ANALYSIS OF GENETIC DETERMINANTS CONTROLLING THE VARIABLE RESPONSE OF ESCHERICHIA COLI TO BACTERIOPHAGE T1

by

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I. INTRODUCTION

Regarding their response to bacteriophage, three types of bacteria have been characterized. Some are resistant, some are sensitive, and others show an intermediate response to phage. A considerable amount of work has been done on the resistant and sensitive types of bacteria, but little information is known about the intermediate response of bacteria toward phage. Cells showing this intermediate type response have been isolated by several groups of workers and have been designated semi-resistant, variably (partially) resistant, or intermediate.

The work done by Carta and Bryson (13) indicated that the T1-variable mutant (B/r/1v) derived from a radiation resistant strain of Escherichia coli B although genetically stable is phenotypically unstable. That is, variable populations are phenotypically heterogeneous with regards to their response to phage T1. These populations consist of cells in the phenotypically sensitive and resistant states. The proportion of cells in either state is growth stage dependent with a predominantly greater number of resistant state cells present during log phase and a predominantly greater number of sensitive state cells present during lag and stationary phases. It appeared

that this T1-variable response is controlled by certain genetic factors. Therefore, for a better understanding of the mechanism of variable resistance, it was felt necessary to determine the genetic factors controlling this type of response. The purpose of this research was to locate and characterize these genetic factors.

II. LITERATURE REVIEW

This review is divided into two major parts. The first part concerns the research that has been done in attempts to characterize various intermediate responses of bacteria to bacteriophage. The second part deals with work related to the mapping of loci controlling various responses of *Escherichia coli* to bacteriophage T1.

A. Studies on Mutants Showing Intermediate Types of Resistance to Bacteriophage

Several bacterial mutants obtained by several groups of workers and showing intermediate types of resistance to bacteriophage have been isolated, but detailed studies on the nature of their characters are mostly lacking. Some preliminary studies concerning the properties of these mutants have been reported, but the mechanism of their resistance and the interrelationship between these mutants still remained unknown.

1. Partially or variably resistant mutants

During studies on spontaneous and induced mutations in radiation resistant cultures of *E. coli* B, Bryson and Davidson (12) found several mutants which they designated as being partially resistant to phage T1. They noticed that this partially resistant strain (B/r/1p) occurred

at a higher rate in populations of induced resistant mutants than in populations of spontaneously arising resistant mutants.

It was found that in broth cultures containing a high titer of phage, the growth rate of the partially resistant bacteria was retarded and that typically resistant cells (B/r/1) arising from the partially resistant cells predominated. However, in lower concentrations of phage the B/r/1p cultures regained their normal growth rate and the population composition of the cultures remained unchanged during the growth stages. In cases of growth on solid medium, if high concentrations of T1 were added, there would be a delayed appearance of bacterial colonies and these consisted of typically resistant cells.

When B/r/1p cells were plated with lower concentrations of phage generally 100 percent efficiency of plating could be observed. The time for colonies of this partially resistant culture to appear on phaged plates was found to be 10 hours later than on unphaged plates. From the above observations, they concluded that the predominating type of cells in the partially resistant cultures are different from the cells in sensitive and resistant cultures.

In 1966 Carta and Bryson reported the isolation of similar types of mutants (B/r/1v) from ultraviolet-irra-

diated E. coli B/r cultures (13). The mutants were isolated by first treating appropriately diluted log phase cultures with ultraviolet light. These diluted cultures were stirred (magnetic stirrer) on an watch glass during the irradiation. After 90 to 99 percent of the population had been killed, one-tenth ml of aliquots of the treated cultures were spread on nutrient agar plates and incubated at 37 C for 2.5 hours. Then o.1 ml of a T1 phage suspension containing 1 x 10^9 plaque forming unit (pfu)/ml was spread on the same plates and incubation was continued for one to two days. Before assaying for the variable character, the colonies were streaked four or five times with intervening subculture on nutrient agar plates in order to eliminate the bacteriophage.

The properties of the isolated mutants were found to be very similar to the wild type B/r strain except in their response to phage T1. Both B/r/1v and B/r strains were able to ultilize lactose, arabinose, xylose, and galactose, were unable to ultilize maltose, and were sensitive to streptomycin and sodium azide.

