Here, as in the case of SB5, the ease with which lysogeny was established argues against phage contamination, while the cytosine requirement excludes the possibility of cellular contamination. What appears to have happened is that one of a few lysogenic cells in the population released phage; the latter rapidly depleted the sensitive cells leading to eventual dominance of the other lysogenic cells. The fact that lysogeny originated here in a xylose fermentative mutant suggests that this change may be related to the change from sensitivity to lysogeny. However, the other sibling variant never produced phage, nor did similar variants isolated from SB⁴ on several other occasions. Further, other lysogenic cultures (SB1 and SB5) produce normal sheened colonies on

EMB-xylose.

The significance of these observations is as follows: First, the circumstances of the origin of SB23 suggest that the lysogenic state derived abruptly from the sensitive state, an inference that could not be made so readily with SB5. Lysogenicity is a compound of two properties - (1) an ability to produce phage detectably, and (2) immunity to that phage. SB5 and SB23 differ from SB4 in both properties and it might be argued, a priori, that the two must be acquired stepwise. But, the ready isolation of SB23 from the lysing population of SB4 argues, on the one hand, that an SB4 cell did not release phage (by a process comparable to the lysis of E. coli B under pressure) and that then SB23 arose by lysogenization (since this is quite inefficient). On the other hand, the ready isolation of SB23 argues that the SB4 population did not contain a large number of merely immune cells from which SB23 arose. Although the circumstances did not permit a decision on this question, it should be noted that both the origin of SB5 and the appearance of plaques on SB4 and SB9 on the supplemented DMA could have involved the abrupt transition from the sensitive to the lysogenic state. Secondly, although the origin of SB23 does not argue against the suspected relationship between the auxotrophic mutations and the origin of lysogeny, it does show that the latter can occur under conditions other than those which peculiarly stress auxotrophs.

c. Deliberate Attempts to Isolate Productive Cells by Selecting for Other Mutants

(1) Selection of Immune Cells

An annoying feature of the indicator strain SB2 since first studied in Adelaide (Bullas, unpublished) has been a change from sensitivity to immunity for phage PSB1. Upon storage of NA slopes at room temperature, cultures of SB2 undergo a gradual deterioration as effective indicators of PSB1. At first, plaques of PSB1 are very distinct; later, they become progressively more hazy and indistinct until finally the culture is of no use as an indicator stock.

This can be traced to a progressive increase in the frequency of genetically immune cells. For example, one culture which had become useless as an indicator after two months storage was diluted and plated on NA to give isolated colonies. Several of these colonies were smeared on L-agar and patch-tested for immunity to PSB1. While 75% showed no lysis with PSB1 and presumably represented immune clones, 25% showed the distinct lysis by PSB1 characteristic of the original SB2.

The immune cells retain the normal ability to adsorb PSB1 (91% adsorbed by one immune isolate, at 37°C for 15 mins.) and thus resemble most of the mutant cells which are refractory to other temperate phages. Such immune cells are generally interpreted as spontaneous mutants present before the application of exogenous phage (e.g. see Lwoff, 1953) but the interpretation is especially clear in this present situation. Furthermore, in the present system, it is easy to isolate immune cells (such as SB47 to be discussed later) with the assurance that they never contacted PSB1.

The immune cells obviously either arise with a very high frequency from SB2 serially subcultured in broth or have a decided selective advantage.

Within two to three days, an initially good PSB1 indicator population will become useless because of an undetermined, but certainly rather high, frequency of immune mutants. The mutation rate to immunity has not been measured, so the reason for the rapid population changes is unclear. But the interpretation based on a 'normal' mutation rate and a high selective advantage of immune cells is, for the moment, the simplest.

Because of the periodic changes just described, it was necessary occasionally to reisolate SB2. This strain designation always refers to a culture upon which PSB1 forms distinct plaques.

Even on the most freshly isolated SB2, plaques of PSB1 are turbid as indicated earlier. It is of interest here to note that although increasing quantities of immune cells in the SB2 culture make the plaques progressively more turbid, most of the plaque turbidity on fresh SB2 is not due to their presence; for when the central growth is emulsified, streaked and tested, most of the cells are still sensitive.

A number of immune cells that arose during storage have been tested for productivity (i.e. lysogenicity) by the Mixed Growth Technique, without positive results. Although most of the immunes thus appear to be non-lysogenic, the possibility exists that a very small minority of them might be productive (lysogenic).

The result of one particular experiment suggested that the production of phage or of lysogenic cells which produced phage might occur in cultures in which the change from sensitivity to immunity was occurring. The heat sterilized supernatants of a series of overnight L-broth cultures of various

S. blegdam derivatives, including an immune strain of SB2 called SB47 (which had never shown any productivity), were assayed for free phage. The inoculum for the SB2 indicator culture came from an NA slope which two months previously had been inoculated from a single phage sensitive colony. The plaques which developed from lysogenic culture supernatants were extremely hazy and it was subsequently shown that the indicator culture then consisted of a mixture of both sensitive and immune cells. SB47, when tested similarly on several other occasions, had never shown the production of plaques. However, on this occasion six plaques, though very hazy, were seen when 0.1 ml of the SB47 supernatant was assayed.

This result first suggested that the immune SB47 was producing some phage though at a very low frequency. When, however, the same supernate (stored overnight in the refrigerator) was retested using as indicator strain, a culture derived from a single sensitive colony from a plating of the mixed indicator on NA, no plaques were produced. This finding indicated that the plaques produced on the first test were more probably derived from the indicator culture itself rather than the SB47. When the top layer was inoculated with the same indicator strain alone (0.2 ml; kept an additional 24 hours at room temperature) and incubated overnight, no plaques were seen, however.

The fact that no plaques were found when the SB47 supernatant was reassayed with a pure sensitive indicator speaks against phage contamination from that source. The top layer agar was also not the source of possible contamination since the same bottle of medium, kept overnight in the water

bath at 45°C, was used for the assay with sensitive indicator. Thus the source of phage remains most probably, the old indicator culture itself.

If indeed the phage originated from the old indicator culture itself, the non appearance of plaques when the culture was incubated without the addition of any supernatant, may be due to the heavy growth of immune cells. It was demonstrated that a known phage preparation, when assayed with the same mixed indicator, produced no visible plaques. The only other known variable in the original test was the incubation period itself. Since it was known that the indicator culture was mixed sensitive/immune, plates were not incubated overnight but after only four hours at 37°C, they were removed and left at room temperature overnight. This method was used to slow the growth rate and thus attempt to prevent overgrowth by immune cells and permit plaques to be seen. This indeed was the outcome for although plaques were visible, they were very hazy. The failure to detect plaques on the overnight incubation plates is therefore not unexpected. In fact, that phage was detected at all with the old indicator culture (25% sensitive and 75% immune) seems to have been entirely fortuitous; due to the heavy population of immune cells, any phage produced by a rare event in occasional cells would stand little chance to survive as a plaque former but be rapidly adsorbed by immune cells and 'lost'. A repeat of the plating and incubation procedure following as closely as possible the method which gave rise to plaques, failed to produce plaques.

The phage propagated on sensitive SB2 and resembled in plaque morphology the previous phages that had been isolated. It was concluded

that in cultures of SB2 which are undergoing the change to immunity (generally old stored cultures), an event leading to phage production by a cell may occur very rarely. The phage is rapidly adsorbed by the surrounding majority population of immune cells and is 'lost' although under ideal conditions which probably allow a greater population of sensitive cells, it may occasionally be detected. The cell which produces the phage is not necessarily the immune type but may represent a cell that has lysed without ever acquiring immunity.

(2) Selection of Auxotrophic Immune Cells

Early results suggested that transition to lysogeny was associated with mutation elsewhere in the genome. Thus it was thought possible that auxotrophic immune cells might automatically be productive or at least that the frequency of change to lysogeny might be higher in auxotrophic immune cultures than in prototrophic cultures, where a secondary mutational event might be necessary before lysogenic transition. The more convenient examination of immune derivatives of an auxotroph was then made. The hypothesis was not supported by an examination of an auxotroph with an unidentified requirement obtained from SB47, an immune derivative of SB2, by the $MnCl_2$ -penicillin method; for this auxotroph was found still to be non-productive.

When culture SB4 was plated out on EMB media during the course of routine tests for sugar fermentation determinations, it was observed that the colonies on EMB-glucose were of two types: the majority were dark and possessed the typical green sheen but a minority were dark and lacked the

sheen. Representative sheened and sheenless colonies were picked into L-broth and tested for lysogenicity and growth requirement. None of the sheenless colonies was lysed by phage but all were non-lysogenic while all of the sheened colonies remained phage sensitive. Both types retained the requirement for cytosine. One sheened sensitive colony and one sheenless immune colony were placed into stock as SB67 and SB56 respectively.

Since a colony type test would provide a very convenient method for distinguishing between sensitives and immunes, similar cultures of the prototrophic SB2 culture were also plated out on EMB-glucose. However, the correlation did not hold here for all colonies were sheened whether sensitive or immune. The curious correlation between the cytosine requirement, sheenless colony on EMB-glucose and immunity is, however, extremely interesting. It is indicated that mutations to glu-sheenless and immunity, previously independent in SB2, have been coupled by the cytosine mutation.

Culture SB56 was tested by the Mixed Growth Technique with sensitive SB2 on several occasions but never showed any phage productivity. Thus the combination of auxotrophy and immunity does not inevitably result in productivity. However, phage production seemed occasionally to be associated with another phenomenon in this immune culture; this is presented in the following section.

(3) Selection of MAL Papillae

When plated out on EMB-maltose the colonies of SB56 were found to produce bright pink papillae on their surfaces which in some colonies became very numerous. This phenomenon had also been seen

previously with some colonies of SB2 and SB4. When picked and plated on EMB-mal the papillae produced raspberry colored colonies which were themselves non-papillating. The papillae appeared only when the appropriate cultures were plated out on EMB-mal medium and not on any other sugar. The physiological difference between the papillae and background is completely unknown.

Since only occasional colonies were papillated in SB2 and SB4 there was a wide variation in maltose mutability within these cultures. But since every colony of SB56 was found to produce papillae the maltose mutability in this culture was much higher than in the sensitive cultures. The heterogeneity of the SB2 and SB4 populations with regard to mal papillation is extremely interesting in itself. In mut⁺ cultures of E. coli, papillation is more frequent than in mut⁻ cultures (Skaar, pers. comm.). But the activity of mut⁺ is quite constant within a culture; i.e. all colonies papillate to the same extent. They are thus like SB56. By contrast, the SB2 and SB4 cultures behave as if there were a movable Ds-like element (see section 4) only affecting the pertinent mal gene in certain clones. SB56 behaves as if the element had stopped moving - an event correlated (perhaps not coincidentally) with the acquisition of immunity.

Since papillae represent easily obtained mutants, a number were examined to test the hypothesis that the origin of productivity occurs more frequently at the time of any other kind of mutation.

Papillae were picked carefully with the use of a binocular microscope and sterile wire and streaked out on EMB-mal. One colony that grew up after incubation was then tested.

All fifty papillae from a sensitive SB2 culture were lysed by PSB1, were non-lysogenic and were prototrophic. All 29 different papillae from an immune derivative of SB2 were immune, non-lysogenic and prototrophic.

From a plate of SB56 colonies on EMB-mal, five papillae at first were picked and tested. Each of the five was found to be cyt⁻, to be immune to lysis by PSB1 and to be lysogenic. Cultures arising from inocula from two non-papillating areas of a colony which produced papillae were cyt⁻, immune but non-lysogenic. It appeared, therefore, that a change from immune, non-lysogenic to lysogenicity had occurred in the five papillae tested. The change was, however, shown not to be a permanent one for two of the lysogenic cultures when tested again three weeks later were found to have returned to the immune non-lysogenic state but still to be cyt⁻. This result is unexplained but could be due to a selective advantage of non-productive mutants arising within the initially productive populations.

Thus additional papillae were tested from SB56. An additional forty papillae from as many colonies were tested but each retained the parental properties of immunity and non-lysogeny.

The prediction that productive cells might more readily be isolated by screening for another unrelated mutation was thus realized. Of particular interest was the fact that the only five productive cultures were isolated in the same experiment. (The records are not clear as to whether all were

from a single colony; but they were certainly from closely related colonies.) This indicates the following sequence of events. An event (possibly the same one which led to immunity) caused SB56 to be permanently mutable for the gene responsible for mal papillation. A second event then occurred in one SB56 cell which increased the probability of origin of productive cells. But the second event tied in with the first event in such a way that productivity originates at the same time as the mutation of the mal gene. The full significance of this peculiar association cannot be assessed at this time. But it is certainly a clue pertinent to the eventual unraveling of the connection between changes in prophage properties and properties of the bacterial genome. The parallel between the coupling of mal and productivity mutations and the coupling between sheen and immunity mutations discussed in the last section is particularly interesting.

(4) Selection of Chloramphenicol Resistant Mutants

The origin of phage producing cultures from mal papillae indicated that the transformation to lysogeny could be associated with mutations other than those affecting auxotrophy. Probably one of the simplest types of mutant to isolate is resistance to antibacterial agents such as the antibiotics. In order to test that antibiotic resistance might also be associated with transition to lysogeny, SB4 which previously had shown that it could give rise to phage and to lysogenic cells was plated out on chloramphenicol NA to obtain resistants.

An overnight L-broth culture of SB4 was centrifuged and resuspended in saline at 5x concentration. Then 0.1 ml of the suspension was spread on NA

plates previously spread with sterile chloramphenicol solution to give a final concentration on the plate of 30 $\mu\text{g/ml}$. Plates were incubated for 48 hours at 37°C. An average of 34 resistant colonies appeared on a plate (which, since 6.6×10^8 cells were plated, gave a frequency of chloramphenicol resistants in SB4 of approximately 2×10^{-7}).

The appearance of the resistant colonies was, however, very unusual. The majority were 'doughnut' shaped consisting of a ring of bacterial growth surrounding a central clear area; the ring-shaped colony was also surrounded by a wide, translucent, flat spreading area of bacterial growth. Some colonies seemed to consist entirely of the translucent area and were quite large (to about 5 mm in diam.).

The appearance of the colonies strongly suggested phage action. The growth from one plate was scraped off and emulsified in 2.5 ml of L-broth and centrifuged. When this extract was spotted on to SB2, one plaque was seen; when 0.1 ml of the extract was grown in top layer with SB2, four plaques were visible. The plaques were similar in appearance to the temperate plaques already described. The emulsified bacteria were also plated out on NA and 25 colonies picked and tested for lysis by PSB1. Each of the 25 colonies was still sensitive and none was immune or lysogenic.

The emulsified suspension of bacteria included cells from the resistant colonies and the background growth although this was very slight. To test whether the phage originated from the resistant colonies, a single 'doughnut' shaped colony was subcultured from the plate into 10 ml of L-broth and incubated overnight. The culture was centrifuged and the clear supernatant

spotted on SB2 and 0.1 ml and 1.0 ml samples also grown in top layer with SB2, incubated and observed for plaques. No individual plaques were seen but the growth of the SB2 in the area of the spot, and on the plate inoculated with 1.0 ml of the supernatant was completely inhibited. The spot of clearing was dissimilar from an area of confluent lysis caused by phage in that the edge was less clearly defined; and tests of dilutions of the supernatant merely showed reduced degrees of clearing rather than individual plaques. The lysed appearance of the chloramphenicol resistant colony seemed, therefore, due not to particulate phage but to a soluble substance which in effect resembled the colicin group of substances (Fredericq, 1957).

The amount of phage present in the original extract was very small and could have originated from any of the resistant colonies or the background bacteria. There may be an intimate relation between the colicin-like substance produced by the resistant colonies and the production of phage, for certain relationships between phages and colicins have been determined. These relationships were reviewed by Fredericq and include (for phage T6 and colicin K) specific, reciprocal cross resistance which behaves as a single marker in genetic crossings, the same receptor on the bacterial surface and identical X-ray inactivation curves. However, colicins do not multiply and their action is not transmissible in series.

Many questions remain concerning the colicin-like substance; the important point here is that after growth on chloramphenicol agar, phage was isolated. Since lysogenic cells were not detected amongst the bacteria

from the plate, the phage may have arisen as the result of lysis of one or few extraordinary cells rather than by the production of a lysogenic cell with the hereditary capacity to produce phage at a low frequency. The result supports the idea that phage production from a sensitive culture is associated with a mutational event not necessarily auxotrophic in nature.

3. The UV Inducibility and Sensitivity of SB1, SB2, SB23 and SB47

The simplest explanations for the origin of phage from a strain initially sensitive to lysis by that phage include - first, that the sensitive strain is actually lysogenic for a functional phage but for which no indicator strain is available, and that detection of the phage from certain substrains reflects a mutational event which has extended its host range; and second, that the sensitive strain carries a defective phage which does not confer immunity.

With some lysogenic strains, irradiation with small doses of ultra-violet light induces the development of bacteriophage and the release of infective particles in some members of the treated population. With other lysogenic strains, however, ultra-violet treatment does not cause phage induction. Thus phages may be grouped into both 'inducible' and 'non-inducible' types. If any of the lysogenic strains studied here were inducible it was thought that the results of ultra-violet irradiation might provide evidence to test the above hypotheses.

To determine first, whether any of the lysogenic strains could be induced by ultra-violet light and the optimal irradiation time for such induction, the following experiment was performed. A volume of 0.1 ml of

an overnight L-broth culture of SB5 was added to 50 ml of L-broth which was incubated at 37°C until the culture reached a concentration of about 10^8 cells/ml. The culture was then divided into 5 ml portions and dispensed into Petri dishes. The separate portions were irradiated for five to 60 seconds at five to 10 second intervals. A 5 ml unirradiated sample was used as control. Each sample, after irradiation, was kept in the dark at 37°C for three hours. They were then centrifuged; the clear supernatant was sterilized by heat at 65°C for 30 minutes and assayed for free phage by the agar layer method with SB2 as indicator strain. The amount of free phage in each sample was then plotted against the time of irradiation and is shown in figure 3. The experiment showed that the phage from SB5 was inducible and that the maximum degree of induction was achieved after about a 15 second irradiation.

Thus, in future experiments, 5 ml of log phase, L-broth cultures of bacteria in Petri dishes were irradiated for 15 seconds, incubated at 37°C for three hours, centrifuged, the supernatants heat sterilized at 65°C for 30 minutes and then assayed for free phage on SB2. Controls consisted of unirradiated samples otherwise similarly treated. The lysogenic strains SB1, SB5 and SB23 were irradiated. The sensitive strain SB2 and the immune derivative of SB2, SB47, were also included to test the possibility that UV treatment might release a phage from these strains, active on unirradiated SB2. Results of the treatment are shown in Table I:

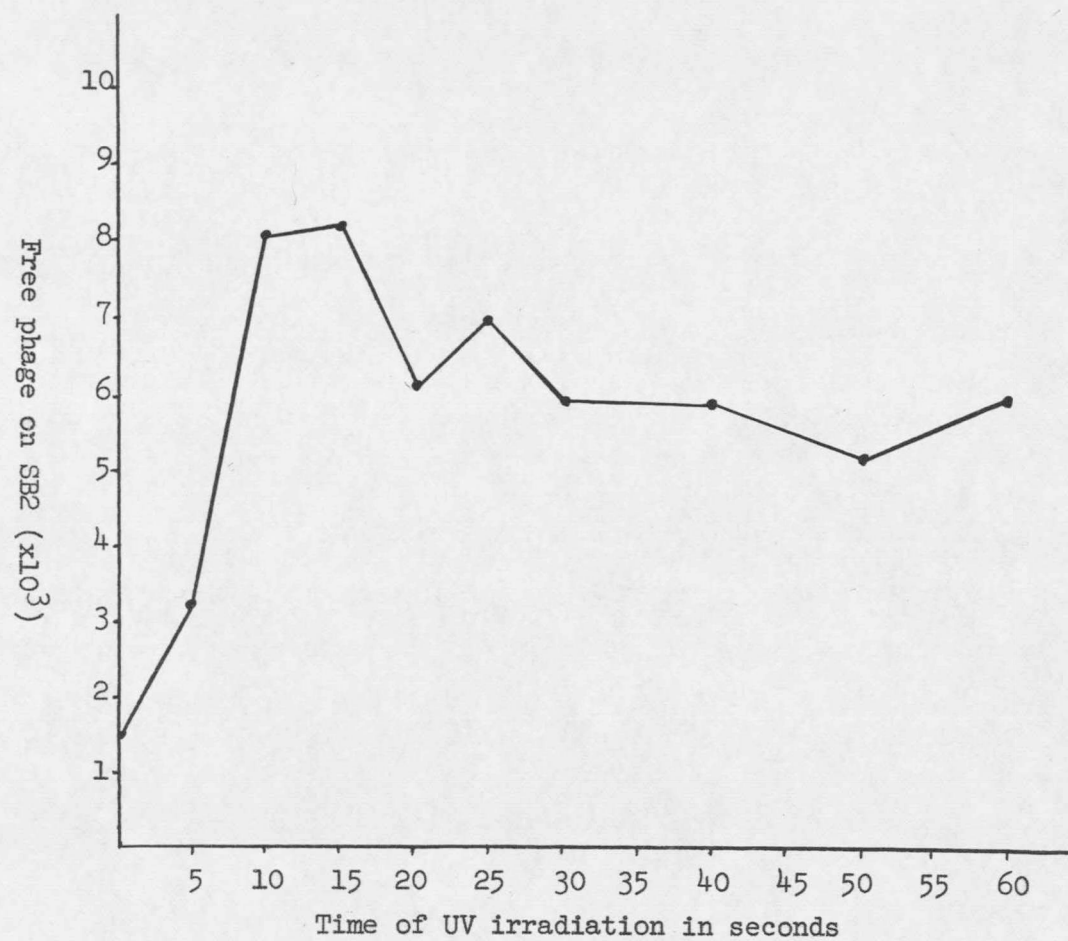


Figure 3. Ultra-Violet Induction of SB5. Irradiation was of log phase cells at a concentration of about 10^8 /ml in L-broth.

TABLE I

UV Induction of SB1, SB2, SB5, SB23, and SB47. Log Phase Cells at a Concentration of 10^8 /ml in L-broth were Irradiated for 15 Seconds and Incubated Three Hours assayed on SB2

Strain	Phage Concentration Without UV	Phage Concentration With UV	With UV/Without UV
SB1	30/ml	8×10^2 /ml	27
SB2	0	0	0
SB5	7.6×10^2 /ml	5×10^4 /ml	66
SB23	8.5×10^2 /ml	3×10^5 /ml	353
SB47	0	0	0

No phage was detected from strains SB2 and SB47. All lysogenic strains were induced but to different degrees. A complication affecting statements of inducibility is the fact, previously stressed, that the different lysogenic strains differ in degree of basal productivity. SB5 and SB23 are probably equivalent, but SB1 is markedly less productive. This difference is repeatable. This leads to two possible ways of expressing inducibility: (1) Increment in phage release provoked by UV. For SB1, this would be 800 less 30 equals 770; for SB5, this would be 50,000 less 760 equals 49,240. Assuming equivalent burst sizes, this means that about 64x as many SB5 cells are induced as are SB1 cells. (2) Factor increase in phage release provoked by UV. These values are given in Table I. On this basis, SB5 is only about 2 1/2x as inducible as SB1. Although there is the possibility of

discrepancies by the use of the two methods of expressing inducibility, both agree here and lead to the conclusion that three levels of inducibility are manifested by these three strains. In view of the finding that each of the phages appear identical by other tests, the differences in degree of inducibility are significant.

Jacob and Wollman (1956) working with E. coli K 12 lysogenized with temperate phages isolated from strains of E. coli of fecal origin, demonstrated that the property of inducibility was correlated with the chromosomal prophage location; the chromosome was therefore divided into 'inducible' and 'non-inducible' regions. The various inducible phages showed some degree of serological relationship to the lambda phage of K12 and were able to undergo some recombination with it. Thus, although each phage was mapped on a different position on the chromosome, all were closely related to lambda. The phages, inducible and non-inducible, were selected from among those that gave no cross immunity. Non-inducible mutants of inducible lambda can also be isolated; the responsible mutation is in the C region.

An inference that may be drawn from the results of the different lysogenic strains of S. blegdam is that in this organism also, prophage may become attached at different positions on the chromosome and that this is reflected in the different degrees of UV inducibility (or even non-inducibility, from results of additional strains tested and reported in a later section). Introduced earlier was the evidence for a correlation between phage production and an apparently unrelated mutational event.

Combining the first inference and the second correlation we have the very interesting suggestion that location of the prophage is correlated with a particular mutation. Since all mutations do not necessarily lead to transition to the lysogenic state, the attachment of prophage in its new location probably does not cause the mutation but the site of the new mutation may become a 'site of attraction' to the prophage to which site it then becomes preferentially attached. The immune strains might represent a situation in which prophage is actually located on a region of the chromosome which results in zero productivity and zero inducibility. In sensitive strains, a similar situation might exist but with the addition of a mutation in the immune region so that the strain lacks the property of immunity. Transition from the sensitive to the lysogenic state would therefore be the result of at least two separate events; one, a mutation which restores the property of immunity and second, a transfer of the prophage from the 'non-inducible, non-productive' region of the chromosome to another 'productive' region.

The UV sensitivity curves of lysogenic strains SB1, SB5 and SB23, sensitive SB2 and immune SB47 were compared. A sample of 5 ml of a log phase culture in L-broth of each strain in a Petri dish was irradiated with UV for total times of 10, 20, 30, 40 and 50 seconds. The number of surviving bacteria for each irradiation time was estimated by plating out appropriate dilutions on NA. A curve for each strain was then drawn of per cent survivors against time of irradiation. The results of these experiments is shown in figure 4 - the curve for SB1 is the average of

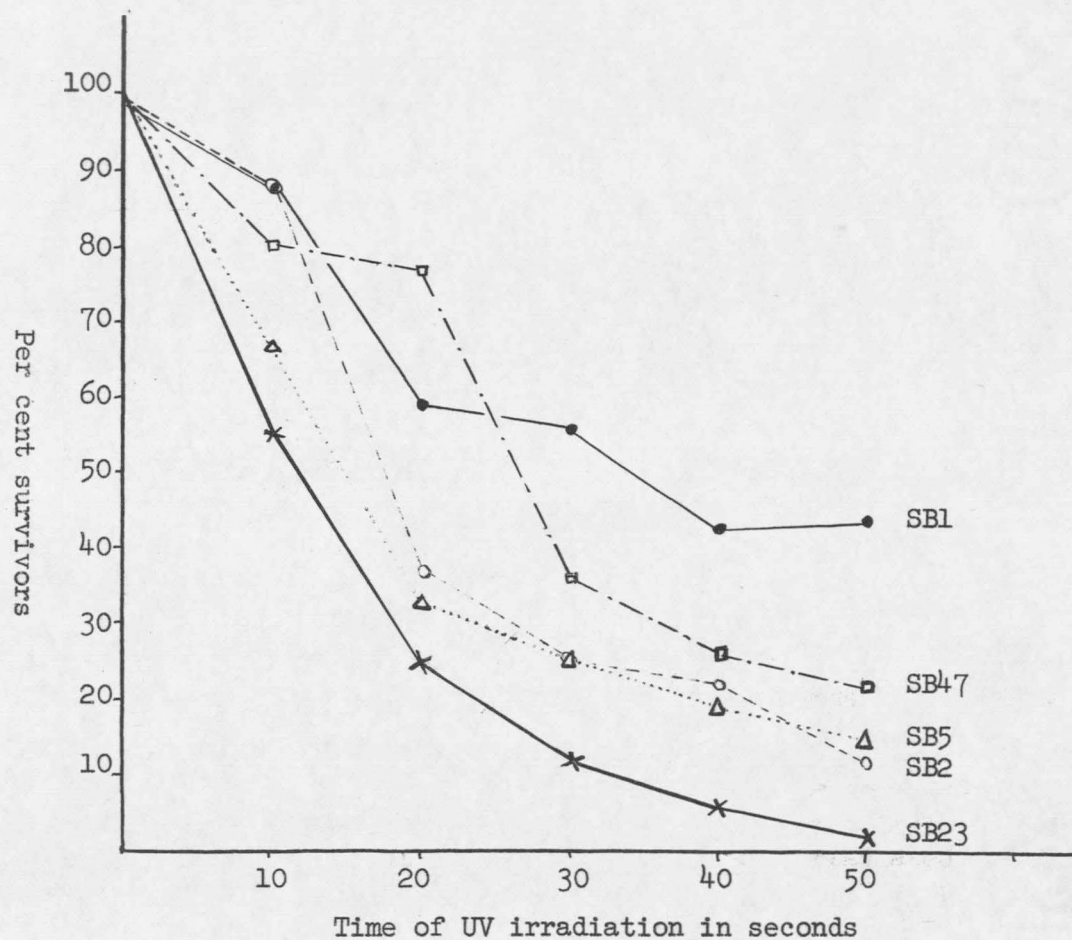


Figure 4. Ultra-Violet Sensitivity Curves of SB1, SB2, SB5, SB23 and SB47. Log Phase Cells at a Concentration of about 10^8 /ml in L-broth were Irradiated.

three separate determinations, of SB2, SB5, SB23 and SB47 of two separate determinations each.

SB23, the lysogenic strain which showed maximum prophage inducibility, gave a curve with a per cent survival after 50 seconds irradiation of only 2 per cent. SB1 the lysogenic strain of least inducibility gave a curve with a per cent survival after 50 seconds irradiation of approximately 50 per cent. SB5 occupies a median position with a per cent survival of 15 per cent. Of particular interest is the finding that SB2 and SB47 give curves that approximate that of SB5 more than SB1 with per cent survivals after 50 second irradiations of 12 per cent and 22 per cent respectively.

Although no phage from strains SB2 and SB47 was detected after the 15 second irradiation when assayed on untreated SB2 (Table I), the curves do strongly suggest that both SB2 and SB47 may have actually been induced to lyse either by a phage for which there was no indicator, or by inducible defective prophage which, it is to be remembered, would have to be unique in not conferring immunity.

4. The Mechanisms of Origin of Lysogenic Cells from SB2

The reasons for the working hypothesis that the lysogenic SB5 arose from SB2 by an intracloal mechanism (rather than by contamination) were discussed in the first section. Subsequently reported data support this hypothesis in the following ways; (1) SB2 itself exhibits the UV sensitivity characteristics of SB5 and (2) phage similar to PSB5 has been isolated from SB2 and its derivatives on six additional occasions. The distribution of these occurrences was not random; instead, in each case,

phage appeared following procedures which could, or demonstrably did, screen for a minority of the original population. In the meantime, hundreds of routine platings of SB2, under conditions which should readily disclose contaminating phage, have been negative.

If (as argued) contamination is excluded as a factor in the origin of any or all of the phage producing derivatives of SB2, then it is clear that SB2 itself carries at least most of the hereditary specifications of a temperate bacteriophage. The basic question then is: In what way does the prophage-like element of SB2 differ from the PSB5 prophage of SB5 and the PSB23 prophage of SB23?

SB5 and SB23 differ from SB2 in two properties: Immunity to and productivity of PSB5 (or PSB23). The rarity of the sensitivity-to-lysogenic transition is consistent with the possibility that two independent steps are involved. The prophage-like element in SB2 might be conceived of as (1) defective in its ability to carry out a very early step in the lytic cycle and (2) defective in its ability to confer immunity, each defect due to a different and revertible mutant gene.

This simplest explanation of the sensitive-to-lysogenic transition, however, is not readily accommodated to two important observations: (1) Although immunity may be acquired independently, there is no unequivocal evidence that productivity can be acquired independently. In fact, two of the seven instances of phage production by SB2 derivatives (the origins of SB5 and SB23) seem to have involved the simultaneous origin of both properties. (2) The origin of lysogeny in SB5 was accompanied by the

origin of a requirement for lysine and the association of other occurrences of phage production with physiologically unrelated mutations argues that this was not a coincidence.

The association of the mutations affecting productivity, immunity and unrelated bacterial functions suggests that the event leading to these hereditary changes involves some kind of position effect due to spatial rearrangements rather than merely to the simultaneous concordance of several "point mutations". A third possibility, that the changes reflect segregation from a diplophase state, although not excluded, appears unlikely at this point.

What are the conceivable ways in which spatial rearrangements of genetic elements could simultaneously affect phage and bacterial genes? A classification of possibilities for which there is some precedent, in observation or theory, follows.

a. The Phage Genome Remains Intact

1. The Phage Genome Remains Fixed to a Definite
Map Attachment Site

(a) Rearrangement of the Classical Type, Involving
Large Blocks of Genes

The chromosomes of higher organisms are distinguished by the presence of two kinds of chromatin - euchromatin and heterochromatin. The functional genes are all included in the euchromatin portion while the heterochromatin is devoid of genes in the usual sense. Although the role of the heterochromatin has been difficult to define,

position effect studies have demonstrated that it exerts a profound effect on the functioning of euchromatic genes placed by rearrangement in its vicinity.

Although heterochromatin-like regions have not been demonstrated in the bacterial chromosome it seems quite possible that such regions do exist. Genes within the vicinity of these regions would be unable to be expressed but may express themselves if transferred to a different position away from the influence of the heterochromatic region. Thus SB2 may be visualized as having the prophage-like element placed very close to a heterochromatic region of the chromosome. As result of a translocation, the positions of lysine and the prophage loci may be traded, so that the cell becomes lys⁻ and lysogenic. The translocation could include a small portion of the heterochromatin so that the translocated gene even in its new position is still nonfunctioning. Later removal of the heterochromatin (e.g. by another translocation) then allows the gene to be expressed. The spontaneous origin of the cyt⁻ lysogenic may represent such a situation.

(b) Rearrangements Which Involve Movement of a Small Element from Locus to Locus

(1) A Dissociator-like Element Which Represses Adjacent Genes

McClintock discovered in a variable and mutable strain of maize, the two unit Activator-Dissociator system (1951). The two loci were shown to be in control of genic action in the course of development. Some of the most significant and pertinent properties of the

Ac-Ds system are:

1. Ac controls both the activity of Ds and the time in development when Ds acts; Ds is inactive when Ac is absent.
2. Ac is unstable and can exist in a number of different states similar to the allelic state of 'ordinary' genes.
3. Ds is unstable in the presence of Ac and can exist in a number of different states.
4. Both Ac and Ds are capable of movement (transposition) within and among chromosomes of the complement.
5. Ds has a dual action in the presence of Ac; first, it is the site of high breakability and second, Ds affects genes lying adjacent to it.
6. Certain loci have been discovered that remain mutable but which require the presence of Ac no longer.
7. Genes affected by Ac and Ds are many and varied in their phenotypic expression and the direction of mutation can be toward lowered or enhanced degrees of expressivity and it can be toward recessiveness or dominance.

Two of the most fundamental properties of the Ac-Ds system are; (1) movement of the Ac and Ds genes, and (2) genes adjacent to Ds are affected.

A system as refined and elaborate as the Ac-Ds system in maize is not postulated as the basis for the changes in the strains of S. blegdam, but there are certain properties of these cultures that are suggestive of the behaviour of a movable locus which affects adjacent genes.

1. The acquisition of the property of lysogeny was acquired only in those strains in which a mutation had occurred elsewhere in the bacterial genome. It can be visualized that were a Dissociator-like element present, occupying a site adjacent to the prophage region, the cell would be sensitive, express no immunity and, as in SB2, be prototrophic. SB5 would therefore represent a culture in which the Dissociator-like element was relocated adjacent to lysine thus releasing the suppression on the prophage, allowing the cell to express its lysogeny and immunity.

2. Mutations at more than one locus were associated with the transition to lysogeny.

Expression of the lysogeny occurred, however, after the acquisition of the cytosine requirement in SB4. This situation could be the result of a 'delayed effect' of the Ds-like gene due either to an activation by an Ac-like gene or to the removal of a suppression or to the delayed relocation of prophage from the 'non-inducible' region.

(2) An Operator-like Element which Activates Genes

Jacob and Wollman (1961) proposed a unique theory for the genetic control of regulatory mechanisms in bacteria. Briefly, the transfer of structural information from genes to protein is regulated by the intermediation of specific repressors synthesized by regulator genes. The repressor acts on an entity which in some way commands the expression of the structural gene and is called the operator. Operators are genetic units and are therefore subject to mutations. Similarly, regulator genes are also subject to mutation. Mutations of the regulator gene which result in the

synthesis of an inactive repressor are recessive in heterozygotes; mutations of the operator which result in decreased sensitivity to the repressor are dominant in heterozygotes. A single repressor may influence the synthesis of several proteins which belong to a single biochemical sequence and thus the operator is a structure common to the systems determining the synthesis of the individual proteins of the sequence. An integrated unit of activity containing the operator locus and the group of structural genes is called the operon.

In the Jacob and Wollman theory, one operator controls the synthesis of the individual proteins in a single biochemical sequence. The possibility that operators may control more than one series of related genes is not entertained. But since there is not yet any interpretation of the role of the operator at the molecular level, it is equally conceivable that one operator may be able to regulate more than one biochemical sequence but does not ordinarily regulate more than one, by reason, for example, of its position on the chromosome. An operator may control sequence A in position A but by translocation also control sequence B in position B. Thus SB5 might represent the transfer of the operator from the lysine operon to a position where it then loses its lysine control but then controls prophage functions. The cell is then lysogenic and lys⁻.

2. Phage Genome is Always Fixed but to Different Sites on the Chromosome

From the results of the UV induction experiments, in particular, phage was visualized as becoming attached to different sites on

the bacterial chromosome. Any part of the chromosome is considered capable of becoming the site of attachment of the prophage. The association between the prophage and a particular site could be that of peculiar attraction, as previously stated (by, for example, reason of 'free' DNA ends at the mutation site). It is also possible that prophage, acting as a mutator-type gene itself, could suppress the activity of the bacterial gene at the site of attachment. Although this explanation can possibly be used to explain the origin of SB5, it is difficult to reconcile with the origin of the cyt-lysogenic strain, SB23, in which the mutation occurred before the acquisition of the lysogeny.

3. Phage Genome is Sometimes Fixed and Sometimes Cytoplasmic

The genetic material of prophage may exist in two distinct states - either integrated into the chromosome or autonomous in the cytoplasm. Bacterial conjugation studies have shown that there exist other genetic elements which behave similarly to prophage and for these elements Jacob and Wollman proposed the term "episomes".(1958). Such episomic elements include, in addition to prophage, the F factor and factors that control colicin production. Although change from the integrated to the autonomous state in lambda prophage is considered by Jacob and Wollman to lead to lysis of the cell this may not be true for all phages. Thus prophage in SB2 and its sensitive derivatives may be cytoplasmically situated and be chromosomally located in lysogenic cells, or vice versa.

b. Phage Genome Undergoes Translocations with the Bacterial Genome

Studies with the lambda phage of E. coli K12 (Kaiser, 1957) and P22 of S. typhimurium (Levine, 1957) showed that the ability of a temperate phage to lysogenize is controlled by a small region in the prophage linkage group called the C region. The C region controls the series of reactions that enable the genetic material of the phage to establish itself as prophage and determine the specific immunity conferred by the prophage upon the lysogenic bacterium. Kaiser and Jacob (1957) demonstrated that the C region also controls the specific location that prophage occupies on the bacterial chromosome.

Recombination between the bacterial genome and the phage genome is known to occur. Prophage lambda can recombine with the adjacent region of the chromosome and incorporate into its linkage group the closely linked markers that control galactose fermentation. The resulting recombinant phage is, however, defective (called lambda dg) but 1/4 to 1/3 of the prophage genome has been replaced by a segment of the bacterial genome (Jacob and Wollman, 1961). The density gradient studies of Weigle, Meselson and Paigen (1959) in which lambda dg was shown to have a higher density than ordinary lambda, added support to the genetic studies that in lambda dg, the defectiveness and the transducing ability of the gal locus could be explained as consequences of the loss of the phage specific DNA accompanied by non-reciprocal acquisition of host specific DNA.

Haploid segregants in which the C region of lambda dg is incorporated into the bacterial chromosome have, however, never been encountered. That is, a segment of the bacterial chromosome may become incorporated into

prophage but the converse does not appear to occur.