The production of T1 by B/r/1v cells was studied. It was found that the burst period of the B/r/1v culture was longer than that of the sensitive culture, and the final phage yield was found to be much higher in B/r/1v than in the sensitive culture.

In micromanipulation experiments, B/r/1v cells were singly isolated from various growth phases and placed into phage T1 medium and observed periodically. The proportions of cells in the resistant state were determined by counting the number of isolated cells that formed clones (which eventually lysed) and those that did not form clones. They found that the highest proportion of cells in the resistant state (those that formed clones) occurred at mid-log phase.

The percentage cloning efficiency of B/r/1v cells was determined by counting the number of clonies formed in the presence of excess T1 and comparing this to the number of clonies formed in the absence of phage T1. The peak value of coloning efficiency was in the range of 43-89% and occurred consistently at mid-log phase.

The above studies indicated that with regards to its phage response B/r/1v cultures consist of phenotypically heterogeneous populations. Also the proportion of phenotypically T1-sensitive cells to the phenotypically T1-resistant cells was growth stage dependent. At lag and stationary phases most of the B/r/1v population was phenotypically sensitive to T1, but at mid-log phase most cells were phenotypically resistant to T1. Therefore, it appeared that the response of a cell toward phage was variable. For better describing this particular response

toward phage T1 the term "partial resistance" was changed to "variable resistance".

2. Semi-resistant mutants

Another intermediate type of mutant was isolated from several wild type bacteria such as Shigella paradysenteriae Y6R, Salmonella interitidis, Salmonella typhimurium and Staphylococcus pyogenes (64) by Wahl and Blum-Emerique in 1952. They designated these mutants as semi-resistant strains (65).

The semi-resistant bacteria could be found arising spontaneously or as the result of induced mutations. Most characters of the mutants were identical to the wild type strain except in their reaction to phage. Immunological studies showed that both wild type and semi-resistant strains were antigenically similar.

One character of a semi-resistant strain derived from Shigella paradysenteriae was that on agar medium the number of plaques formed by phage S13 on this strain was always smaller than the number of plaques formed by the same number of phage S13 on the sensitive strain. Also, the size of the plaques was generally smaller and the shape was more irregular than plaques formed on the sensitive strain.

In liquid medium, the adsorption of phage by an early log phase sensitive strain was 96% whereas the

adsorption by the semi-resistant strain was very low (66). Only 0.7% of the bacteria of semi-resistant cultures were able to be infected by the phage. The authors designated the fraction of bacteria which were able to adsorb and support the propagation of phages as being in a receptive state, while those uninfected bacteria were considered to be in a refractory state. There was an equilibrium between these two states. Each different semi-resistant strain was characterized by their proportion of refractory and receptive bacteria in the population (62,67). In general the lower the proportion of receptive bacteria in a semi-resistant population, the smaller was the size of the plaque. Also, in the presence of phages, the receptive bacteria continue to appear as descendents of refractory bacteria. Therefore, from the point of view of its reaction to phage, the semi-resistant culture is a heterogeneous population.

Since there was usually only a small portion of the semi-resistant population in the receptive state, the rate of multiplication of phage was usually slower than the growth of bacteria. During the multiplication of the phage on the semi-resistant strain, the phage first infected the small portion of the receptive bacteria in the population then infected new receptive bacteria descending from initially refractory bacteria. Therefore,

no massive lysis could be observed because of the slow phage proliferation. The yield usually was very low and a high titer could not be obtained.

For supporting the propagation of phage S13 in both the sensitive and the semi-resistant bacteria, it was found that the absolute and relative concentrations of $PO_4^{=}$ and Ca^{++} ions in the medium were very important (68). The phage would only grow in a certain range of Ca^{++} concentration with the $PO_4^{=}$ concentration lower than a certain maximum. The growth of phage S13 on semi-resistant strain of Shigella paradysenteriae was found to require more calcium and tolerate less phosphorus than on the sensitive strain.

Finally, the authors used the term "reactivity" (63) instead of "virulence" to describe the different reactions of the very similar strains toward the same type of phage under the same condition. The reactivity of a strain is related to the frequency of appearance of the receptive state bacteria as descendents of refractory bacteria. Under given condition the greater the number of receptive bacteria in a semi-resistant population, the higher its reactivity.