The lysogenic SB2 derivatives may, however, represent such a reciprocal exchange. If SB2 does indeed possess most of the genetic structure of prophage but lacks the region which controls the reactions enabling the phage to establish itself as prophage and to express its specific immunity, the cell, although possessing most of the phage genome, would be phage sensitive and non-lysogenic. At some time in its ancestral history, SB2 may have been normally lysogenic but either a reciprocal exchange occurred or the prophage 'donated' or 'lost' this region to the bacterial genome (without exchange). The change from sensitivity to lysogeny would thus represent a reinsertion into the prophage genome of the C region carrying with it an adjacent part of the bacterial genome (such as the genes for lysine or cytosine synthesis). Conversion to lysogeny is therefore coincident with mutation at another locus.

The change to lysogeny by the cyt⁻ auxotroph, SB4, occurred, however, after the occurrence of the mutation in which the cell remained sensitive. In this event, the region from the bacterial genome may have become detached creating a mutation due to a deletion. The chromosomal segment then remained cytoplasmic, replicated independently but at a similar pace to the host, and only occasionally became integrated into the prophage region thus converting the cell to lysogenic.

The major headings in this classification are sufficiently general to include the final explanation for the change from sensitivity to lysogenicity. Data presented up to this point do not permit a decision between them,

however, and it is not argued that the special possibilities cited as examples are true. The classification, however, provides a useful theoretical framework upon which later observations can be discussed.

B. The Origin of Alternate Auxotrophs

1. The Origin of Phenylalanine Requiring Cells in SB5

Culture SB5 had been placed into stock on NA slopes and kept at room temperature for a period of two years with two serial subcultures. When tested on DMA after this period, it was noted that washed suspensions grew appreciably better than when first isolated. To check for the suggested accumulation of prototrophic revertants, an overnight L-broth culture of SB5 was diluted and plated out to obtain isolated colonies on NA. These plates were then replicated to DMA. Approximately 1,000 colonies were tested. Two prototrophs were indeed found; but surprisingly, the most numerous new type (about 2%) was found to be an auxotroph which required phenylalanine.

The pa⁻ cultures derived from appropriate colonies were shown to be lysogenic for a phage indistinguishable from the phages previously isolated (from SB1 and SB5) and described in the preceding section. The phage isolated from one pa⁻ culture (SB21) and called PSB21, had a similar plaque morphology to PSB1, the same host range (SB2 and SB4 lysed, SB5 and SB23 not lysed), the same heat resistance (surviving 30 minutes at 80°C) and identical serology (88% PSB21 neutralized in 30 minutes by antiserum prepared against PSB1).

Although SB21 (and the other pa⁻ bacteria) was like SB5 in the phage it

carried, there was a noticeable difference between lys⁻ and pa⁻ colonies. When plates were either incubated for two to three days at 37°C or were kept at room temperature for a similar period, pa⁻ colonies developed a thick, raised wall of gummy material which was not shown by lys⁻ colonies. This phenomenon appears to be similar to that cited by Wilson and Miles (1955) as being described by Muller in 1910 and was common in freshly isolated strains of Salmonella. Since Muller used this characteristic to distinguish between certain Salmonella strains, he called it the "Schleimwall-Versuch" (slime wall test). Its appearance was fairly common amongst freshly isolated strains of S. paratyphi B and was rare in S. typhimurium. The mucoid material was reported by Birch-Hirschfeld (1935) to contain a polysaccharide which was antigenically the same no matter by what type of Salmonella it was formed.

The possibility that SB21 and the other pa⁻ cells were contaminants may be excluded since (1) these cultures carry a phage similar to PSB5 and (2) no other pa⁻ Salmonella was in use in the laboratory. Further, and even more compelling reasons are implicit in the observations detailed below.

It therefore appears that a lys⁻ strain (SB5) gave rise to cells with a new and alternative requirement for phenylalanine. Since the syntheses of these two amino acids are not known to be related, it does not seem fruitful to entertain the idea that the two mutant genes are alleles. On classical precedent, it should be simplest to argue that the pa⁻ cells arose in two steps: First, the reversion of lys⁻ pa⁺ to lys⁺ pa⁺, followed by the origin of lys⁺ pa⁻ mutants from these. An alternative sequence is lys⁻ pa⁺ to

lys⁻ pa⁻ leading to lys⁺ pa⁻. In view of the high frequency observed of the end product, it would be necessary to assume strong selection for both the first and second step mutants.

The existence of cells expected to result from the first step (that is, the prototrophs) in the old culture SB5 is consistent with the first interpretation given above. On the other hand, the relative rarity of these prototrophs and the absence of any detectable lys⁻ pa⁻ mutants raise the alternative possibility that pa⁻ cells may derive directly from lys⁻ cells by some kind of novel mutational event, similar to or identical with the event which it has been suggested gave rise to the lysogenic, lys⁻ strain (SB5) from the non-lysogenic prototroph (SB2).

2. The Conditions of Origin of Alternative Auxotrophic Mutants

To examine the possibility that lys⁻ cells may change directly into the alternatively auxotrophic pa⁻ cells, the following series of experiments was performed.

a. An Upper Limit to the Frequency of Origin of pa⁻ Cells Under Standard Conditions of Growth

From a plating of the old SB5 culture on NA, one lys⁻ colony was picked into L-broth and incubated overnight. The culture was then washed twice in saline, concentrated 2x and 0.1 ml plated on DMA and on DMA-phenylalanine. The plates were incubated for two days. About 50-100 revertant-type colonies appeared on both plates. It was expected that some of the colonies on the supplemented DMA would be of the new type provided the frequency of the alternative auxotrophic change were sufficiently high.

However, on replication of the DMA-phenylalanine plates to DMA, all colonies proved to be prototrophic. Since approximately 10^9 bacteria were plated, the frequency of alternative auxotrophs within the plated culture was less than 10^{-9} . The conditions of this test, however, do not duplicate those under which the pa⁻ mutants were originally found; i.e. storage at room temperature. Thus, the alternative auxotrophic change may either arise more frequently under the other conditions, or (more probably, in the absence of other information) the high frequency of the new type may be due to new and strong selection of rare mutants under the conditions of storage.

b. The Repeated Occurrence of pa⁻ Auxotrophs in Stored Cultures

In view of the preceding results, an experiment was performed which attempted to duplicate, though over a shorter time interval, the cultural conditions under which pa⁻ alternative mutants had been initially observed.

Ten well isolated colonies of lys⁻ were picked from a plating on NA of the old SB5 culture and plated out on individual NA plates. The plates were incubated overnight and tested for growth requirements. This latter test was done by removing a sample of the growth from each plate, emulsifying it in saline, diluting and plating out on NA to give isolated colonies; these colonies were then replicated to DMA, DMA-lysine and DMA-phenylalanine. All cultures tested (about 100 colonies for each of the ten cultures) were lys⁻.

The ten cultures were then allowed to remain at room temperature for

two weeks. After this two-week storage period, samples of each culture were inoculated into L-broth, incubated overnight, and again plated out on NA to give isolated colonies. The plates were then tested for growth requirements as before. Of these ten lys⁻ cultures, two contained detectable pa⁻ cells (four of a total of 152 in one culture and one out of 123 in the other). In the remaining cultures, the frequency of pa⁻ cells, if present, was below the level of detection (i.e. less than 1%). These results are shown in Table IIa.

c. The Instability of pa⁻ Cultures

The preceding experiment, although very suggestive, does not exclude rigorously the two-step origin of the pa⁻ cells. In fact, if the frequency of origin of these cells is as rare as is indicated by the first experiment above, the two step hypothesis would be very difficult to exclude. However, another pertinent piece of information was obtained by observations on the instability of the pa⁻ derivatives.

At the same time that the ten lys⁻ cultures were studied (reported in the preceding section), ten pa⁻ cultures derived from isolated colonies of the old (and mixed) SB5 culture were treated in the same way as the ten lys⁻ cultures. When first tested, only pa⁻ colonies were seen but after two weeks storage, three of these cultures were found to be predominantly lys⁻ (Table IIb). One culture also contained a (detectably) large number of prototrophs, but again no lys⁻ pa⁻ cells were found. To explain the accumulation of these large numbers of alternative auxotrophs in initially pa⁻ cultures, one could again postulate a two-step mutational sequence with

TABLE II

a. Production of Alternate Auxotrophs in lys^- Cultures Stored for Two Weeks

Culture	Total cols. tested	lys^-	pa^-	prototrophs	other
lys^-1	152	148	4	0	0
lys^-2	123	122	1	0	0
lys^-3-10	~100	~100	0	0	0

b. Production of Alternate Auxotrophs in pa^- Cultures Stored for Two Weeks

Culture	Total cols. tested	lys^-	pa^-	prototrophs	other
pa^-1	273	183	68	2	20
pa^-2	95	69	26	0	0
pa^-3	113	74	39	0	0
pa^-4-10	~100	0	~100	0	0

strong selection for the products of both steps. But it is a remarkable coincidence, on this basis, that the new auxotroph which appears in these cultures is of the same type from which the pa^- itself was derived.

Furthermore, such an explanation would be hard pressed to explain the apparent, strong selective advantage of pa^- cells over lys^- cells at one time, and the reverse at another time.

In fact, this contradiction does not so much argue against the two step nature of the transitions from lys^- to pa^- and vice versa, but rather

argues against selection as the sole factor responsible for the high frequencies of these new types. It is suggested that some process occurs with a frequency much greater than that of 'normal' mutations under the conditions of storage at room temperature. This process could be either (1) the direct transition of pa⁻ to lys⁻ and lys⁻ to pa⁻ or (2) the transition from prototrophy to either lys⁻ or pa⁻. On the second explanation, it may be argued that prototrophic revertants of both pa⁻ and lys⁻ cells occur with mutational frequency but that these prototrophs are extremely unstable and change in certain predictable directions; i.e. to pa⁻ or to lys⁻.

A decision between the above two possibilities cannot be made on the basis of the present data alone, but it should be pointed out that later experiments indicate that prototrophs isolated from the old SB5 culture are unstable in a curious way. Furthermore, the second explanation has the advantage that it does not need to invoke a higher rate of change under conditions of storage than under ordinary cultural conditions. All of the prototrophic revertants observed on direct plating of even young cultures may have contained large numbers of derivative pa⁻ or lys⁻ cells.

One other result that developed from the tests with the stored, originally pure pa⁻ cultures from SB5, was the discovery in one culture, not only of lys⁻ cells, but also of prototrophs and other auxotrophs (Table IIb). These other auxotrophs were found to possess the double requirement of phenylalanine and tyrosine. Thus, for the moment, it is appropriate to discuss these pa⁻tyr⁻ cells which are by no means of merely incidental interest.

d. The Properties of the $pa^{-}tyr^{-}$ Derivatives of pa^{-} Cells

The colonies of the $pa^{-}tyr^{-}$ cultures differed from the pa^{-} colonies in lacking the gummy wall. It was also noted that some $pa^{-}tyr^{-}$ cultures, when transferred to L-broth and plated out on L-agar after overnight incubation, appeared 'phage infected' - i.e. the overnight growth of such cultures was covered with temperate-type plaques. Sixteen separate $pa^{-}tyr^{-}$ cultures were picked from a plating on NA of the culture in which they originally appeared (now designated SB7). These sixteen colonies, after overnight growth in L-broth, were tested for lysis by phage PSB1 and lysogenicity on SB2. It was found that nine were sensitive, two were immune and productive while five gave the 'phage infected' appearance but were lysed by PSB1.

On two other separate occasions, $pa^{-}tyr^{-}$ colonies were again picked directly from platings of SB7 and tested for sensitivity to PSB1 and lysogenicity on SB2. Of 25 colonies picked in one test, 16 were immune and productive, six were sensitive while three were 'phage infected'. Of 32 colonies picked in the later test, 25 were immune and productive, seven were sensitive and none was 'phage infected'.

In section A of the Results it became apparent that changes from sensitivity to lysogeny could occasionally occur but the change usually seemed to be associated directly or indirectly with another physiologically unrelated mutation. Some of the $pa^{-}tyr^{-}$ cultures appear to represent the reverse change from lysogenicity back to sensitivity, the change once again being associated with another physiologically unrelated mutation.

The simplest interpretation of the pa⁻tyr⁻ mutation, at this point, would be that a second mutation has occurred at a tyr locus, followed by selection for the double mutant. However, in view of the alternative auxotrophic mutations already detected in this system, it is appropriate to consider the possibility that the change observed here is also of this type. The alternative conditions here would be, on the one hand, non-function of a pa⁻ locus and, on the other hand, non-function of another locus concerned with an earlier step in the synthesis of these two related aromatic amino acids (c.f. Davis 1952). A decision between these two interpretations of the origin of the pa⁻tyr⁻ cells should be possible by determining whether the two deficiencies are lost together as a result of either mutation or recombination. Unfortunately, the two tests do not give completely unambiguous results.

(1) The Mutational Test

Washed suspensions of pa⁻tyr⁻ cultures were plated out on DMA, DMA-phenylalanine and DMA-tyrosine and incubated for 48 hours to test for reversion. No colonies were found on the DMA or the DMA-phenylalanine plates but many colonies (at a frequency of 1×10^{-5}) came up on the DMA-tyrosine plate. By the replication tests, these colonies were all shown to require tyrosine only. Results were the same whether the pa⁻tyr⁻ culture were lysogenic or non-lysogenic. The tyr⁻ revertants of of lysogenic pa⁻tyr⁻ cultures were similarly lysogenic, those of non-lysogenic pa⁻tyr⁻ cultures were also non-lysogenic, and those of 'phage infected' pa⁻tyr⁻ cultures were lysogenic. When plated out on DMA, tyr⁻

cultures produced no revertants.

Thus pa⁻tyr⁻ cultures, although showing no detectable reversion to prototrophy, very easily revert to tyr⁻. This result is consistent with the hypothesis that pa and tyr are separate loci.

(2) The Recombination Test

When phage PSB1 was used as a transducing vector it was found that pa and tyr were transduced together in 100% of transductants tested. For the transduction, 0.5 ml of a sterile preparation of phage PSB1 (propagated on SB2, titer 4.5×10^8 /ml) was mixed with an equal volume of an overnight L-broth culture of a non-lysogenic pa⁻ tyr⁻ culture (SB33). After 15 minutes at 37°C, 0.1 ml aliquots of the phage/cell mixture were plated out on DMA and DMA-phenylalanine and incubated 48 hours. The numbers of prototrophic colonies that developed were counted. The culture, plated out without phage, constituted the control. Results of two separate experiments are shown in Table III:

TABLE III

Transduction of pa and tyr to SB33 with PSB1

Cells plated	Phage titer	Mult. of infection	Spontaneous revertants on DMA	Cols. on DMA	Cols. on DMA-phenylalanine
8.5×10^7	4.5×10^8	.53	0	8	36
1.0×10^8	4.5×10^8	.43	0	15	36

Fewer transductants appeared on the unsupplemented DMA plates than on DMA supplemented with phenylalanine. However, replica platings showed that colonies on both types of plate were prototrophic. Thus the pa and tyr loci have so far proved inseparable by recombination.

These results are reconcilable with the mutational test, of course, simply by arguing that the pa and tyr loci are distinct but very closely linked. In other words, it is simplest to infer that the pa⁻ to pa⁻ tyr⁻ change was not an alternative auxotrophic mutation.

e. The Relationship of the tyr⁻ Mutations to Lysogeny

It is now appropriate to examine the interesting reversion to sensitivity characteristic of some of the pa⁻tyr⁻ cultures. It should be recalled that the pa⁻tyr⁻ colonies derived from SB7 were of three types: immune and productive (lysogenic), non-immune and non-productive (sensitive) and non-immune and non-productive ('phage infected'). The relative frequency of these types changed with time, the sensitive type decreasing while the lysogenic type increased.

This can be explained by arguing that all pa⁻tyr⁻ mutants are initially sensitive, i.e. that the acquisition of the tyr⁻ mutation is accompanied by a loss of both properties of lysogeny. In a mixed population containing a majority of lysogenic cells (the lys⁻ and pa⁻ cells) the sensitive cells would tend to become infected leading eventually to a predominance of secondary lysogenic cells.

However, there are several aspects of the situation that do not readily fit this simple picture: (1) How did sensitive cells (liable to

infection) come to be so numerous in the first place? (2) Why are immune non-productive cells, so frequent in SB2, not observed here? (3) How does one explain the delayed appearance of phage in the 'phage infected' clones?

In attempts to answer the first question, it could be argued that there is some very strong selective advantage of the pa⁻tyr⁻ cells over other cells in the population or that (as suggested for the origin of pa⁻ and lys⁻ cells) there is some event which occurs with much higher than mutational frequency. Again, this might involve the instability of a prototrophic antecedent.

In approaching the second and third questions, it can be noted that in at least some pa⁻ cells, there has occurred a coupling of the mutations to tyr⁻ with loss of lysogenic properties, similar in all general respects to the occasional coupling of mal mutation and phage property mutations discussed in an earlier section. The observations upon which the second and third questions are based both argue against the origin of the 'phage infected' clones via infection. The following observations are closely related to the problem.

It was found the 'phage infected' cultures on storage became immune to lysis by PSB1. Plated out on L-agar the stored cultures no longer showed the 'phage infected' appearance. Thus lysogenic cells are readily obtained from these basically sensitive cultures without the obvious appearance of immune, non-productive cells. The situation is very comparable to that in which SB23 arose and the same inferences are

possible, that lysogenic cells arose mutationally in these populations (thus acquiring simultaneously the properties of immunity and productivity) which account for their 'phage infected' properties.

It is significant to note that the lysogenic cells derived from the 'phage infected' pa⁻tyr⁻ clones are still pa⁻tyr⁻. Thus, the initial coupling of mutational events at the tyr⁻ and phage property loci is no longer in effect once the coupled mutations have occurred. Furthermore, reversions at the pa locus do not alter the phage properties of an initially pa⁻tyr⁻ bacterium regardless of whether it is sensitive or lysogenic.

The fact that some of the pa⁻tyr⁻ cultures were stably sensitive while others were unstable (the 'phage infected' cultures) deserves comment. We seem to be presented here with a situation comparable in many ways to that illustrated by mal papillation. Some of the tyr⁻ mutations seemed to have rendered the cell more 'mutable' for the reversion to lysogeny. However, the possible significance of this and its relationship to the mal papillae situation will not be discussed here.

f. The Productivity and Inducibility of lys⁻, pa⁻, pa⁻tyr⁻ and tyr⁻ Strains

In view of the intimate relationship between lysogeny and the tyr⁻ mutation, the lys⁻ and pa⁻ alternative auxotrophs were examined more closely for associated differences in phage properties. Specifically, SB5 and SB21 were compared with regard to level of productivity and UV-inducibility. The lysogenic pa⁻tyr⁻ (SB35) and tyr⁻ (SB29) derivatives

were also examined for these properties.

TABLE IV

Productivity and Inducibility Values of SB5, SB21, SB35 and SB29

Culture	Growth req.	Free phage without UV	Free phage with UV	With UV/ without UV
SB5	<u>lys</u> ⁻	7.6×10^2	5×10^4	66
SB21	<u>pa</u> ⁻	40	1.1×10^3	28
SB35	<u>pa</u> ⁻ <u>tyr</u> ⁻	2×10^2	3.6×10^4	180
SB29	<u>tyr</u> ⁻	7.1×10^3	1.8×10^5	25

From these results it is clear that changes in phage properties accompany both of the principal auxotrophic changes discussed here. The change from lys⁻ to an alternative pa⁻ requirement is coupled to a lowering of both productivity and the inducibility ratio. The change from pa⁻ to pa⁻tyr⁻ is coupled either to a loss in both productivity and immunity or (directly or indirectly) to a heightened productivity and inducibility. The change from pa⁻tyr⁻ to tyr⁻ in lysogenic cells is accompanied by an increase in productivity but a lowering of the inducibility ratio. It therefore seems very likely that both sets of unorthodox phenomena are intimately related and due to a common underlying process.

3. The Mechanism Underlying Unorthodox Auxotrophic Changes and Associated Changes Affecting the Lysogenic State

There are several reports in the recent literature pertinent to

the changes described here. Kaplan (1961), working with Serratia marcescens, strain CV, observed the occurrence of alternative auxotrophic mutants and was able to demonstrate that direct changes from one auxotrophic type to another could occur without reversion first to prototrophy. By means of a special auxanographic technique in which auxotrophs which required substance A were plated out on minimal medium supplemented with substance B, Kaplan found that among 60 monoauxotrophs, there were five which spontaneously mutated to an auxotrophic state requiring substance B, coincidentally losing the need for the first one (i.e. substance A). To this occurrence Kaplan gave the name "Auxotrophiesprungmutation" (auxotrophic leap mutation). He demonstrated that the auxotrophic leap occurred in one step without the mediation of prototrophy. Examples of such auxotrophic leaps were $\underline{\text{arg}}^- \rightleftharpoons \underline{\text{nic}}^-$, $\underline{\text{nic}}^- \rightleftharpoons \underline{\text{met}}^-$, $\underline{\text{pro}}^- \rightleftharpoons \underline{\text{his}}^-$, $\underline{\text{leu}}^- \rightleftharpoons \underline{\text{ad}}^- + \underline{\text{hyx}}^-$. He postulated that a mutation at one locus suppressed mutations at other loci, that the information on the specificity of an enzyme could be stored not only in a single gene but in several. This theory, however, is at variance with the "one gene-one enzyme" hypothesis.

Another example of auxotrophic changes was reported by Grigg and Sergeant (1961) with Neurospora crassa. Certain back mutations of a particular mutant were associated with a high frequency of simultaneous "switch mutations" of another character in the same genome. Two examples of switch mutations of a his-3 mutant were $\underline{\text{his}}^- \rightarrow \underline{\text{his}}^+ \underline{\text{pan}}^-$ and $\underline{\text{his}}^- \rightarrow \underline{\text{his}}^+ \underline{\text{c}}^-$ (c an unidentified auxotrophic character). To explain the high frequency of partial and intermediate back mutations a model based on

unequal sister strand exchange in multi-repeat loci was proposed. The recovery of complementary products of metabolism was cited as evidence for the plausability of the proposed model.

Ephrati-Elizur, Srinivasan and Zamenhof (1961) found that among nine his⁻ mutants obtained by heating spores of an indole⁻ strain of Bacillus subtilis, three lost or altered this indole requirement simultaneously with acquisition of the his⁻ requirement. Zamenhof and de Giovanni-Donnelly (1962) who extracted the DNA from two of these strains were able to transform the parent his⁺indole⁻ strain. They interpreted their results to indicate that various "non-linked" segments of the DNA molecule may not be mutationally independent but gave no suggestion of a possible mechanism of this dependence.

In a strain of Proteus mirabilis, Bohme (1961) found that reversion from streptomycin dependence to streptomycin independence was associated with a change in auxotrophy in 72% of revertants. The remaining 28% were prototrophic. The majority (70%) of the auxotrophs required the amino acids isoleucine and valine. Bohme hypothesized that the prototrophic revertants originated by mutation of the allele str-d (streptomycin dependence) to str-s (streptomycin non-dependence); the streptomycin non-dependence of the auxotrophic revertants was assumed to be the consequence of a suppressor mutation, the genotypical designation of which was str-d; sur-str.

A search of the older literature would probably uncover many more examples similar to these presented above, possibly all due to the same,

novel mechanism. For example, in E. coli, Lederberg (1957) found that the change from Lp_2^S to Lp_2^R (affecting the property of phage adsorption) was sometimes coupled with a change from mal^+ to mal^- : Richter (1958) found that the change from F^+ to Hfr in an E. coli K12 derivative, was, for a particular type of Hfr, always associated with a change from mal^+ to mal^- ; and Anderson (1946) found that T1 resistant, T5 sensitive mutants of E. coli B always required the amino acid tryptophan. This latter example suggests a relationship between phage-bacterium properties and unrelated properties of the host.

All the situations that have been cited here are suggestive of the same kind of positional, mutational event postulated in Part A of the Results to account for the present observations.

Assuming that a common mechanism underlies both the shifts in lysogenic state and the associated, physiologically unrelated mutations, the results presented in this section argue quite strongly against any explanation based upon reductions from a diplophase state, for both the alternative, auxotrophic changes and shifts in lysogenic state are readily reversible under appropriate conditions.

C. Atypical Changes Affecting H Antigens and Their Relation to Other Mutations

1. H Antigen Changes in pa^- Cells and Their Derivatives

The origin of pa^- alternative auxotrophs from SB5 (lys^-) has been described in the preceding section. The reasons for excluding contamination as an explanation for their appearance may again be

summarized: (1) The pa⁻ cells are lysogenic for a phage indistinguishable from PSB5 (2) no other pa⁻ Salmonella was in use in the laboratory (3) the origin of pa⁻ cells is a repeatable phenomenon and (4) the pa⁻ cells, under appropriate conditions, 'revert' to lys⁻. These reasons are sufficiently convincing to allow one to tolerate the remarkable additional observations reported below, which (in themselves) suggest contamination.

An overnight L-broth culture of the old SB5 culture was diluted and plated out on NA to obtain isolated colonies; then the isolated colonies were replica plated to identify their requirements. Twenty-two pa⁻ colonies and 25 lys⁻ colonies were subcultured to L-broth and incubated overnight. The resulting cultures were then tested for H agglutination using antisera prepared against SB2. The SB2 antiserum gave a tube agglutination titer of 1/20,480, final serum dilution (FSD), when tested with H suspensions of SB2. Since S. blegdam is monophasic for the antigens gmq the antiserum was called gmq antiserum. In order to simplify agglutination reactions with other cultures, the gmq antiserum was pre-diluted in saline to three different dilutions - 1/20, 1/1,000 and 1/10,000 and agglutination tests were done using only these three dilutions. Agglutination with all three dilutions was recorded as +++ and was taken to indicate that the culture was serologically identical with SB2, with 1/20 and 1/1,000 as ++ and 1/20 only as +.

All 25 lys⁻ cultures gave +++ agglutination; but the pa⁻ cultures were markedly different. Five of these gave + agglutination, while 17 were not agglutinated at all.

Microscopic examination and tests on motility medium showed that all pa⁻ cultures were motile, implying that a new flagellar antigen had been substituted for gmq.

In an attempt to identify the H antigens of the 22 pa⁻ cultures, all were tested against an array of commercial Salmonella H antisera in addition to the gmq antiserum prepared against SB2; the 25 lys⁻ cultures were similarly tested. The Lederle antisera were used only at the recommended 1/1,000 dilution and recorded as either + or -. Three of the pa⁻ cultures agglutinated only with the i antiserum, three with 1, 2, 3, 5, 6, 7, (hereafter abbreviated to 1, 2..) and 16 with both i and 1, 2... One only of the lys⁻ cultures agglutinated with the i antiserum in addition to the +++ agglutination with gmq; all other lys⁻ cultures agglutinated only +++ with gmq. Since a minority of the pa⁻ cultures (six of the 22) agglutinated only with i or 1, 2.. antisera and the majority agglutinated with both antisera, it was assumed here that all pa⁻ cultures were actually diphasic.

Thus the remarkable shift from lys⁻ to an alternative requirement for pa appears to be coupled to an equally remarkable shift from a monophasic gmq condition to a diphasic i: 1, 2.. condition. The latter antigens have never been described for S. blegdam and the i: 1, 2 serotype is characteristic of other Salmonella strains (notably S. typhimurium). S. typhimurium strains were in stock in the laboratory. Although the possibility of contamination has been ruled out satisfactorily on other grounds, it is appropriate here to note that these S. typhimurium strains were not in

current use, were all prototrophs and were all non-motile.

The coupling of auxotrophic and antigenic changes parallels the coupling of other changes reported earlier. Two features of these other couplings, however, do not seem to be characteristic of the present one: (1) That coupled mutations (e.g. mal papillation and gain of productivity) occur only in certain clones and (2) that once coupled mutations have occurred (e.g. lys⁻ and gain of lysogenicity) reverse mutations are not necessarily coupled. The following experiments illustrate this.

Twenty-six pa⁻ cultures that arose independently in the originally lys⁻ cultures isolated from SB5 and which appeared after the two week storage period (Table II) also agglutinated with either i and/or 1, 2.. antisera and only five gave + agglutination with gmq. Twenty-seven lys⁻ cultures which arose independently from originally pure pa⁻ cultures agglutinated +++ with gmq antiserum alone.

Thus, insofar as can be told from these observations, the lys⁻ and the pa⁻ properties are invariably associated with the diverse, antigenic properties of gmq and i: 1, 2.. respectively.

The possibility of antigenic changes associated with the origin of pa⁻tyr⁻ cells was then examined. A total of 47 separate pa⁻tyr⁻ cultures was tested at different times by the methods given for the pa⁻ and lys⁻ cultures. Results are shown in Table V.

TABLE V

Agglutination Tests with pa⁻tyr⁻ Strains

<u>gmg</u>	<u>Antisera</u>		<u>Number</u> <u>lysogenic</u>	<u>Number</u> <u>sensitive</u>	<u>Number</u> <u>'phage infected'</u>
	<u>i</u>	<u>1, 2..</u>			
++	-	-	22	8	3
++	-	+	2	1	0
++	+	+	7	1	0
+++	-	-	<u>3</u>	<u>0</u>	<u>0</u>
		<u>Total</u>	34	10	3

Clearly, another antigenic change is coupled with the tyr⁻ mutation. The antigenic change parallels that associated with the pa⁻ to lys⁻ change, but there are consistent differences in detail. It is especially noteworthy that most of the pa⁻tyr⁻ cultures do not react with anti-SB2 serum to the same titer as SB2 (or SB5). In 11 cases, this is attributable to the occurrence of other antigenic types in the culture (i and/or 1, 2..). These cultures appear to be triphasic although the existence of three distinct cell types (rather than the possession of more than one antigen by one cell) is only a likely inference.

In the majority (36) of the cultures, however, no i nor 1, 2.. antigens could be demonstrated. A routine check of these strains with an array of standard antisera was not made, so it is entirely possible that these strains are not monophasic (as SB2 and SB5 appear to be) but may exhibit H antigens so far unrelated to the system.

The serotypes of tyr⁻ cultures was then examined. Twenty-three separate cultures were obtained from revertants appearing on DMA-tyrosine of pa⁻tyr⁻ cultures. They included 24 that were lysogenic and nine that were non-lysogenic. Each culture agglutinated +++ with gmq and was not agglutinated by either i or 1, 2... The reversion of the pa⁻ gene therefore also appears to be coupled to an antigenic change, which might be interpreted as a restoration of the monophasic condition.

Representative lys⁻, pa⁻, pa⁻tyr⁻ and tyr⁻ cultures were also tested for agglutination with the SB2 O antiserum by the slide agglutination method. All were agglutinated similarly to the SB2 culture. There is therefore no evidence for a change in the O antigens of these auxotrophs although fractional changes (e.g. from the 9, 12 condition of S. blegdam to the 4, 12 condition of S. typhimurium) have not yet been excluded.

2. Attempts to Direct the Antigenic Changes

Alternative Salmonella H phases may be revealed by swarming cells through motility medium in which has been incorporated antiserum against the phase one wishes to suppress. An attempt was made therefore, to uncover i and 1, 2.. antigens in SB2 and in a lys⁻ culture. SB5 and SB2 were passaged three times through motility medium in which had been incorporated 0.1 ml of SB2 H antiserum. The swarming of both cultures was almost completely inhibited on the first plate but the size of the swarms gradually increased on serial passage. On the third passage the swarm extended to about 1 cm from the site of the original inoculum. On transfer to motility medium without the added antiserum both cultures again swarmed

well to the full diameter of the plate. The lysine requiring culture still retained the lysine requirement and SB2 was still prototrophic. Yet, neither culture when tested, agglutinated with i or 1, 2.. antisera and both agglutinated only + with the gmq antiserum. Thus the antigens i and 1, 2.. were not uncovered by antiserum passage although the titer with the gmq antiserum was altered so that it was similar to the titer of some pa⁻ cultures. Since both cultures showed good motility when finally removed from gmq antiserum, it may be suspected that unidentified H antigens were present.

However, the results do not allow the conclusion that SB2 and SB5 are diphasic gmq:x. (where x represents the unidentified antigen). On the contrary, the fact that good swarming on anti-gmq serum was never seen argues against a diphasic condition. What is suggested is that the changes are in the relative amounts of two or more antigens present on the flagella of all cells. For example, the change involved may be equivalent to the (hypothetical) conversion of S. blegdam (monophasic gmq) to S. rostock (monophasic gpu) or to S. enteritidis (monophasic gm). Precedent for this type of change is the conversion by growth in anti-d serum of S. salinatis (deh:denz₁₅) to S. sandiego (eh:enz₁₅) (Edwards and Bruner, 1942). An understanding of the changes involved here must await further serological tests.

For the present, the important point is that under 'standard' conditions of growth, the event which leads to the i:1, 2.. serotype is of the same order of rarity as the associated event leading to the pa⁻ condition;

neither has been detected despite rather powerful selective techniques. It may be noted here, however, that although transfer through specific antiserum is a highly sensitive test for the uncovering of other H antigens, all phases (of multiphasic Salmonella) are not necessarily freely reversible by this technique (e.g. see Bruner and Edwards, 1941).

In view of the changes in flagellar antigens accompanying auxotrophic variation discussed in the preceding section, it was thought that reversion back to prototrophy of pa⁻ cells might also be accompanied by a change of the flagellar antigens from i:1, 2.. back to gmq. Pa⁻ cultures reverted readily to prototrophy (frequency of revertant cells about 1×10^{-6}) as detected by plating out on DMA. Five different pa⁻ colonies were picked from a plating of SB5 on NA. An overnight, L-broth culture of each culture was washed twice in saline and 0.1 ml plated out on DMA plates and incubated for two days. A total of 85 prototroph colonies (representing a minimum of five independent reversions) were then picked into L-broth and tested for agglutination with antisera against antigens i, 1, 2.. and gmq. Eighty-four cultures agglutinated only with i and/or 1, 2.. but one culture gave +++ agglutination with the gmq antiserum. Thus the majority of prototrophic revertants of pa⁻ cells retained the H antigenic structure of the parent cell but one possessed antigens typical of SB2. Although the frequency of antigenic reversion was low it does show that pa⁻ cultures have not lost irrevocably the potentiality for the gmq genotype.

3. Significance of the Antigenic Changes

Salmonella are generally either monophasic or diphasic with

reference to their H antigens. That is, a given strain either consistently possesses one particular H antigenic complex or the cells of a strain exist in two alternative forms, each characterized by a particular H antigen complex. The diphasic condition is remarkable in two respects: (1) The probability of "mutation" from one phase to the other is frequently quite high (up to 10^{-3}) and (2) the two phases, though often serologically distinct, are mutually exclusive. Both of these properties suggest that phase variation is not an example of a classic mutational process. In fact, there is a strong similarity to that serotypic variation in Paramecium which is under cytoplasmic control. However, the studies of Lederberg and Edwards (1953) and Lederberg and Iino (1956) reveal through transduction experiments a unique basis for the determination of phase in Salmonella. The essential features of their explanation are as follows.

Each of the two phases is controlled by a specific locus - H₁ for phase 1 and H₂ for phase 2 - and that alternation of these phases depends on interrelationships between these two loci. The allele present at the H₂ locus may alternate between two states, one epistatic to H₁ in which the H₂ phenotype is expressed and the other hypostatic to H₁ which results in expression of the H₁ phenotype. The alternation of states could be interpreted by a variety of mechanisms. For example, one advanced by Jacob and Wollman (1961) was the alternation of a cytoplasmic element from the integrated state (epistatic) to the autonomous state (hypostatic).

Exceptions to the general rule that Salmonella strains are either monophasic or diphasic have been known for some time. Frequently, however,

the existence of third or fourth phases can only be demonstrated by the application of powerful selection methods - e.g. swarming through soft agar containing homologous antiserum.

The exceptions are of two general types. The first type of exception is exemplified by the selection of S. salinatis (deh:denz₁₅) of cells diphasic for eh:enz₁₅. Many other examples of this type of change may be cited (e.g. gm → gp, mt → gt, mt → gms, gmq → gm). Here, the new phases are closely related serologically to the old.

Lederberg and Iino postulated that antigenic variations of this type represented normal point mutations of either the H₁ or H₂ loci, and were therefore alleles of the same locus. As discussed earlier, the observed effects of anti-gmq serum upon SB2 may be interpreted in this way.

On the other hand, the second type of exception is much more difficult to accommodate to the Lederberg-Iino model without introducing important additional concepts. In these cases, the new phases observed represent profoundly different antigens than those initially present. Examples of this type of situation may be cited.

Edwards and Bruner (1939) described a strain of the normally monophasic S. abortus-equi (-; enx) with three phases: (a:enx:z₅) Bruner and Edwards (1941) further described a strain of S. paratyphi A with four serologically different phases (the three 'extra' phases were unnamed) rather than the normal one (-: a). The natural occurrence of four reversible and serologically unrelated phases in cultures of S. mikawashima (y:enz₁₅:z₄₇:z₅₀) was described by Edwards, Sakazaki and Kato (1962).

LeMinor and Edwards (1960) described three phases for S. goerlitz (z₂₇:eh:1,2). S. goerlitz cultures could be transformed by passage in appropriate antiserum motility medium to the diphasic form eh:1,2 but from such cultures the z₂₇ form could no longer be recovered by further appropriate antiserum passage. Taylor, Lee, Edwards and Ramsey (1960) described a culture monophasic for z₄₃ which changed irreversibly to the diphasic form lw:z typical of S. worthington. These workers also described many strains which when first isolated were diphasic for z₄₃ (z₄₅ or z₄₆) and gst which could be readily changed to the monophasic form gst characteristic of S. senftenberg but from which the z₄₃, z₄₅ or z₄₆ phase could no longer be recovered.

An anomaly to the typical H₁H₂ genetic control of phase variation was later found by Lederberg himself (1961) with a normally monophasic strain of S. paratyphi b (CDC-157). This strain normally possessed only the a antigen but rare reversions to 1, 2 occasionally occurred. Genetic analysis of this strain revealed that the 1,2 antigen was determined by an allele of the H₁ locus rather than the H₂ as is usual for this antigen and the situation was explained by arguing that CDC-157 was carrying a "diploid" H₁ locus.

Spicer and Datta (1959) reported on a strain of S. typhimurium in which the H antigens i:1,2 were altered by transduction to the form b:enx. The H antigens reverted in the first or second phases giving rise to organisms with the antigenic formulae b:1,2 and i:enx. Transduction of the b and enx antigens therefore had not replaced or substituted for the

original antigens (as originally postulated for transduction in general by Lederberg and Edwards, 1953). The results here may also be interpreted as the result of a diploid condition of the H₁ and H₂ loci. Under certain unspecified conditions one of the H₁ loci would be dominant over the other, and similarly for H₂.

Clearly, the situation observed here in the present experiments is an exception of this second more perplexing type. It seems unlikely that the genes for i and/or 1,2 specificity arose by point mutation from the gene for gmq. Instead, what is suggested is that most, if not all, of the hereditary specification for the i and 1,2.. antigens is carried by all SB2 strains but that these specifications are in some way prevented from being used. Only by some event, possibly positional, can these specifications be used.

The present system is unique in that associated changes affecting other bacterial properties have been studied simultaneously. The following points argue that all of these changes are related to the same underlying mechanism of change - and are significant to the eventual understanding of that change.

(1) There is a close parallel between the lysogenic traits and other bacterial traits in SB2 derivatives and the antigen system. As examples of a second or 'reserve' phase which is not expressed and which would parallel the non-expression of i and 1,2.. may be cited the lack of immunity and productivity in SB2 and the latent capacity to synthesize phenylalanine in SB21 and lysine in SB5. The immunity-to-productivity

change may be visualized as another example of 'phase variation' (although of course, the reverse change in single cells, from productivity to immunity alone is physically impossible) as also the $\text{lys}^- \text{pa}^+$ to $\text{lys}^+ \text{pa}^-$ change. The different degrees of productivity of the lysogenic derivatives may indicate variations in 'mutability' in different cultures homologous to the variations in mutability of mal fermentation in different SB2 cultures (papillae formation) previously discussed. Although it has been assumed that all cultures that exhibit either the i or the 1,2.. antigens are actually diphasic, it is possible that those which have (so far) exhibited only one may represent variations in mutability between the states $\text{i}^- \text{1,2..}^+$ and $\text{1}^+ \text{1,2..}^-$. Additional work will be necessary to determine this.

(2) There is a coupling of the antigenic changes to all of the previously mentioned traits. Antigens i and 1,2.. are expressed in cultures which require phenylalanine but are not expressed in cultures which require lysine. The $\text{pa}^- \text{i:1,2..}$ cultures have a lower productivity and inducibility ratio than $\text{lys}^- \text{gmq}$ cultures (Table IV) which indicate variations in mutability.