3. Intermediate type resistant mutants

In 1962 Bendigkeit et al. (9) obtained several mutants from E. coli B showing intermediate resistance

to bacteriophage T5. They found that this resistance was quite stable for each individual mutant but the degree of resistance among mutants differed. In liquid or agar media, the growth rates of intermediate,sensitive, and resistant cells are all identical in the absence of T5. But in the presence of phage T5, colony formation of the intermediate cells was delayed in comparision to the resistant cells. In high concentrations of phage T5, colony formation was prevented.

It was found that the intermediate mutants as well as the sensitive cells, could not be killed by the phage which had been previously inactivated by heat, by sonic oscillation or by antiserum but could be infected by the ultraviolet light treated phages. They also found that iron could protect the intermediate type cells but not sensitive cells against the action of phage T5 or UV-treated T5. Microscopic study showed that in the presence of T5, the death and lysis of mutant cells were reduced by iron. When iron was added at the adsorption period, the multiplication of phages was greatly reduced.

From the above results the authors proposed that this intermediate type of resistance is primarily due to a low rate of adsorption.

B. Mapping of the Loci Controlling Resistant/Sensitive Response to Phage T1 in E. coli

1. The tonB locus

During the isolation of tryptophane requiring (try^-) mutants, Yanofsky and Lennox (71) found that phage T1 resistant cells could be easily obtained that also had the try^- mutation. These data indicate that the gene controlling resistance/sensitivity to phage T1 (tonB) is closely associated with tryptophane (try) region and that deletions may simultaneously cause mutations in both regions. During further study on the accumulation of the intermediates in tryptophane synthesis produced by these deletion mutants, they found that the tonB is closely linked to the try gene and is on the same side as the leucine (leu) marker.

In 1966 Singner (58) further established that $att_{\phi 80}$ (attachment site for prophage $\phi 80$) is also closely linked to T1 gene but is on the opposite side of try region, which means that the T1 marker (tonB) lies between the try and $att_{\phi 80}$ markers on the bacterial chromosome.

2. The tonA locus

A second locus which controls the resistance/sensitivity response to phage T1 was mapped on bacterial chromosome by several workers (17,23,51). This locus was found to control not only the resistance/sensitivity to

phage T1 but also the cells' response to phage T5. This T1,T5 controlling gene was named tonA locus (21).

The transduction experiments done by Demerec *et al.* (23) showed that the cotransduction frequency of azi-pan (azi, resistant/sensitive to azide; pan, genes determining and regulating pantothenic acid biosynthesis) with tonA was about 23.1 to 61%. This result indicated a very close linkage relationship between tonA and azi-pan markers.

Data obtained from conjugation mapping gave more information about the position of this tonA locus on the bacterial chromosome. Maccuro and Hayes (51) found that the tonA locus is about 2 minutes (in time of transferring) apart from azi and on the same side with lac (lactose fermentation) region. Curtiss(17) found that the tonA gene is about 4 minutes away from pro (genes determining and regulating proline biosynthesis) and on the same side with azi marker.

From the above date, it is clear that the tonA gene is located between pro and azi markers on the *E. coli* chromosome.

A chromosomal map indicating the positions of both tonA and tonB loci is given in Fig. 2.

III. MATERIALS AND METHODS

A. Reagents and Media

Xylose and all the L-amino acids were obtained from Nutritional Biochemical Corporation, Cleveland, Ohio. The nutrient agar, nutrient broth, agar, yeast extract, penassay broth, tryptone, and bromothylmol blue were from Difco Laboratories, Detroit, Michigan. Streptomycin sulfate and potassium penicillin G were obtained from Eli Lilly Company, Indianapolis, Indiana. Ethyl methanesulfonate (EMS) was obtained from Eastman Kodak Company, Rochester, N. Y. All the other reagents were reagent grade and were purchased from J. T. Baker Chemical Company, Phillipsburg, New Jersey.