Taylor et al. (1960) have shown a correlation between the occurrence of the z₄₅ antigen and rapid fermentation of tartrate in Salmonella. This and other similar correlations may be explained in terms of coupled mutations similar to those described here.

D. Segregation-type Phenomena and Associated Origin of Novel Genotypes

1. Changes Involving a Lysogenic Prototroph Derived from SB5

One of the prototrophic colonies detected and isolated directly from platings of SB5 (see Section B, part 1; prototroph hereafter referred to as Prototroph 1) was immune to phage PSB1 and lysogenic on SB2. It was unusual in its agglutination reactions with the H antisera, however; it gave +++ agglutination with gmq antiserum but also agglutinated with antisera against antigens i and 1,2..; i.e. it behaved as a triphasic culture. In view of the two extra phases found to be possessed by pa⁻ cultures this strain was therefore considered especially significant. The culture was first tested to see if it were prototrophic or were actually composed of a mixture of lys⁻ and pa⁻ cells.

An overnight L-broth culture of Prototroph 1 was diluted and plated out on NA to obtain isolated colonies. After overnight incubation, 10 colonies were picked into L-broth and investigated. Four of the 10 cultures were found to be prototrophic, lysogenic and to be agglutinated by both i and 1,2.. antisera but not by gmq; five required lysine, were lysogenic, did not swarm on motility medium but agglutinated +++ with the gmq antiserum only; and one was an auxotroph which required phenylalanine, was lysogenic and agglutinated by i and 1,2.. antisera only. Thus Prototroph 1 did appear to be a mixture of pa⁻ and lys⁻ cells although four of the 10 cultures which originated as single cells tested as prototrophic. These observations suggest that Prototroph 1 is an unstable recombinant between lys⁻ and pa⁻ cells.

However, an additional and remarkable observation is not readily consistent with this interpretation. The four lys⁻ cultures were non-motile whereas the lys⁻ cultures isolated directly from platings of SB5 had been motile. Flagellar staining showed that the non-motile cells were actually peritrichously flagellated with from six to eight flagella per cell, and therefore could be described as "paralyzed" (Stocker, Zinder and Lederberg, 1953) or mot⁻. If Prototroph 1 had actually resulted from a rare sexual recombination between a lys⁻ and a pa⁻ cell it was to be expected that the segregant lys⁻ could be also motile, as the parent lys⁻.

The four prototrophic cultures from the first plating of Prototroph 1 were picked and tested further. Grown up in L-broth overnight, they were diluted and plated out on NA to obtain about 100 colonies per plate which were then replicated to DMA, DMA-lysine and DMA-phenylalanine. Each of the four cultures was now composed entirely of a mixture of lys⁻ and pa⁻ cells and no prototrophs were found. Thus 'segregation' to lys⁻ and pa⁻ of the original prototrophic colonies had occurred again. Fifteen lys⁻ colonies from a plate of each of the four prototrophic cultures tested were tested for swarming ability on motility medium. A total of 58 were non-motile and two were motile. Five non-motile lys⁻ cultures from each of the four original cultures were shown to be lysogenic as well as the two motile strains. All cultures agglutinated +++ with gmq antiserum and did not agglutinate with either i or 1,2.. antisera. Similarly, 20 pa⁻ cultures were lysogenic, motile, agglutinated with i and 1,2.. antisera

and gave no agglutination with gmq antiserum.

Thus, on five independent occasions Prototroph 1 has readily given rise to both pa⁻, i;1,2.. and lys⁻, gmq cells. On all five occasions the majority of the latter were mot⁻. The high frequency of the change, the complementarity of the two types, the predictability of the new types and pre-existence of most of the properties in the previous history of Prototroph 1 all argue that it is an unstable diploid. On the other hand, the non-motility of the lys⁻ derivatives raises serious questions about this interpretation. At best, it would have to be argued that Prototroph 1 arose by recombination involving a rare mot⁻ mutant in the original SB5 culture.

If the segregation explanation is valid, the association of the non-motility and the lys⁻ suggests that the two genetic loci might be closely linked and therefore transduce together. To test this, motility was transduced to a non-motile lys⁻ culture with phage PSB1 and resulting swarms tested for an associated change to prototrophy. Similarly, lys was transduced and resulting prototrophic transductants tested for an associated change to motility.

Equal volumes of a sterile preparation of phage PSB1 (titer 4.5×10^8 /ml) and an overnight L-broth culture of a non-motile lys⁻ strain (SB44) were mixed together and placed at 37°C for 15 minutes. Loopfuls of the phage/cell mixture were then streaked through a plate of MM and incubated to test for swarm production. The transductant mixture was also washed twice in saline and 0.1 ml plated out on DMA plates which were

incubated for 48 hours. For controls, SB44 without added phage was similarly treated and plated out in MM and on DMA.

On overnight incubation the culture streaked through MM showed many swarms while the control plate showed none. After 48 hours incubation, the transductant mixture on DMA showed many prototrophs while the control plate showed only few. Inocula taken from 10 different areas of the swarm (since well isolated swarms were not seen) were tested for growth requirement. Each of the 10 cultures still required lysine. Twenty different prototrophic colonies from the DMA transductant plate were picked and tested for motility and all found to be still non-motile.

Thus the genes for lysine utilization and motility do not appear to be closely linked on the bacterial chromosome as would be suspected on the segregation hypothesis. On the other hand, that hypothesis is not excluded by this negative information.

2. Significance of the Apparent lys^- to pa^- Segregation

It is very unlikely that Prototroph 1 is a normal prototrophic revertant obtained by direct selection of revertants appearing on DMA of either lys^- or pa^- cultures. Although not extensively tested for, this segregation-type behaviour was not shown by normal prototrophs of either auxotroph. There are two other possible explanations for the origin of Prototroph 1;

- (1) by recombination between a lys^- and a pa^- cell,
- (2) or as a representative of an 'intermediate stage' not involving diploidy in the transition to either lys^- or pa^- .

The first explanation also seems unlikely since it does not account for the occurrence of non-motility. Further, artificial mixtures in L-broth of lys⁻ and pa⁻ cultures have shown only lys⁻ and pa⁻ colonies on plating out. A more critical examination might show the presence of few recombinants; for recombination within some members of the Salmonella group has been shown to occur (Baron, 1960), at a very low frequency (1×10^{-8}).

The second explanation that Prototroph 1 may represent an intermediate stage in the production of alternate auxotrophs seems to be the most acceptable explanation at this stage. If this is indeed true the mechanism of production of alternate auxotrophs would therefore include a prototrophic stage. Kaplan demonstrated that the "auxotrophic leap mutation" with Serratia marcescens CV apparently occurred without the intermediacy of a prototrophic stage. The frequency of change in the SB2 derivatives has, however, been shown to be much less than the frequency in S. marcescens and may therefore represent a different mechanism. The finding that the lys⁻ cells that result from this particular prototroph are usually non-motile is merely evidence of another coupled mutation many of which have already been described earlier. However, the "memory" of earlier requirements exhibited by the derivatives of Prototroph 1 is remarkable.

3. Changes Involving an Immune, Non-productive Prototroph Derived from SB5

The second prototroph which was isolated from platings of SB5

(hereafter referred to as Prototroph 2) was immune to lysis by PSB1 but was non-productive in the Mixed Growth Test with SB2. It therefore resembled neither the lys⁻ nor the pa⁻ cultures but did resemble the immune prototrophs that arose in old cultures of the indicator strains, SB2. It was agglutinated with 0 antiserum prepared against SB2 and gave +++ agglutination with gmq antiserum, and was not agglutinated by either i or 1,2.. antisera. Ten independent cultures which were inoculated from single colonies on an NA plate of Prototroph 2 were similarly shown to be immune to PSB1, non-productive and agglutinated +++ by gmq antiserum and to be prototrophic. Prototroph 2 therefore did not resemble Prototroph 1 which readily 'segregated' to lys⁻ and pa⁻ auxotrophs. It also differed in being non-productive but the possibility must be recognized that Prototroph 1 may also have been non-productive - that only the segregants gave rise to phage. Two of the cultures which arose as single colonies from Prototroph 2 were placed into stock as SB37 and SB38 and are the cultures on which further work was done.

a. Segregation-like Phenomena in Derivatives of SB37

Soon after being placed into the stock collection, SB37 was retested for growth characteristics and lysogenicity. A total of 91 colonies on NA was replicated to DMA and shown to be prototrophic. Ten single colonies chosen from the NA plate were further tested for lysogenicity and phage sensitivity and shown to be immune to lysis by PSB1 and lysogenic by the Mixed Growth Test with SB2. Each of the 10 colonies was passaged through MM to prepare them for agglutination tests and each

swarmed well. Cultures inoculated from the edge of the swarm were agglutinated +++ with gmq antiserum. But in addition, seven were also agglutinated by i antiserum and three by both i and 1,2.. antisera. The i and 1,2.. agglutinations were therefore extra to the pure gmq agglutination originally obtained. Since each of the cultures originated as a single prototrophic colony, the agglutination reactions appeared to represent either a diphasic or triphasic situation in single clones similar to that described for some pa⁻tyr⁻ strains.

An additional unusual phenomenon was observed on the motility plates containing the swarms of the 10 individual isolates of SB37. These plates had been inoculated at the center and swarmed on overnight incubation at 37°C to about half the diameter of the plate. They had then been left on the bench at room temperature. After two days at this temperature it was observed that from the edge of the swarm many fan-like 'secondary swarms' had developed with the apex towards the edge of the primary swarm. After three days at room temperature these secondary swarms had grown out to a distance of about 1 cm from the site of origin. A possible interpretation of the phenomenon was that these 'secondary' swarms represented a selection for cells with the ability to swarm better at room temperature. This phenomenon had not been observed with other strains plated out in MM. Because of its unique nature the phenomenon was thought worthy of further investigation.

In the first test, four secondary swarms, each from a different primary swarm, were chosen. With a sterile wire, tubes of L-broth were

inoculated from the outside edge of the fan-like secondary swarm, and incubated. Cultures were then tested for growth requirements, for lysis by phage PSB1, for lysogenicity and agglutination with the H antisera. Three of the cultures were shown to be prototrophic but one was an auxotroph with the requirement for phenylalanine. Each was immune to lysis by PSB1 but lysogenic in the Mixed Growth Test with SB2. One prototroph agglutinated with i and 1,2.. antisera, a second prototroph agglutinated with i and 1,2 and gave ++ agglutination with gmq, the third prototroph agglutinated with i and 1,2.. and + with gmq. The pa⁻ auxotroph agglutinated with i, 1,2.. and gmq.

The secondary swarms therefore showed some remarkable and unexpected differences from the parent cultures:

(1) Each was lysogenic, whereas the parent cultures were immune but non-productive.

(2) One was an auxotroph and required phenylalanine.

(3) All showed some agglutination with i and 1,2.. antisera in contrast to SB37 and the 10 separate isolates of SB37; one prototrophic culture had completely lost its agglutinability with gmq antiserum.

Because of the unexpected nature of these four secondary swarms, additional secondary swarms were tested. Plates of MM were inoculated centrally with the 10 separate cultures previously isolated from SB37 and incubated overnight at 37°C. They were then allowed to remain on the bench at room temperature for an additional two days. A total of 45 additional secondary swarms was picked into L-broth and tested as previously. Results of the

tests for all secondary swarms are shown in Table VI.

Sixteen of the secondary swarms were prototrophic, 10 resembled the parent culture in being immune and non-productive but none of these agglutinated with gmq antiserum; 15 of these prototrophs agglutinated with either i or 1,2.. antisera and one was agglutinated by neither. This latter culture possibly represented a strain with a fourth, unidentified antigen. Twenty-nine of the swarms were auxotrophic. Twenty-eight were pa but a most surprising result was the isolation of a mutant with the completely new auxotrophic requirement of arginine. Each of the auxotrophs was lysogenic but none was agglutinated by gmq antiserum although all showed some agglutination with i and/or 1,2.. antisera.

The secondary swarm phenomenon therefore resulted in some unexpected developments. Except for two cultures of the first set of four secondary swarms tested, none of the cultures showed any agglutination with gmq antiserum although the original Prototroph 2 isolated directly from the SB5 plate and the 10 immediate single colony isolations from this culture gave +++ agglutination with gmq antiserum. In going from the original Prototroph 2 culture there seems to have been a progression from completely gmq through triphasic gmq:i:1,2.. to an almost total loss of gmq agglutinability with the gain of i and 1,2... The antigenic change was not completely correlated with acquisition of the auxotrophy for the prototrophic cultures had also lost gmq agglutinability.

A second unexpected result from the secondary swarms was the change from non-productivity in Prototroph 2 and SB37 to lysogeny in the secondary

TABLE VI

Agglutination Reactions of Prototroph 2 and Prototroph 2 Derivatives

Strain		gmq gmq;i	gmq;l,2..	gmq;i;l,2..	i l,2..	i;l,2..	Fourth antigen	No antigen	Not tested	Total
Prototroph 2, IM		10								10
SB37 passaged in MM	IM	7		3						10
<u>Secondary swarms from SB37</u>										
<u>1st Test</u>										
Prototroph	L			2	1					3
pa ⁻	L			1						1
<u>2nd Test</u>										
Prototroph,	L				2	4				6
Prototroph,	IM				3	2	4	1		10
pa ⁻ ,	L				12		16			28
arg ⁻	L						1			1
<u>Isolated cols. of SB42, after storage.</u>										
Prototroph,	IM	3		1	2				4	10
pa ⁻ ,	L			1	1			3		5

L=lysogenic

IM=immune

swarm cultures. Ten of the 16 prototrophs remained immune and non-productive but six were lysogenic and all the auxotrophs were lysogenic. The plaque produced by all lysogenic cultures resembled that already described for PSB1.

The third development was the discovery of a secondary swarm with the completely new auxotrophic requirement of arginine. Since phenylalanine and arginine appear to have separate biosynthetic pathways there appears to be no biochemical connection between the two auxotrophic types.

One pa⁻ culture which had arisen as a secondary swarm, which agglutinated with both i and 1,2 antisera, was selected and placed into stock as SB42. When plated out on EMB-maltose after approximately one month after being placed into stock, colonies of two types were seen - small, dark and sheenless and some larger pink colonies. The smaller colony predominated with about one large pink colony per 40 small dark colonies. Representative small and dark colonies were picked directly from the EMB medium into L-broth and tested for growth requirement, lysogeny, motility and H agglutinability. Results of these tests are also shown in Table VI.

When it is remembered that SB42 had its origin as a lysogenic, pa⁻ secondary swarm which developed from SB37 which was an immune, non-productive prototroph, the results with the SB42 derivatives are certainly unexpected. The results include:

- (1) The presence of prototrophic, immune, non-productive cultures

which were agglutinated with i and/or 1,2.. and + with gmq. These therefore resemble very closely the original culture from which the secondary swarm arose. The prototrophic cultures produced both small, dark colonies and large, pink colonies on EMB-maltose. These colonies appear to represent a 'reversion' back to the original type rather than reversions of the normal type which would be expected to be lysogenic.

(2) The presence of pa⁻ cultures which were lysogenic but which were non-motile (first detected by their inability to produce swarms in MM). No flagella were seen when two non-motile strains were appropriately stained. They were therefore non-motile because of the complete absence of flagella and in this respect differed from the lys⁻ cultures which 'segregated' from Prototroph 1, which possessed paralyzed flagella. In the genetic notation of Stocker et al. (1953) the non-motile, non-flagellated pa⁻ strains may be represented as fla⁻.

(3) The + agglutination with gmq antiserum of both prototrophic and auxotrophic cultures. The original SB42 agglutinated only with i and 1,2.. antisera. This reaction also represents a tendency to return to the property of the original parent culture and Prototroph 2.

(4) The non-motile pa⁻ cultures were typically 'rough' strains of Salmonella. In L-broth they produced a heavy floccular growth which settled at the bottom of the tube leaving a perfectly clear medium above, rather than the even turbidity of normal pa⁻ cultures. They were also found not to agglutinate with the O antiserum prepared against SB2 and produced rough colonies on NA. Non-motile, pa⁻ cultures also produced

small, dark colonies and large, pink colonies on EMB-maltose.

The prototrophs present in SB42 are obviously different from the typical prototrophic revertants of pa⁻ cells since these revertants retain the property of lysogenicity of the auxotroph. The SB42 prototrophs are immune and non-productive and therefore resemble more the parent culture from which SB42 originally developed as a secondary swarm. These results seem to indicate that the changes which occur in going from the primary to the secondary swarm are quite unstable and more or less reversible.

b. Segregation-like Phenomena in Derivatives of SB38

Soon after being placed into the stock collection but after the experiments with SB37, SB38 was also retested in a similar manner. An overnight L-broth culture of SB38 was plated out on NA to obtain isolated colonies which were then replicated to DMA. Of a total of 107 colonies replicated, 99 were prototrophic and eight were auxotrophs. Seven of these auxotrophs required phenylalanine but the eighth was of a new type and was found to require the vitamin nicotinic acid. A representative 10 prototrophs and all the auxotrophs were tested for lysogeny. Each of the prototrophs was immune to PSB1 and non-productive and therefore resembled the original parent, Prototroph 2. The pa⁻ and nic⁻ auxotrophs were both lysogenic. Tested serologically, all derivatives agglutinated with the SB2 0 antiserum; the prototrophs agglutinated with i and 1,2.. and +++ with gmq antisera; each of the pa⁻ cultures agglutinated with i and 1,2.. while the nic⁻ auxotroph agglutinated with i and + with gmq.

The results of the tests with these derivatives of SB38 are shown in Table VII.

TABLE VII
Auxotrophic, Lysogenicity and Agglutination Tests of
SB38 and its Single Colony Isolations

Culture	Number tested	Growth req.	Lysogenicity	Agglutination with		
				<u>i</u>	<u>1,2..</u>	<u>gmq</u>
SB38	1	Prototroph	immune	-	-	+++
SB38 derivatives	7	Prototroph	immune	+	-	+++
	3	Prototroph	immune	+	+	+++
	7	<u>pa</u> ⁻	lysogenic	+	+	-
	1	<u>nic</u> ⁻	lysogenic	+	-	+

Culture SB38 thus behaved somewhat differently from the SB37 culture which on plating out produced only prototrophic colonies. With both strains, the prototrophs remained immune and non-productive and gained the i and 1,2.. agglutination reactions in addition to agglutination with gmq of the original Prototroph 2. The pa⁻ cultures from SB38 resembled the pa⁻ cultures derived as secondary swarms from SB37. They therefore may have arisen by a similar genetic mechanism but without going through the swarming procedure that was necessary for SB37. Similarly the nic⁻ culture which appeared spontaneously could also have arisen in a similar manner to that which gave rise to the arg⁻ secondary swarm in SB37. Secondary swarms were produced in a similar manner to the SB37 prototrophs by the SB38

prototrophs but were not tested further. Thus the genetic mechanisms which gave rise to auxotrophs and which allowed also the 'unmasking' of lysogeny in some derivatives are probably identical for SB37 and SB38.

One of the SB38 prototrophic single colony isolations (SB64) was further tested. Subcultured to L-broth, incubated overnight and plated out to obtain isolated colonies on NA, SB64 was shown to 'segregate' further to prototrophs and pa⁻ auxotrophs. The prototrophs (three only tested) were immune non-productive and agglutinated with i and 1,2.. antisera; the pa⁻ cultures (two only tested) were lysogenic and agglutinated with i and 1,2.. antisera but not with gmq antiserum.

4. Significance of the Segregation-like Phenomena with Prototroph 2

Prototroph 2 differs from Prototroph 1 in several ways: (1) Prototroph 1 was productive, Prototroph 2 was immune but non-productive; (2) Prototroph 1 gave rise to non-motile lys⁻ and pa⁻ auxotrophs, Prototroph 2 gave rise to other prototrophs (both productive and non-productive types) and auxotrophs which were predominantly pa⁻ but included also the new types of arg⁻ and nic⁻. No lys⁻ auxotrophs were found. (3) The secondary swarm phenomenon was exhibited by Prototroph 2 but was not exhibited by Prototroph 1. (4) In Prototroph 1, one of the 'segregant' types (the lys⁻) retained the gmq agglutinability of the original parent culture, although non-motile; in Prototroph 2, gmq agglutinability was almost completely lost in all 'segregants'. (5) In at least one of the pa⁻ auxotrophs from Prototroph 2, reversion to the parent type was found to occur; reversibility to the parent type was not detected in any of the

Prototroph 1 'segregants'.

The overall picture of changes occurring in Prototroph 2 and its derivatives is far less suggestive of segregation than that described for Prototroph 1. In fact, the reversibility of the changes argues strongly against this interpretation. It is significant therefore, to note that novel genotypes arose in both lines of descent - and that the origin of these novel genotypes was coupled with changes in other cell properties. In Prototroph 1, the origin of mot⁻ was coupled with the origin of pa⁻ and the i: 1,2.. antigens. In Prototroph 2, the origin of the arg⁻ and the nic⁻ cultures was coupled with increasing productivity and the origin of the i and 1,2.. antigens; the origin of fla⁻ was coupled with the origin of another novel property, O⁻, and both to the origin of pa⁻ and (in one case) decreased productivity.

E. Mutational Origin of a Unilinearly Inherited Gene

The non-motile pa⁻ cells derived from SB42 were devoid of flagella and cultures failed to swarm when inoculated into motility medium. They did exhibit, however, another phenomenon which was thought worthy of further investigation. Two non-motile, pa⁻ cultures had been placed into stock as SB90 and SB91. Both cultures were lysogenic. The work reported in this section was done with SB90 although SB91 also exhibited the same reactions.

SB90 did not produce swarms on MM but growth was not restricted to the line of the original inoculation. After 24 hours incubation, many lines of small colonies which resembled the trails in abortive transduction

of motility to non-motile strains (Stocker, Zinder and Lederberg, 1953) were formed and extended to a distance of at least 10 mm into the medium. Because of the similarity in appearance of these lines of colonies to the "trails" of abortive transduction and of naturally occurring non-motile strains (e.g. S. dublin SW-553, Lederberg, 1956) they also will be referred to here as trails.

Three serial subcultures in L-broth failed to produce any apparent reduction in the numbers of trails that the culture produced in motility medium. A reduction was expected were the trails produced by a unilinear transmission similar to that postulated for abortive transduction. Moreover, after the third subculture, several fine swarms were produced. When tested, these swarms produced even growth in broth, were found to be motile with peritrichous flagella, were still pa⁻, immune to PSB1 and lysogenic in mixed culture with SB2 and were agglutinated with 1,2.. antiserum. In addition they had regained the SB2 O antigen. Six separate swarms were tested with identical results. This latter result was of particular interest for the S to R variation which has been described for many different groups of bacteria has been found to be independent of the H to O variation, since rough variants are often flagellated. The R variation has been found to be often associated with roughness of colony on solid medium, loss of virulence, instability in saline, loss of the O antigenic component and alteration in the method of cellular division. The S to R change is relatively irreversible under the ordinary conditions of cultivation in the laboratory and the R to S change seldom if ever has

been observed in earlier work. It is very difficult although not impossible to induce the R to S change by a specific stimulus when the original S to R change has been complete. Partly degraded SR variants occur and reversion to the normal S form may be more easily induced in these strains (Wilson and Miles, 1955).

At the center of each swarm and therefore at the presumed origin, was a pyramidal shaped area of dense micro-colonies with the apex towards the site of original inoculation. These resembled the "flares" described by Stocker et al. (1953) for non-motile strains of Salmonella to which motility had been transduced. Lederberg (1956) has described similar "flares" in transductional platings. He has argued that they have no special relationship to the transductional process as they can be observed also in replatings of motile colonies both of transductional and stock origin. They were especially prominent in platings of rough Salmonella. They were therefore considered by Lederberg to be a phenomenon related to auto-agglutination and he mentioned no relationship between flares and motile swarms. However, since the flares of SB90 were always associated with a swarm and flares did not appear without accompanying swarms, it appeared that these flares were intimately related with the changes from non-motility to motility.

Since phage PSB1 had previously been shown to transduce motility to the non-motile lys- cultures from Prototroph 1, an attempt was made to transduce motility to SB90. This was not possible, however, due probably to the loss of the specific O antigen resulting in low phage adsorption

(only 22% adsorbed in 15 minutes at 37°C).

Since serial subculture caused no reduction in number of trails formed by SB90, it seemed important to test the trail forming ability of the individual colonies making up the trails themselves. Accordingly, 10 single colonies from the trails produced by plating out SB90 in MM were dissected out with a sterile wire and replated into fresh MM. Plates were incubated for 24 hours at 37°C. Each culture produced many trails. In turn, 10 other trail colonies resulting from the originally plated colonies were also picked and replated to MM. These colonies also produced many trails. In addition, a number of motile swarms were produced. The swarms were found to consist of cells with peritrichous flagellation, which had regained the SB2 O antigen and adsorbed phage PSB1 (93% in 15 minutes at 37°C). Thus although the trails gave the appearance of unilinear transmission, the trail producing property was inherent in all the cells of the culture.

Since no report of a similar experiment with reference to the trails of abortive transduction of motility or of a naturally occurring non-motile strain could be found in the literature, motility was transduced to an available non-motile strain of S. typhimurium with PSB1. As the result of abortive transduction some trails were produced. Fresh MM plates were inoculated with cells from the center of colonies in these trails and incubated. No trails were produced. There therefore seems to be a fundamental difference between the trails of abortive transduction of motility and the trails produced by SB90.

It is pertinent to note that the mot⁻ lys⁻ cells derived from Prototroph 1 do not form trails. Mutant swarms appear directly and (as described earlier) this property can be transduced to these strains.

The most distinctive features of the properties of SB90 can now be summarized:

(1) SB90 arose as a non-motile variant from a culture which arose in turn as a secondary swarm 'segregant' from a derivative of Prototroph 2. The secondary swarm culture was one in which, presumably, there was an enhanced motility in MM media at room temperature.

(2) SB90 in MM produced large numbers of trails; serial subculture from individual colonies in the trails resulted in the production of further trails and eventually normal swarms.

(3) SB90 is devoid of the O antigen and can therefore be described as rough. Spontaneous reversion to motility results also in the gain of the O antigen.

(4) SB90 differs from the non-motile lys⁻ cultures which arose as 'segregants' from Prototroph 1 in being non-flagellated and rough. The lys⁻ variants are flagellated and smooth.

(5) The auxotrophic property of pa⁻ was not affected by the properties of the non-motility.

In devising a model to help explain the situation in SB90 there are two evident differences from the situation described for abortively transduced cells which must be kept in mind. The first is that in SB90, the ability to form trails (and hence motile cells) is retained by the

cells of the colonies in the trails themselves. Thus, although the trail formation may be taken as evidence of a unilinear inheritance type of phenomena, this explanation is not sufficient to explain the situation in SB90. And second, in SB90, fla⁻ appears also to be coupled to 0⁻. The fla⁻ condition disappears in one of two ways - (a) by trail formation (which will hereafter be referred to as 'abortive mutation') which is relatively an unstable condition and (b) by stable reversion which is coupled to a reversion of the 0⁻ condition.

From these two observations it may be inferred that the fla⁻ to fla⁺ to fla⁻ changes (including the abortive mutations) do not result from either (1) point mutations involving base changes in the fla cistron or (2) segregation from a diplogenic condition. Rather, these observations infer that the changes involve shifts in relative position of an intact fl⁺ cistron.

Autonomous replication is a characteristic property of cytoplasmic genes. This assumption is supported by the work of Jacob and Wollman (1961) on episomes. Autonomous replication implies a lack of synchrony with chromosomal gene replication and either may be faster or slower. Cytoplasmic elements may be autonomous in one of two clearly distinct ways: (1) They may not replicate at all or (2) they may be capable of more rapid replication than the chromosomal genes. The first property may be described as rep⁻ and the second as rep⁺. With this terminology, the trail forming cells may be described as containing a rep⁻ cytoplasmic fla⁺ gene which is transmitted unilinearly to one line of descendants. There is,

however, the added observation that the colonies formed in the trails must still possess a fla⁺ gene since the cells in these colonies retain the capacity to produce either further trails or stable swarms.

From this latter observation the inference may be made that the trail initial cell has a rep⁻ cytoplasmic gene associated with only one of the four nuclei. This inference would predict that the first two trail colonies should still be fla⁺ but later colonies be fla⁻, but this is not so. The majority of single colonies that were picked from the trails themselves for subculture to MM were taken from the ends of trails which were composed of at least 10 colonies, and yet these colonies still produced trails. On the other hand, the trail forming cells may contain two fla⁺ genes; one is rep⁻, cytoplasmic and functional, the other is rep⁺ chromosomal but non-functional. Although trail forming colonies have a rep⁻ cytoplasmic fla⁺ gene which does not multiply per se, the fla⁺ gene does replicate but every new copy ends up rep⁺, chromosomal and non-functional.

On the basis of the foregoing observations and inferences it is now possible to construct a model of the possible events leading to the formation of trails in SB90. In constructing this model the auxotrophic change is ignored since this does not seem to be affected here.

There are four main assumptions upon which the model to be presented is based. The first three assumptions are closely allied to the ideas advanced by Adelberg and Burns following their work on the F episome in E. coli (1960).

(1) Structural genes may occasionally move to a position where synchronous replication is impossible. That is, chromosomal genes may occasionally become cytoplasmic (or episomal).

(2) If another copy of the gene is present (i.e. in other nuclei of multinucleate cells) the cytoplasmic gene cannot multiply and is diluted out.

(3) If no other copy of the gene is present the cytoplasmic gene may multiply at a rate faster than the cell so that there may eventually be more than one cytoplasmic gene per nuclear gene.

(4) Structural genes in the autonomous position may move back to a position on the chromosome. Reinsertion into the correct position leads to a restoration of the function of the gene but reinsertion into a wrong position does not permit the gene to function at all.

The series of possible events in SB90 is diagrammatically represented in figure 5, on the basis of the above four assumptions, and illustrates the following hypothesis.

The motile parent (Prototroph 1 derivative) had the fla⁺ gene situated in the normal position on the chromosome; fla is linked to 0. As the result of an event which will be called an abortive mutation, the fla and 0 cistrons are detached from the chromosome and become cytoplasmic. In the cytoplasm they cannot replicate because of the presence of similar genes on the other nuclei of the cell, and it is not until after two cellular divisions that the cytoplasmic genes have the opportunity of being present in a cell none of whose chromosomes possess similar genes.

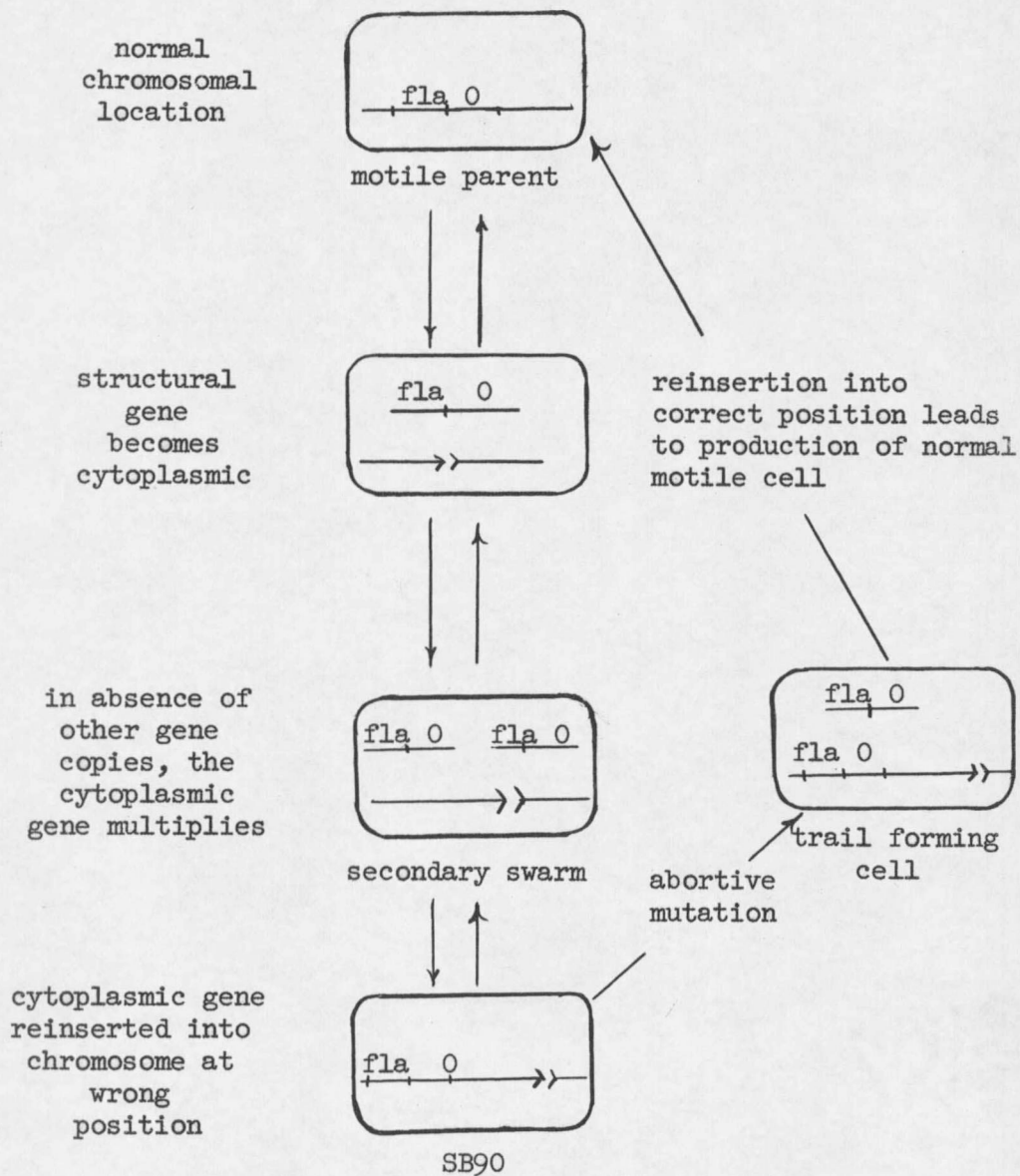


Figure 5. Diagrammatic Representation of Possible Events Leading to Trail Formation in SB90.

In the absence of identical genes the episomic particle replicates at a faster rate than the cell so that many copies of the gene may occur within the cytoplasm. This condition may describe the situation in the cells of the secondary swarms which possessed enhanced motility at room temperature.

The genes may be reinserted into the chromosome. Reinsertion at the correct position causes the cell to return to the smooth, motile condition. The replication of the cytoplasmic particles is inhibited and these are eventually diluted out by cellular division. But reinsertion may also be at an incorrect position. In this position the genes cannot exert their effect and the cell is therefore phenotypically fla⁻0⁻. This may describe the condition in SB90. As the result of a second abortive mutation the genes may again become episomal. They cannot replicate, however, because of the presence of similar genes (though non-functional) in other nuclei but the cell in which it occurs is able to produce flagella and is therefore motile. As long as the episomic genes are transmitted in cellular division to cells which contain similar genes they are "unilinearly inherited" and trails are produced in motility medium. Thus cells in the trail colonies, although phenotypically fla⁻ 0⁻ because of the incorrect position of the genes on the chromosome, nevertheless retain the potentiality to produce further trails by the mechanism of abortive mutation. The model as presented in figure 5 specifies that the incorrect position is chromosomal. This is, of course, the most obvious interpretation but it may not necessarily be the correct one. Further, even if it is chromosomal it may be attached in a different way

(e.g. laterally) rather than be a complete reinsertion as illustrated.

Occasionally, however, the episomic particle may succeed in being transferred to a cell in which no other copies of the gene occur. It may then replicate faster than the cell so that there may be many copies per cell after several cellular divisions. Eventual reinsertion into the chromosome at the correct position produces smooth, motile cells. This more rapid multiplication of the episomic particle leading to the eventual production of normal motile cells may be the mechanism behind the formation of the flares previously described, from which normal swarms seemed always to originate.

The model may also be used to explain the results of abortive transduction. Through the mediation of phage, a fla⁺ gene is incorporated into a fla⁻ cell. The fla⁺ gene remains episomal but cannot express itself nor does it multiply because of the presence of the fla⁻ gene in the nuclei. The fla⁻ gene is allelic to fla⁺ and they can be considered identical to each other as far as repression of similar episomic particles is concerned. The fla⁺ episome is transmitted unilinearly and leads to the formation of the trails. The cells comprising the trail colonies are thus all genetically fl⁻ (in contrast to the cells of the trails of SB90 which were only phenotypically fl⁻) and will not produce further trails on subculture. Lederberg (1956) considered the possibilities that the unilinearly transmitted fragment in abortive transduction was either a non-replicating chromosomal fragment or an intermediate product of its action and concluded that either was possible. The hypothesis presented here would support the

idea that the unilinearly transmitted particle is indeed a non-replicating chromosomal fragment.

F. Mutability to Streptomycin Resistance

Some of the situations that have already been described in earlier sections appear to be or may be described as instances of altered mutability. The production of papillae by colonies of some derivatives of SB2 when plated out on EMB maltose medium appears to result from an altered mutability to maltose fermentation. This increased mal mutability was found to be a property of some single colony isolations of SB2 (e.g. SB10); and the immune derivative, SB56, of the sensitive, cyt⁻ culture, SB4, was found to have an increased mal mutability. The increased probability of the change from sensitivity to lysogeny in auxotrophs may also be a reflection of increased mutability. The production of alternate auxotrophs may actually be the result of an increased mutability at different loci.

In another system, streptomycin mutability has proven to be an especially convenient indicator of mutability at other loci. Treffers, Spinelli and Belser (1954) and Skaar (1956) showed that an increased mutation rate from streptomycin sensitivity to streptomycin resistance in E. coli K 12 was also associated with increased mutability at other, unrelated loci.

Since streptomycin resistant mutants are especially convenient for studies on mutability, a selection of SB2 derivatives was surveyed for possible differences in mutability to streptomycin. In Section A of the

Results it was shown that the origin of lysogeny (or phage, the product of lysogeny) was associated with the change from chloramphenicol sensitivity to chloramphenicol resistance. Thus a secondary purpose of testing for streptomycin mutability was to see if the appearance of lysogeny was also associated with the change from streptomycin sensitivity to streptomycin resistance.

Bacteria were grown in 5 ml of L-broth overnight. They were then centrifuged and resuspended in 1 ml of saline. NA plates in which were incorporated streptomycin to a final concentration of 250 µg/ml, were then spread with 0.1 ml of the bacterial suspension (about 7×10^8 bacteria per plate) and incubated at 37°C for 48 hours. The numbers of streptomycin resistant colonies that then appeared were counted. The results of the streptomycin mutability tests, together with some of the properties of the strains tested, are shown in Table VIII.

The purpose of testing for an associated change to lysogeny in streptomycin resistant clones which arose from phage sensitive cultures was defeated because of the complete absence of streptomycin resistant cells in all the sensitive cultures tested. But this result is itself an extremely interesting one. Some immune cultures (SB47 and SB56) produced streptomycin resistant colonies but these were not tested to see if they were now productive.