The concentrations of each different aqueous stock solution are listed below:

L-amino acids	5,000 γ/ml, when 50 γ/ml concentration was used in the medium 50 γ/ml, when 0.5 γ/ml concentration was used in the medium
D-glucose	20%
Streptomycin	20,000 γ/ml
Penicillin G	40,000 units/ml
$CaCl_2$	2.5×10^{-1} M

The composition of 10X strength minimal salts stock solution is shown as follows (19):

K_2HPO_4	105.0 g
KH_2PO_4	45.0 g
Na citrate . $2H_2O$	4.0 g
$MgSO_4$. $7H_2O$	1.2 g
$(NH_4)_2SO_4$	10.0 g
H_2O	1 liter

In preparing the above medium, magnesium sulfate was added last after all the other chemicals had been dissolved. This stock solution was divided into several 100 ml-bottles and autoclaved. Minimal broth medium was made by diluting the stock salts solution appropriately with distilled water, sterilizing, and then adding the proper amount of sterile stock glucose (20% solution), and in some cases, the appropriate sterile stock amino acid solutions. For solid minimal medium a sterile agar solution was added to the minimal broth medium so as to give a final agar percentage of 1.5. The concentration of glucose and amino acid in the medium were 0.2% and 50 γ/ml respectively.

The following media were used for the isolation of mutants, and in conjugation and transduction experiments:

L Broth (LB) (49):

Bacto-tryptone	10 g
Yeast extract	5 g

NaCl	5 g
Glucose	1 g
Water	1 liter

The pH was adjusted to 7.0 with 1 N NaOH.

Bromo thymol blue-xylose agar:

Bromo thymol blue	60 mg
Xylose	10 g
Nutrient agar	23 g
Water	1 liter

L Broth Agar (LA) (49): LB contained 1.2% agar. The sterile $CaCl_2$ was added to final concentration 2.5×10^{-3} M before pouring plates.

L Broth Soft Agar (LSA) (49): LB contained 0.6% agar. Before distributing 2 ml portions of soft agar(LSA) into sterile tubes, $CaCl_2$ was added to give a concentration of 2.5×10^{-3} M. This soft agar was maintained at 45 C before using.

SAH (Soft Agar with 0.39% agar) (25):

Nutrient broth powder	8 g
NaCl	5 g
Agar	3.9 g
Water	1 liter

The pH was adjusted to 7.4 with NaOH.

Tryptone Magnesium Medium (Try-Mg) (25):

Tryptone	10 g

NaCl	10 g
$MgCl_2 \cdot 6H_2O$	2 g
Water	1 liter

This medium was used without pH adjustment.

B. Bacterial Strains and Bacteriophages

The E. coli B/r (radiation resistant and T1 sensitive), B/r/1v (T1 variable), bacteriophage T1, and phage P1kc were obtained from this laboratory. The donor strains HfrB1, HfrB2, and HfrB3 were kindly supplied by Dr. Herbert Boyer (Department of Microbiology, University of California Medical Center, San Francisco, California). The phage P1bt used for transduction experiments was kindly donated by Dr. W. B. Dempsey (Department of Biochemistry, University of Texas, Southwestern Medical School, and Basic Research Unit, Veteran's Administration Hospital, Dallas, Texas).

All bacterial broth cultures were grown at 37 C on a reciprocating water bath shaker (New Brunswick Scientific Company, New Brunswick, New Jersey) and, the cultures on solid media were incubated at the same temperature. The stock bacterial cultures were maintained at 4 C and transferred to fresh media every six months.

The phage T1 was propagated as follows: A four hour log phase culture of E. coli B/r was mixed with a small amount of stock phage T1 and incubated at 37 C with aeration.

The ratio of phage to bacteria was 1:10. After the mixture was clear, the lysate was centrifuged and a few drops of chloroform were added to the supernatant. The mixture was shaken at 37 C to evaporate the chloroform and then assayed for plaque forming units.

The assay procedure for phage involved adding 0.1 ml of a serially diluted phage suspension into 2.5 ml soft agar (0.7%) at 45 C, and adding a drop of log phase B/r cultures into the phage agar mixture before it was evenly spread on the surface of nutrient agar plates. After 6 hours incubation time, the well separated plaques were counted and the phage titer was calculated.

C. Isolation of Bacterial Mutants

1. Amino acid requiring mutants

Amino acid requiring mutants were isolated by following the procedure described by Gorini and Kaufman (30). Five ml of an overnight stationary phase culture was transferred to 45 ml of fresh nutrient broth and grown for 3 hours. A portion of this log phase culture was spun down by using a clinical centrifuge (International Equipment Company, Boston, Massachusetts) at its maximum speed for 10 minutes. The precipitated cells were then resuspended in an equal volume of minimal salts medium and 0.1 ml of ethyl methanesulfonate was added to every 10 ml of culture suspension. The culture

was incubated with aeration by shaking for another hour and then centrifuged. The precipitate was resuspended in an equal volume of minimal salts solution then 50 ml of minimal medium containing 0.2% glucose and a growth factor amino acid (50 γ/ml) were added. The cells were grown overnight, centrifuged and resuspended again in minimal salts medium. This overnight culture was diluted with 9 volumes of minimal salt medium containing 0.2% glucose but without the amino acid growth factor. After three hours incubation, 2,000 units of penicillin G per ml were added and the cells were grown with aeration for another two hours. The penicillin treated cells were centrifuged and resuspended in minimal salts medium. The culture was assayed on nutrient agar, and then 0.1 ml of a suitable dilution of the cell suspension was spread on minimal agar plates containing 0.2% glucose and 0.5 γ/ml any particular growth factor. The growth factor requiring auxotrophic suspects appeared as small colonies on plates, whereas prototrophs appeared as large colonies.

Small colonies were replicated using the velveteen method (46) on minimal salts agar with 0.2% glucose and with or without amino acid (50 γ/ml). Any colony which did not grow on a plate without amino acid but on plate with a particular amino acid was considered a mutant requiring that particular amino acid.

Further purification was accomplished by streaking

the mutant clones on minimal salts agar containing 0.2% glucose and 50 γ/ml of the required amino acid. Single, well isolated colonies were picked up and transferred to nutrient agar slants. These slant cultures were used as stock strains for all experiments.

2. Streptomycin resistant mutants

The streptomycin resistant mutants were isolated by growing ethyl methanesulfonate treated cells overnight in nutrient broth and then screening these by plating 0.1 ml of a suitable dilution on nutrient agar containing 200 γ streptomycin per ml. Cells were treated with the same concentration of EMS as described previously. After the one hour incubation with EMS the cells were spun down, resuspended in an equal volume of nutrient broth, incubated overnight, and screened.

3. Xylose non-utilizing mutants

Xylose non-utilizing (xyl^-) mutants were isolated from stationary phase culture that were treated with EMS as described above. These EMS treated cells were spread on bromothymol blue-xylose-nutrient agar. The xyl^- colonies which appeared blue were picked up and further purified on bromothymol blue-xylose-nutrient agar. Mutants were maintained on nutrient agar slants.

D. Spotting Test

The original HfrB3 strain gave very low recombination

frequency presumbaly because it had reverted to a predominantly F^+ population (33) after repeated transfers in various laboratories. Therefore, a spotting test was used to re-isolated the HfrB3 strain from the predominantly F^+ culture. The isolation procedure followed the method described by Taylor and Adeleberg (60). Colonies of the reverted donor culture were spotted on a lawn of F^- recipients that had been prepared on media selective for recombinants. The faster growing Hfr clonies which appeared on the selective media were picked up and used as donor strains for all the conjugation experiments.

E. Method for Assaying the Variable Response to Bacteriophage

The assay method was based on the streaking test of Carta and Bryson (13). Cells from each individual colony were picked up and streaked on nutrient agar plates. After 12 to 14 hours incubation time milky saline suspensions from each colony were cross streaked on nutrient agar against a phage T1 (ca. 5×10^{10} pfu/ml) streak and incubated overnight. Resistant and sensitive strains were cross streaked in the same manner and served as controls. The sensitive cultures showed no growth after the point of contact with the phage T1 except for resistant mutant colonies which appeared later. Resistant strains grew uniformly in the whole area of the bacterial streak. Variable mutants showed

a much reduced amount of growing beyond the point of contact with the phage streak (Figure 1).