Increased mutability to streptomycin resistance was, however, observed in some strains. Mutable strains were not restricted to prototrophs or to auxotrophs but were scattered throughout the 25 strains tested. The most

TABLE VIII

Mutability of Derivatives of SB2 from Streptomycin
Sensitivity to Streptomycin Resistance

Culture	Growth req.	Origin	Lysogeny	Cols. on strep. agar
SB1	prototroph	natural lysogenic	L	685
SB2	prototroph	natural sensitive	NL	0
SB10	prototroph	<u>mal</u> mutable SB2	NL	0
SB14	prototroph	SB2 lysogenized with PSB1	L	0
SB15	prototroph	SB2 lysogenized with PSB1	L	95
SB47	prototroph	immune SB2	IM	90
SB5	<u>lys</u> ⁻	MnCl ₂ -penicillin auxotroph	L	0
SB43	<u>lys</u> ⁻	NM strain from Prototroph 1	L	0
SB21	<u>pa</u> ⁻	alternate auxotroph from SB5	L	730
SB90	<u>pa</u> ⁻	NM trail former from Prototroph 2	L	Resistant plus 250
SB94	<u>pa</u> ⁻	trail colony from SB90	L	Resistant plus 110
SB92	<u>pa</u> ⁻	swarm from SB90	L	Resistant plus 300
SB32	<u>pa</u> ⁻ <u>tyr</u> ⁻	auxotroph from SB7	NL	0
SB33	<u>pa</u> ⁻ <u>tyr</u> ⁻	auxotroph from SB7	NL	0

(continued)

TABLE VIII (continued)

Culture	Growth req.	Origin	Lysogeny	Cols. on strep. agar.
SB34	<u>pa</u> ⁻ <u>tyr</u>	auxotroph from SB7	L	0
SB35	<u>pa</u> ⁻ <u>tyr</u> ⁻	auxotroph from SB7	L	67
SB36	<u>pa</u> ⁻ <u>tyr</u> ⁻	auxotroph from SB7	L	Resistant
SB8	<u>tyr</u> ⁻	UV auxotroph from SB2	NL	0
SB29	<u>tyr</u> ⁻	<u>pa</u> ⁻ <u>tyr</u> ⁻ revertant of SB34	L	313
SB31	<u>tyr</u> ⁻	<u>pa</u> ⁻ <u>tyr</u> ⁻ revertant of SB32	NL	0
SB4	<u>cyt</u> ⁻	MnCl ₂ -penicillin auxotroph	NL	0
SB23	<u>cyt</u> ⁻	lysogenic derivative of SB4	L	0
SB56	<u>cyt</u> ⁻	immune SB4	IM	151
SB57	<u>cyt</u> ⁻	immune SB4	IM	0
SB41	<u>nic</u> ⁻	Prototroph 2 derivative	L	Resistant plus 45
SB49	<u>arg</u> ⁻	Prototroph 2 derivative	L	Resistant plus 140

L=lysogenic, NL=sensitive, IM=immune

striking observation was that mutable strains were all immune or lysogenic although all immune or lysogenic strains were not mutable. The original sensitive SB2 was not mutable but one strain artificially lysogenized with phage PSB1 (SB15) was mutable although another lysogenized strain (SB14) was not mutable. And SB47, the immune derivative of SB2 was also mutable.

Amongst the auxotrophs, neither of the two lys⁻ cultures tested (SB5 and SB43) was mutable although both were lysogenic. The phenylalanine derivatives were particularly interesting. SB21, an alternate auxotroph derived from the old culture SB5, was mutable. All pa⁻ cultures derived from SB42 (which arose as a secondary swarm from Prototroph 2) including the non-motile trail formers and the swarm culture which arose from these after serial subculture, produced a heavy background of streptomycin resistant growth in addition to large numbers of colonies. Thus the pa⁻ derivatives of SB42 differed in this respect from the alternative pa⁻ auxotrophs obtained directly from SB5.

One lysogenic culture of pa⁻tyr⁻ (SB35) was mutable while another lysogenic pa⁻tyr⁻ (SB36) was streptomycin resistant (with no large colonies). None of the three non-lysogenic pa⁻tyr⁻ cultures tested was mutable.

Of the two tyr⁻ cultures tested, only the lysogenic culture (SB29) was mutable. And SB56, the immune derivative of the sensitive SB4 culture was the only cyt⁻ culture to be mutable. The nic⁻ strain (SB41) and the arg⁻ strain (SB49), derived from Prototroph 2, resembled the pa⁻ cultures derived from SB42 for they produced a background of streptomycin resistant growth in addition to many colonies.

Thus increased mutability to streptomycin resistance was restricted to cultures which were either immune or lysogenic. A second finding was that some cultures exhibited not only an increased mutability to streptomycin resistance but contained an extremely high proportion of already resistant colonies (which produced the background growth). Thus, for example, SB36 appeared to consist wholly of resistant cells while SB41, SB49, SB90, SB92 and SB94 consisted of resistant cells and of other cells that produced larger colonies. The possibility that these colonies may have been streptomycin dependent was not tested. The appearance of the two types of growth in these strains, on the other hand, may indicate that there were two levels of resistance in the culture but this also was not tested. It is to be noted that the level of resistance used by Treffers et al was 100 $\mu\text{g/ml}$ whereas the level used here was 250 $\mu\text{g/ml}$. Other mutants resistant only to lower levels of streptomycin may actually exist in the present system but were not tested for.

The higher rate of mutation to streptomycin resistance exhibited by some SB2 derivatives was not necessarily coupled with a higher rate of mutability to other factors. For example, SB10, which exhibits a high rate of mal mutability, did not show a high mutability to streptomycin resistance. Thus a mutability system similar to that in Treffers' et al. E. coli K 12 derivatives is probably not involved here. The finding that some of the derivatives do have a high rate of mutability to streptomycin resistance does indicate, however, that a mutability factor is concerned here also.

A type of genetic determinant for instability at the Sm locus different from that described by Treffers et al. was discovered and studied by Gundersen, Jyssum and Lie (1962). It differed from the Treffer mutator gene for it was not found to follow the chromosomal factors in genetic crosses. Since the character could be spontaneously lost and transferred to a mutator-negative cell by infection, it appeared to be episomic in nature. Another difference between the Treffers' mutator gene and the Gundersen factor (which was found in hospital strains of E. coli and not in K 12 derivatives) is that the episomal factor appears to influence only the Sm locus, for increased mutability at other loci was not detected.

The results with the SB2 derivatives would indicate that the factor associated with increased streptomycin mutability may be similar to the factor discovered by Gundersen et al. although permanent loss of the property of mutability and the ability to transfer the property by infection have not yet been tested. The mutability factor in SB2 derivatives seems to influence only the Sm locus although it is often found associated with immune or lysogenic cells. This latter result suggests that the mutator factor may occasionally undergo some form of recombination with the determinant of immunity (possibly the prophage itself). Since the hypothesis has already been stated that prophage may occur cytoplasmically it would follow that the factor responsible for streptomycin mutability is also cytoplasmic.

The Japanese workers, Watanabe and Fukasawa (1960) found that the actual determinants of drug resistance among the various Enterobacteriaceae

were contained upon episomic-like elements (called R factors) which could be transferred to sensitive bacteria by cell conjugation and by transduction. Subsequent work showed that many kinds of R factor existed (Mitsuhashi, Hashimoto, Harada, Egawa and Matsuyama, 1961) which carried various combinations of multiple drug resistance. Mitsuhashi, Harada, Hashimoto, Kameda and Suzuki (1962) found that recombination between two different types of R factor could occur.

It is possible that more than one type of factor may be concerned in the strains tested here. An episomal mutator gene may be carried by the strains which exhibit the increased mutability to streptomycin resistance whereas an element analogous to the R factor of the Japanese workers may be concerned in those strains which are already streptomycin resistant. Both factors could be concerned in those strains which exhibit both the resistance and the mutability.

One striking correlation from the results in Table VIII is that the auxotrophic change from lys⁻ to pa⁻, always associated with the H antigenic change, is here also associated with a mutability shift. Lys⁻ cultures were not mutable but all pa⁻ cultures were mutable.

In the immune strains SB47 and SB56 the change to mutability appears to have been coincident with the change from sensitivity to immunity but with other strains it cannot be certain exactly when in their ancestry the mutability arose. For example, the mutability of the Prototroph 2 derivatives could have arisen in the Prototroph 2 itself, in the secondary swarms or perhaps even in a pa⁻ ancestor similar to SB21.

Mutability in the SB2 derivatives may be pictured as controlled by episomal elements which may change position thereby affecting the probability of activation or inactivation of certain genes. Specific interpretations or applications of this general picture depend yet upon additional answers. For example, it is not yet known whether streptomycin resistance in the SB2 derivatives is dominant or recessive to streptomycin sensitivity. In E. coli K 12 heterozygotes resistance is recessive to sensitivity but in the episomal transfer of Mitsuhashi et al. it appears to be dominant.

G. Ultra-Violet Inducibility as an Indicator of Prophage Location

Recombination in bacteria by cell to cell contact was discovered first in 1946 by Lederberg and Tatum and has since been extensively studied in the K 12 strain of Escherichia coli. Some strains were designated F^+ and were fertile with other F^+ sublines, and others were designated F^- . F^- strains were not fertile with each other but only with F^+ strains. Recombination between F^+ and F^- bacteria is, however, very rare and even under the most favorable conditions is never greater than 1×10^{-5} . The discovery of donor strains with a much higher frequency of recombination (called Hfr strains) by Cavalli-Sforza (1950) and Hayes (1953) with which 1,000 times more recombinants were obtained when mated with an F^- strain, opened the way to the quantitative study of bacterial recombination.

Since the initial discovery of Hfr strains, many other similar strains of K 12 were discovered. Each Hfr type was characterized by the nature of the different characters it transmitted at high frequency and by the order

in which these characters were transmitted. The linkage group of a particular Hfr strain could be represented as an oriented linear structure with a proximal extremity (the origin) and a distal extremity. For any Hfr strain the order in which the genetic characters was arranged was the same whether determined by the relative frequency of the transmission or by the relative time of their entry into the zygotes.

When the linkage groups of many different Hfr types were compared, it was found that the linkage relationships between characters were constant and uniform except for those characters which, in a given Hfr type, were linked either to the origin or to the distal extremity of the Hfr chromosome. To construct a genetic map of E. coli K 12, the information gained from the study of many Hfr strains was taken into account. This led Jacob and Wollman (1961) to represent the chromosome of E. coli K 12 as a closed curve. From this representation, the linkage group of any Hfr could be deduced by opening the closed curve at a defined point and inserting the origin 0 at one of the two extremities thus formed.

F^+ strains of E. coli can transmit the F^+ character to F^- cells by cell-to-cell contact. This infectious property of F^+ strains led to the postulation of an F factor as the determinant of the F^+ character. The F factor also shares certain other properties with other bacterial derivatives. The F factor, colicine agents and temperate bacteriophages are all hereditary determinants of bacterial properties which can be gained from other bacteria by infection and can be lost. Each is non-essential for the existence of the cell. Each may behave as either "integrated" by incorporation into the bacterial chromosome (in which

state it multiplies at the same pace as the host) or "autonomous" when it loses its association with the bacterial chromosome (and it multiplies at a pace different from that of the host). For heritable factors of bacteria with these properties, Jacob and Wollman (1958) proposed the name "episome", although only the property described in the last sentence is here used to define the term.

Episomes for the determination of colicinogeny have been shown to be transmissible by cell contact in the genus Salmonella as well as in E. coli. This finding led to the suggestion that conjugation and chromosomal recombination in Salmonella might occur during transmission of colicinogeny. Indeed colicine agents have been shown to induce chromosomal recombination both in S. typhimurium (Stocker, 1960) and in E. coli (Clowes, 1961).

Smith and Stocker (1962) applied the method of colicine induction of chromosomal recombination to map the chromosome of S. typhimurium LT-2. When strains used as parents differed in several characters, recombinants selected for possession of one selective marker from each parent differed with respect to various unselected markers. By methods analogous to those used for the mapping of the E. coli K 12 chromosome, these workers inferred the order of the chromosome of the various loci concerned. Although comparatively few loci were mapped by this method the map was shown to be homologous with that of E. coli K 12 in that (1) the order of loci of the two species was similar and (2) the linkage group could better be represented as a single closed loop rather than as a single straight line.

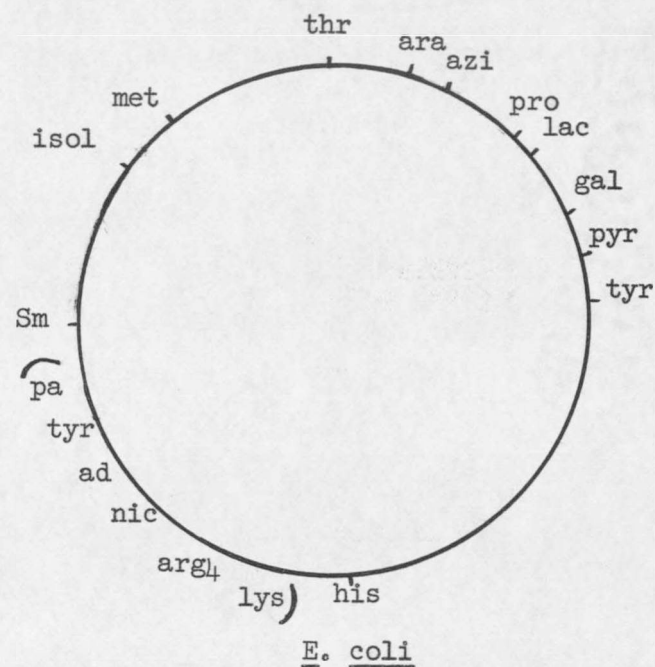
There are additional reasons for postulating a close homology between the chromosomes for E. coli and S. typhimurium and even members of other genera of the Enterobacteriaceae. As the result of transduction experiments the chromosomes of both E. coli and S. typhimurium have been shown to contain identical clusters of tryptophan genes (Demerec and Hartman, 1956; Skaar and Davidson, 1956); and a close linkage between the cysteine and tryptophan loci has been demonstrated for both species (Demerec, 1956; Yanofsky and Lennox, 1959). Generalized transduction of nutritional, fermentative and antigenic characters has been shown to be widespread amongst many Salmonella types (see Zinder and Lederberg, 1952; Iseki and Sakai, 1953; Bailey, 1956).

Recombination between bacteria classified in different genera of the Enterobacteriaceae (Escherichia and Shigella) was first demonstrated by Luria and Burrous (1957). Subsequently, recombination between other genera has been shown to occur (Baron, 1960). These intergeneric crosses include recombination between E. coli and S. typhimurium, S. typhi and Serratia marcescens.

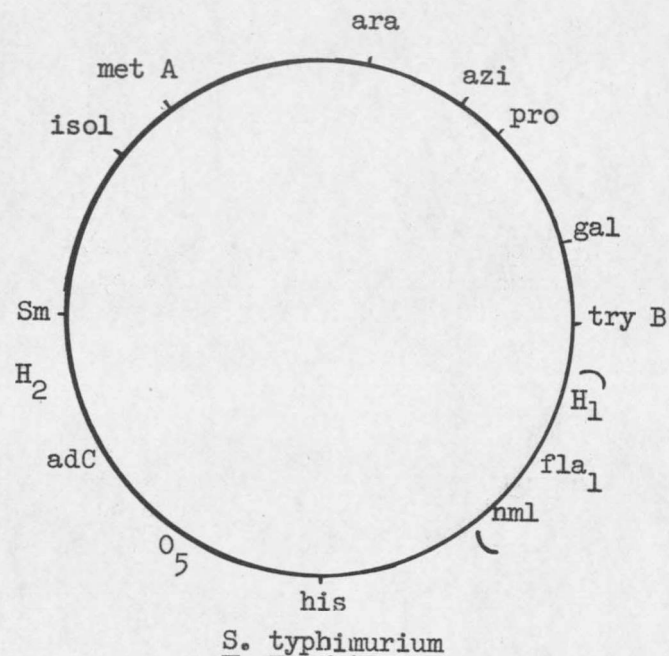
There is thus very strong additional evidence that the chromosomes of many members of the Enterobacteriaceae may be closely homologous.

Representations of the chromosomes for E. coli K 12 and S. typhimurium LT-2 are shown in figure 6, adapted from those published by Jacob and Wollman (1961) and Smith and Stocker (1962) respectively.

Because of this close homology between the E. coli and S. typhimurium chromosomes and for the additional reason that there appear to be close



E. coli



S. typhimurium

Figure 6. Schematic Representation of the Linkage Groups in E. coli and S. typhimurium;
Adapted from Jacob and Wollman (1961) and Smith and Stocker (1962).

Symbols used correspond to synthesis of threonine (thr), proline (pro), pyrimidines (pyr), tryptophan (try), histidine (his), arginine (arg), lysine (lys), adenine (ad), tyrosine (tyr), phenylalanine (pa), methionine (met), isoleucine (isol), nicotinic acid (nic); to fermentation of arabinose (ara), lactose (lac), galactose (gal) N-methyl lysine (nml); resistance to sodium azide (azi), streptomycin (Sm); location of genes controlling flagellar production (fla), flagellar phase (H₁ and H₂), antigen O₅ production (O₅). Symbols in brackets indicate that the order of included markers has not yet been determined.

homologies among other members of the family Enterobacteriaceae, a theoretical 'hybridization' of the chromosomes for E. coli and S. typhimurium into one composite chromosome could be tentatively accepted as a model for a chromosome representative for the whole Enterobacteriaceae group of bacteria. By reference to the E. coli chromosome in figure 6 it can be seen that the genetic loci of characters which were studied in the S. blegdam SB2 strain and its derivatives are all located on a segment of the chromosome between about gal and Sm. A comparison of the two chromosomes shows that some characters specific for Salmonella (e.g. H₁, H₂ and O₅) are mapped in regions that are located between certain loci that are common to both bacteria (e.g. gal and his). An attempt at a theoretical 'hybridization' to form a composite chromosome representative for the Enterobacteriaceae group is shown in figure 7 for that region between gal and Sm only, showing only selected markers.

Of particular interest amongst the genetic characters which were studied in E. coli K 12 was the location of different prophages. It was found by Jacob and Wollman (1956) that the ultra-violet inducible phages were located on a segment of the chromosome extending from the galactose region to a region beyond the histidine locus. All ultra-violet non-induced phages were located distally to his. With reference to the E. coli chromosome shown in figure 7, it is seen that the region of change from inducible to non-inducible or the 'cut-off region', is located approximately in a median position of the region between gal and Sm, that portion of the chromosome which includes the loci studied in the SB2

Sm (pa,tyr,ad,nic,lys,arg) his try pyr gal

E. coli chromosome

Sm H₂ ad O5 his (fla₁, H₁) try B gal

S. typhimurium chromosome

Sm (pa,tyr,ad,nic,lys,arg) his (fla₁, H₁) try B pyr gal
H₂ ad O5

composite chromosome

Figure 7. Schematic Representation of a Composite Chromosome from the Chromosomes Published for E. coli and S. typhimurium.

derivatives and which we illustrated in the composite chromosome.

It has already been shown that different mutant, lysogenic derivatives of S. blegdam SB2 vary in degree of ultra-violet inducibility (Tables I and IV). Because of the division of the E. coli chromosome into inducible and non-inducible regions and because of the already demonstrated differences in degree of inducibility of different lysogenic, mutant derivatives of SB2, it was of particular interest to test the UV inducibility of those other strains not yet tested. (i.e. the arg⁻ and nic⁻ strains); especially since these two strains have mutations the loci of which are included in that region beyond his where the non-inducible region starts in the E. coli chromosome. The UV inducibility of the arg⁻ and nic⁻ mutants was therefore tested. In addition, prototrophic revertants of all mutants which reverted to prototrophy were similarly tested to see if reversion altered inducibility. Table IX shows the results of all UV inducibility tests and includes some results previously placed in Tables I and IV.

Cultures with the same requirement or derived from strains with the same requirement have been grouped together in such a way that the results of the induction ratio tend to show a graded variability from high induction to no induction.

From the results in Table IX it is seen that the nic⁻ and arg⁻ auxotrophs showed no detectable UV inducibility. This result supports the original idea that the SB2 chromosome also has inducible and non-inducible regions which correspond to similar regions in the K 12 chromosome. And

TABLE IX

Ultra-Violet Induction of Lysogenic Derivatives of SB2

Strain		Phage/ml without UV	Phage/ml with UV	With UV/ without UV
SB23	<u>cyt</u> ⁻	8.5×10^2	3×10^5	353
SB5	<u>lys</u> ⁻	7.6×10^2	5×10^4	66
SB44	<u>lys</u> ⁻ , <u>mot</u> ⁻	2.1×10^2	6×10^4	286
SB35	<u>pa</u> ⁻ <u>tyr</u> ⁻	2×10^2	3.6×10^4	180
SB29	<u>tyr</u> ⁻	7.1×10^3	1.8×10^5	25
SB21	<u>pa</u> ⁻	40	1.1×10^3	28
SB90	<u>pa</u> ⁻ , <u>fla</u> ⁻	1×10^2	1.6×10^2	1.6
SB92	<u>pa</u> ⁻	70	50	0.7
SB94	<u>pa</u> ⁻ , <u>fla</u> ⁻	2.4×10^2	4.8×10^2	2
SB41	<u>nic</u> ⁻	7	5	0.7
SB49	<u>arg</u> ⁻	7	10	1.4
<u>Prototrophic revertants</u>				
SB76	(<u>cyt</u> ⁻)	2.1×10^3	7×10^5	333
SB77	(<u>cyt</u> ⁻)	2.2×10^3	8×10^5	364
SB61	(<u>lys</u> ⁻)	1.5×10^3	2×10^5	133
SB62	(<u>lys</u> ⁻)	3.8×10^3	3×10^5	78
SB58	(<u>pa</u> ⁻)	9	1.4×10^2	16
SB59	(<u>pa</u> ⁻)	10	30	3
SB78	(<u>nic</u> ⁻)	9	9	1
SB79	(<u>nic</u> ⁻)	9	10	1
SB73	(<u>arg</u> ⁻)	9	9	1

again from Table IX, the UV induction ratios of the prototrophic revertants are not significantly different from the values obtained from the auxotrophs themselves. The order of mutants from highest inducibility to no inducibility is cyt, lys, pa tyr, tyr, pa, (nic, arg).