F. Acridine Orange Test

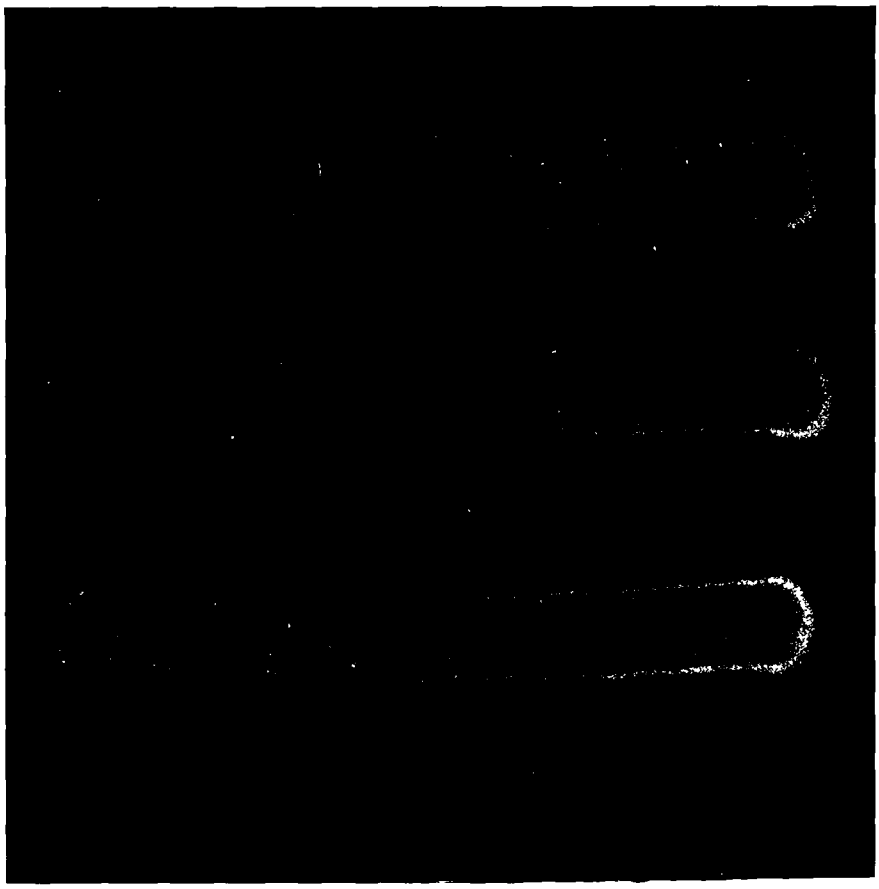
Hirota's method (39) was used for this test. The variable culture was grown overnight in penassay broth (pH 7.4) plus 30 µg/ml acridine orange. The treated cells were streaked on nutrient agar plates and each individual colony developing on the plates was used for assaying the variable response.

G. Conjugation

The transfer of chromosomal material from one bacterium to another by direct cell to cell contact was discovered in 1946 by Lederberg and Tatum (48). This process, called conjugation, has been shown to occur in many bacteria (7,8, 10,18,27,40,50,52,73,74).

Bacterial conjugation involves random collision of cells of opposite mating type and the formation of an effective contact between them, followed by the unidirectional transfer of genetic material from donor to recipient cell (33,34,35, 44,55,57). There are two successive stages in bacterial conjugation (34,36). The first is the formation of zygotes. Three steps are involved in this process: the formation of specific pairs; the conversion of specific pairs to effective

Figure 1. Streaking test for assaying the bacterial variable response to bacteriophage T1. Portion of a Petri dish containing nutrient agar upon which different saline suspensions of E. coli have been cross-streaked against bacteriophage T1: (top to bottom) sensitive (B/r), variably resistant (B/r/1v), and resistant (B/r/1) to T1. The direction of the bacterial streaks which developed is from right to left across the vertical phage streak.



pairs; and the transfer of genetic material. The second stage of conjugation, the formation of recombinants, involves two steps: the integration of the transferred material into the recipient chromosome and the segregation of the recombinant chromosome.

In Hfr x F^- cross, the transfer consists of the progressive penetration of a Hfr chromosome into the F^- cell (55). Penetration always starts from the same extremity or origin in all cells of a given Hfr type, and proceeds in a predetermined sequence. The time required for transferring the whole chromosome is approximately 2 hours (34,70).

Genetic transfer has been studied experimentally by stopping mating at intervals by violent agitation to break up the mating pairs (70). In this way the order of transfer of markers may be determined by examining which and how many markers have been transferred and recombined in a given time period. A genetic map can be constructed in units of transferring time (36).

The mapping of a chromosome can also be accomplished by determining the cotransfer frequencies of unselected markers among the selected recombinants (36). The cotransfer frequency of an unselected marker is dependent upon the distance between this marker and the selected marker. That is, the further the unselected marker is from the selected marker, the lower is the probability of transfer. On the

other hand, the closer the distance between the two markers, the higher the probability is for cotransfer. By comparing the cotransfer frequencies of unmapped markers with other unselected markers of known locations, the position of the unmapped markers can be determined.