In section A of the Results, it was suggested that sites of mutation on the chromosome might be also the sites of attachments of prophage to the bacterial chromosome. The inference to be drawn from the results in this section add support to this idea.

Although the phages from all the lysogenic strains appeared very similar by the simple tests of heat stability, host range and serology, yet in different auxotrophs they exhibit a remarkable order in degree of UV inducibility.

Mixed Growth Tests of each of the lysogenic cultures with each of the other lysogenic cultures showed that all lysogenic strains were immune to the phages carried by the other lysogenic strains.

Since Jacob and Wollman (1961) found that different phages occupied different sites on the bacterial chromosome and that these locational differences were reflected in whether a phage was inducible or non-inducible, the results here also suggest that the SB2 chromosome has inducible and non-inducible regions and that prophage may occupy different sites on the chromosome within these two regions.

While the correct map order for lys, pa, tyr, nic and arg has not been determined their known occurrence around the 'cut off' region is highly significant. Whether a prophage is induced or not induced is

determined by its location in one or the other segment of the chromosome. Although the number of markers is small and the number of UV induction tests with each strain limited, the order of markers from high inducibility to non-inducibility may actually be correlated with the order of markers on the chromosome. Thus the order cyt, lys, pa tyr, tyr, pa, (nic, arg) may actually represent the order of markers of the chromosome. On the chromosome map the pyr locus occurs well within the inducible region and it is the cyt⁻ auxotroph which is induced to the greatest degree. (Since cyt⁻ auxotrophs are also satisfied by uracil the pyr locus may well be the one involved in the cyt⁻ strains here). The greater inducibility of pa⁻tyr⁻ mutants over both pa⁻ and tyr⁻ strains may actually indicate that different pa and tyr loci are concerned here.

The similar orders of markers for map location and degree of inducibility very strongly suggest a correlation between the site of mutation and location of prophage. The simplest correlation is that prophage is located at the mutational site.

The UV inducibility results of Table IX indicate that the degree of inducibility of inducible phages varies. When this result is compared with the map order of markers, it is suggested that the degree of inducibility varies with distance from the 'cut off' point. This latter result may also be inferred for the E. coli phages from the results published by Jacob and Wollman (1956). Inducibility was measured as the maximal fraction of infective centers per 100 irradiated bacteria that were induced to lyse by the UV treatment, rather than the amount of free

phage after treatment compared with amount of free phage before treatment to give a value of 'degree' of inducibility. Yet the highest induction (95%) was achieved for phages lambda and 434 which are located well in the inducible region close to the gal locus; phage 381 has less than half this value (45%) and is located closer to the 'cut off' region close to the try locus; phages 424 and 466 located distal to his have an even lower value (25%); while phage 370 is well into the non-inducible region with a value of less than 1%. Thus it appears that a similar graded series exists for the E. coli phages and that degree of inducibility may be a function of distance from the 'cut off' region.

A restatement of the ideas that have developed from a consideration of the results of the ultra-violet induction tests will close this section.

A composite chromosome produced by theoretically 'hybridizing' the reported chromosomes for E. coli and S. typhimurium may be taken as a tentative model for an Enterobacteriaceae chromosome. In SB2 prophage may become attached at different points on the chromosome. Because of a correlation between the degree of inducibility and the map position of the mutation on the chromosome the position of attachment is probably at the site or very closely linked to the site of the mutation. Once attached to a particular site, prophage remains attached at that site even if the cell loses its mutation by reversion back to prototrophy. The chromosome has both inducible and non-inducible regions and the closer a prophage is located to the 'cut off' region the lower the degree of inducibility. Prophage located in the non-inducible region is not induced.

DISCUSSION

Many factors were affected by the changes which occurred in the derivatives of SB2. These changes are summarized in Table X.

The first finding of importance was that lysogenic cells may arise from initially sensitive cells by a mechanism involving some kind of positional effect due to spatial rearrangements.

The reasons for this important inference have already been discussed at length in Section A of the Results. Briefly, these include the two reasons (1) that the sensitive strain SB2 exhibits a UV sensitivity similar to its lysogenic derivative SB5 rather than the relative insensitivity of the naturally occurring lysogenic strain SB1 and (2) phage similar to PSB5 was isolated from SB2 and its derivatives on seven known occasions following procedures which either could or demonstrably did screen for a minority of the original population which showed other genetic changes. The implication that a lysogenic strain may arise directly from a sensitive strain without the presence of exogenous phage is far reaching for it implies that the sensitive strain carries at least most of the hereditary specifications of a temperate prophage even though it exhibits no immunity, and under normal conditions produces no phage. The only known precedent for a similar situation in another bacterial strain is the demonstration by Rutberg and Hedin (1960) that pressure treatment of sphaeroplasts of E. coli B results occasionally in the release of a phage to which the untreated culture remains sensitive.

In the light of later results, the mechanism originally proposed in Section A to explain the change from sensitivity to lysogeny may now

TABLE X

Summary of Pertinent Changes Involving Derivatives of SB2

Culture	Immediate Ancestor	PSB1 sensitive	productive	non-productive	lysine	phenylalanine	tyrosine	cytosine	arginine	nicotinic acid	H antigens gmq	H antigens i:1,2..	O antigen	UV Inducibility ratio	maltose mutability	streptomycin mutab.	mot gene	fla gene
SB1		-	+	-	+	+	+	+	+	+	+	-	+	21	-	+	+	+
SB2		+	-	-	+	+	+	+	+	+	+	-	+	-	-	-	+	+
SB4	SB2	+	-	-	+	+	+	(-)	+	+	+	-	+	-	-	-	+	+
SB5	SB2	-	(+)	-	(-)	+	+	+	+	+	+	-	+	(66)	-	-	+	+
SB7	SB5	-	+	-	+	(-)	+	+	+	+	-	(+)	+	?	?	?	+	+
SB10	SB2	+	-	-	+	+	+	+	+	+	+	-	+	-	(+)	-	+	+
SB14	SB2	-	(+)	-	+	+	+	+	+	+	+	-	+	?	-	-	+	+
SB15	SB2	-	(+)	-	+	+	+	+	+	+	+	-	+	?	-	(+)	+	+
SB21	SB5	-	+	-	+	(-)	+	+	+	+	-	(+)	+	(28)	-	(+)	+	+
SB23	SB4	-	(+)	-	+	+	+	-	+	+	+	-	+	(353)	-	-	+	+
SB29	SB34	-	+	-	+	(+)	-	+	+	+	+	-	+	25	-	+	+	+
SB31	SB32	+	-	-	+	(+)	-	+	+	+	+	-	+	-	-	-	+	+

() = property which differs from immediate ancestor

TABLE X (cont'd.)

Culture	Immediate Ancestor	PSB1 sensitive	productive	non-productive	lysine	phenylalanine	tyrosine	cytosine	arginine	nicotinic acid	H antigens gmq	H antigens i:1,2..	O antigen	UV Inducibility ratio	maltose mutability	streptomycin mutab.	mot gene	fla gene
SB32	SB7	(+)	-	-	+	-	(-)	+	+	+	+	-	+	-	-	-	+	+
SB33	SB7	(+)	-	-	+	-	(-)	+	+	+	+	-	+	-	-	-	+	+
SB34	SB7	-	+	-	+	-	(-)	+	+	+	+	-	+	?	-	-	+	+
SB35	SB7	-	+	-	+	-	(-)	+	+	+	+	-	+	(180)	-	+	+	+
Prototroph 1		-	+	-	(+)	+	+	+	+	+	+	(+)	+	?	?	?	+	+
Prototroph 2		-	-	+	(+)	+	+	+	+	+	+	-	+	?	?	?	+	+
SB37	Pro. 2	-	-	+	+	+	+	+	+	+	+	(+)	+	?	?	?	+	+
SB38	Pro. 2	-	-	+	+	+	+	+	+	+	+	(+)	+	?	?	?	+	+
SB41	SB38	-	+	-	+	+	+	+	+	(+)	-	+	+	0.7	-	+	+	+
SB42	SB37	-	+	-	+	(-)	+	+	+	+	-	+	+	?	?	?	+	+
SB43	Pro. 1	-	+	-	(-)	+	+	+	+	+	+	-	+	?	?	-	(-)	+
SB44	Pro. 1	-	+	-	(-)	+	+	+	+	+	+	-	+	286	?	?	(-)	+
SB47	SB2	-	-	(+)	+	+	+	+	+	+	+	-	+	-	-	(+)	+	+

TABLE X (cont'd.)

Culture	Immediate Ancestor	PSB1 sensitive	productive	non-productive	lysine	phenylalanine	tyrosine	cytosine	arginine	nicotinic acid	H antigens gmq	H antigens i:1,2..	O antigen	UV Inducibility ratio	maltose mutability	streptomycin mutab.	mot gene	fla gene
SB49	SB37	-	+	-	+	+	+	+	(-)	+	+	+	+	1.4	-	+	+	+
SB56	SB4	-	-	(+)	+	+	+	-	+	+	+	+	+	-	+	(+)	+	+
SB58	SB21	-	+	-	+	(+)	+	+	+	+	+	+	+	16	?	?	+	+
SB59	SB21	-	+	-	+	(+)	+	+	+	+	+	+	+	3	?	?	+	+
SB62	SB5	-	+	-	(+)	+	+	+	+	+	+	-	+	78	?	?	+	+
SB73	SB49	-	+	-	+	+	+	+	(+)	+	-	+	+	1	?	?	+	+
SB76	SB23	-	+	-	+	+	+	(+)	+	+	+	-	+	333	?	?	+	+
SB77	SB23	-	+	-	+	+	+	(+)	+	+	+	-	+	364	?	?	+	+
SB78	SB41	-	+	-	+	+	+	+	+	(+)	-	+	+	1	?	?	+	+
SB79	SB41	-	+	-	+	+	+	+	+	(+)	-	+	+	1	?	?	+	+
SB90	SB42	-	+	-	+	-	+	+	+	+	-	+	-	1.6	-	+	-	-
SB92	SB42	-	+	-	+	-	+	+	+	+	-	+	+	0.7	?	+	+	+
SB94	SB42	-	+	-	+	-	+	+	+	+	-	+	-	2	?	+	-	-

be re-examined. The results of the UV inducibility ratios were interpreted to indicate that prophage is a movable element and may locate at different sites on the chromosome. This hypothesis therefore excludes all ideas based on the supposition that the phage genome remains fixed to one definite map attachment site. Phage may become attached to a site on the chromosome but this site is different in different auxotrophs, for the location of prophage appears to be correlated with the site of a particular bacterial mutation. That prophage may locate at different positions implies that the prophage genome is sometimes fixed and sometimes cytoplasmic although the cytoplasmic existence does not necessarily imply multiplication.

The results do not entirely exclude the possibility that translocations between the bacterial genome and the prophage may occur. The coupling of bacterial and prophage properties may actually represent a translocational type of mechanism. However, it is not necessary to invoke translocations, for the hypothesis that prophage may locate on different sites on the chromosome contains the implication that the prophage is entire and that the lysogenicity and inducibility properties are due to its specific location rather than to bacterial-prophage translocations.

The second important finding was the ability of SB5 to give rise to another auxotroph with the alternate auxotrophic requirement of phenylalanine. The lys⁻ to pa⁻ change was accompanied by a lowering of the productivity and the UV inducibility ratio and was coupled to a

change in expression of H antigens. The change was found to be reversible. The reciprocity of the production of alternate auxotrophs is strongly suggestive of a positional mutational event and argues against a mechanism that involves a reduction from a diplophase state. The simplest mechanism that can be invoked is one that involves a mutating element which moves between the lys and pa loci so that when one function is suppressed the other is gained. Since it has already been argued that prophage is located at these specific sites in these auxotrophs, the possibility that prophage itself might be the mutating element is to be considered. This idea seems to be excluded, however, by the demonstration that the prototrophic revertants of both pa⁻ and lys⁻ cultures have the same productivity and level of UV inducibility as the respective auxotrophs. Prophage has apparently not changed its site of attachment yet the cell is prototrophic.

There is additional evidence that prophage itself is not the mutating element. The origin of lysogeny in the cyt⁻ derivative occurred after the mutation which affected the nutritional requirement; and pa⁻tyr⁻ derivatives may be either lysogenic or non-lysogenic. Auxotrophy may therefore arise independently of lysogeny.

The moving element may take the form of a mutator-type genic system (similar to the Ac-Ds system in maize) or of a controlling element similar to an episome that may move freely between the chromosome and the cytoplasm under appropriate circumstances. Unstable reversions at the su-leu A locus in S. typhimurium were interpreted by Dawson (1962) also as

evidence of a movement to and fro from the leu locus of units which he called "controlling episomes" which induced mutation at any su-leu A site to which they were attached.

To this episomic-like element the symbol S will be applied. S may move and become attached to any point on the chromosome and induce mutations. It may move its location on the chromosome to another locus thus causing the production of alternate auxotrophs. But once attached to particular loci it preferentially attaches to the chromosome at these positions; thus the cell has a 'memory' of previous mutations in its genealogical history.

One of the outstanding features of so many of the changes that have involved lysogenic, auxotrophic, antigenic and other bacterial properties has been the coupling of one change with another apparently unrelated one. These coupled changes can be seen by reference to Table X.

Some of the most outstanding of these coupled changes will be cited here:

a. The origin of lysogeny accompanies an auxotrophic or other physiologically unrelated mutation; e.g. requirement for lysine or cytosine, chloramphenicol resistance.

b. Increased mal mutability (papillae formation) is occasionally coupled with the change from immunity to productivity.

c. Production of alternate auxotrophs or auxotrophs with new requirements is accompanied by many other apparently unrelated changes:

1. The change from lys⁻ to the alternative pa⁻ requirement is

coupled with the loss of the gmq H antigens and the gain of antigens i and 1,2..; it is also coupled to a lowering of productivity and degree of inducibility.

2. The change from pa⁻ to pa⁻tyr⁻ is coupled to a gain of the H antigens gmq and loss of i,1,2..; and also either a complete loss in productivity and immunity or to a heightened productivity and UV inducibility.

3. The change from pa⁻tyr⁻ to tyr⁻ in lysogenic cells is accompanied by an increase in productivity and a lowering of the UV inducibility ratio.

4. The production of nic⁻ and arg⁻ auxotrophs from Prototroph 2 was accompanied by a loss in UV inducibility.

d. The production of the lys⁻ auxotrophs from Prototroph 1 was accompanied by a mutation to mot⁻.

e. 'Segregation' from Prototroph 2, immune and non-productive, resulted in the simultaneous gain of auxotrophy and productivity.

f. The loss of flagella by pa⁻, SB⁴2 derivatives was accompanied by a loss of the O antigen; reversion to motility resulted also in the gain of the O antigen.

g. 'Reversion' to prototrophy in some SB⁴2 derivatives was coupled to a loss in productivity.

h. The change to immunity in the cyt⁻, SB⁴ culture was accompanied by a loss of the ability to produce a sheen on EMB-maltose.

i. The acquisition of immunity in the SB⁵6 culture, derived from SB⁴

was coupled to an increase in mal mutability.

j. Many of the SB2 derivatives which became immune or lysogenic also gained an increase in mutability to streptomycin resistance.

The coupling of so many diverse properties indicates that a common mechanism may be concerned throughout.

On the other hand, changes that were originally coupled were not necessarily permanently coupled. The production of the alternate pa⁻ auxotrophs was originally coupled with a change to expression of the H antigens i and 1,2..; but pa⁻ cultures readily revert to prototrophy and while the majority of prototrophic revertants are also i;1,2.. very few revert back to gmq. The lys and mot genes were coupled mutationally in the 'segregants' of Prototroph 1 but were shown to be separable by recombination for they could be independently transduced. Spontaneous revertants to prototrophy were non-motile and spontaneous swarms which occasionally arose were lys⁻. Lysogenic cells derived from the 'phage infected' pa⁻tyr⁻ clones were still pa⁻tyr⁻. Thus the initial coupling of mutational events at the tyr locus and the phage property loci was no longer in effect, once the coupled mutations occurred.

Many of the coupled changes could be described as phase changes. Thus the lack of immunity and productivity in SB2 and the gain of immunity and productivity in some derivatives may be visualized in this way. The immunity to productivity change itself (especially evident in the Prototroph 2 derivatives) may also be visualized to occur as a phase change. The lys⁻pa⁺ to lys⁺pa⁻ change and the changes to different degrees

of productivity may also be visualized as phasic.

Phase changes in higher plants were reviewed by Brink (1962) in terms of an hypothesis in which it was assumed that in addition to the genes which are stable, the chromosome also contain "self-perpetuating accessory materials that undergo para-mutation.....as an essential aspect of a nucleo-cytoplasmic system of morphogenetic determination."

The presence of so many coupled changes very strongly supports the idea that a movable mutating element is responsible for the observed changes. Coupling of different factors may occur if some loci remain mutable after S has shifted location or if S has an affect which extends to adjacent genes. More than one episomic-like factor may actually be involved and these become associated by a form of recombination. S may also undergo recombination with prophage. Linked to prophage, bacterial mutations become coupled to prophage mutations; linked to other S-like factors additional loci may be affected. Precedent for recombination between episome-like elements is the recombination that has been shown to occur between the different R factors of antibiotic resistance discovered by Mitsuhashi et al. (1962).

Occasionally, S may make a 'mistake' in locating on the chromosome and it locates at a different position, thus causing the production of new genotypes. Thus, for example, the arg⁻ and the nic⁻ mutants could have arisen in this way.

SUMMARY

Three independent derivatives of Salmonella blegdam, strain SB2, have exhibited the capacity to produce a temperate bacteriophage which lyses SB2. On seven occasions, lysogenic cells were isolated directly from these derivatives. The event leading to lysogeny or phage production occurs at mutational frequency. It was concluded, therefore, that SB2 carries at least most of the hereditary specifications of a temperate bacteriophage to which it is sensitive. Further mutational changes in phage related properties observed in SB2 derivatives included changes from sensitivity to immunity, changes from lysogeny back to sensitivity and changes from one level of lysogenic productivity and ultra-violet inducibility to another.

Most of the changes in phage related properties were accompanied by mutational changes affecting other unrelated bacterial properties which included nutritional requirements, motility, flagellar and somatic antigens, fermentation, colony morphology and antibiotic sensitivity. The prevalence of these coupled mutations argues that the changes in phage related properties are position effects rather than "point mutations".

Coupling between two or more mutations affecting non-phage related properties also occurs. Sometimes coupling is maintained for both forward and reverse mutation. This type of coupling usually involves a reciprocity - while one mutation restores a function, the coupled mutation abolishes another. In other situations, the coupling is lost once the mutations have occurred. Events occur which increase the probability of specific mutations. Some coupled mutations are "abortive" for they lead

to functional genes which do not replicate. The cell has a "memory" for previous mutation so that reciprocal mutational changes occur between loci which were previously mutated.

Different auxotrophs showed different levels of UV inducibility. Examination of the degree of UV inducibility of particular auxotrophs led to the hypothesis that the site on the chromosome of a particular mutation was correlated with the location of prophage. Thus prophage is visualized as a movable element which can locate at many sites on the bacterial chromosome but is particularly 'attracted' to sites of mutation.

The hypothesis was advanced that the coupled mutations were controlled by an episomal element to which the symbol S was applied. This element can change position on the chromosome and suppress the activity and alter the mutability of adjacent genes. Prophage and S may occasionally become linked thus coupling bacterial mutations with phage property mutations.

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