1. Experimental

The parental strains used in conjugation study were routinely checked for their genetic markers a few days prior to using them in experiments. The matings between donor and recipient strains consisted of mixing in a 250 ml Erlenymer flask 0.5 ml of a log phase culture of donor cells grown in penassay broth with 5 ml of recipient cells grown similarly. This mixture was incubated for 1 hour at 37 C. The surface area-bolume relationship of this mixture allowed for aeration without shaking and the 1:10 ratio of donor to recipient cells allowed the donor cells a better chance to make effective pairing with recipient cells. Serial dilutions of this mating mixture were made and then 0.1 ml portions of the mixture were plated on medium selective for recombinants. The unmated donor and recipient cells were spread on the same type of medium and served as controls. Well isolated recombinant colonies were picked and purified by streaking on selective agar plates.

2. Interruption of conjugation

Three slightly different approaches were tried for the

interruptive conjugation experiments:

(i) Mixed samples (4ml) of the donor and recipient cultures were removed from a flask at various time interval and transferred into a 5 ml glass cup of a Virtis blender (Virtie Company Inc., Gardiner, N.Y.). Cell pairs were separated by blending the samples for 2 minutes at maximum speed (23,000 rpm). The blades and the cup were cleaned between samples by washing several times with sterile distilled water. The blended mixture was immediately poured into a cooled sterile empty tube and diluted with saline in an ice bucket. Duplicate 0.1 ml samples of the blended material were plated on various selective media for scoring the different recombinant classes.

(ii) The second method for interrupted mating was carried out as above except a Sorvall Omni-mixer (Ivan Sorvall, Inc., Norwalk, Connecticut) instead of a Virtis mixer was used to blend pairing cells and the blended material was diluted 5 fold right after separation.

(iii) The third procedure for interruptive conjugation was based on the method described by Haan and Gross (32). Both donor and recipient cultures were inoculated separately into 5 ml of nutrient broth and incubated overnight without aeration. Then, a 0.5 ml aliquote of donor culture was diluted tenfold into 4.5 ml fresh nutrient broth and incubated in a shaker for 1.5 hours. The young donor cells (0.2 ml) and the overnight culture of recipient cells (1 ml)

were added to 1 ml nutrient broth and shaken for 5 minutes in a water bath. The mixture was diluted by adding 0.5 ml of the cells to 200 ml prewarmed nutrient broth. Both the diluted and the undiluted mixtures were then plated on selective media after incubating for one hour without aeration.

H. Transduction

In transduction, a bacteriophage acts as a genetic vector transferring bacterial genetic material from one bacterial strain to another. This mode of genetic exchange has been extensively investigated by many workers (47,49,59, 72,75). Phages accomplishing generalized transduction appear capable of incorporating any portion of the bacterial genome, including both chromosomal genes and episomal factors (2,42).

In general, during conjugation, the donor bacterium can contribute a very substantial part, or even all, of its DNA to the related recipient cell. The process of transfer of DNA requires direct contact between the donor and recipient cells (34,36). In transduction, genetic transfers occur indirectly from donor to recipient bacteria by means of a bacteriophage vector. However, for obvious reasons only a small portion of the bacterial genome can be incorporated into the phage. Therefore, only those genes which are close

together on the bacterial chromosome will be simultaneously transduced by a phage particle (49,75,6,22,24). The higher the cotransduction frequency of two markers, the closer the distance between them. Usually, no cotransduction can be obtained when two markers are two minutes or more apart. The correlation of cotransduction frequency with map distance in terms of transferring time (minute) has been summarized by Taylor and Trotter (61).

1. Experimental

Methods of Lennox (49) and Dempsey (25) were used in the transduction experiments.

(i) Lennox's method

Phage P1kc was used as gene carrier and the titer of the phage obtained by this method was usually very low.

a. Phage assay

An overnight culture of 1 ml indicator strain (x289) in LB was added to 9 ml LB (containing 2.5×10^{-3} M $CaCl_2$) and grown for 1.5 hours with aeration. The log phase culture (1.8 ml) was added to 0.2 ml of P1kc phage lysate at different dilutions in sterile tubes. After 20 minutes for preadsorption, 0.2 ml of the mixture was added to 2 ml LSA and poured on LA plates. After the top layer of soft agar had been solidified, the plates were inverted and incubated for 6 hours. The plaques were counted and the phage titer was calculated.

b. Phage lysate preparation

The donor strain was grown as above. To 1.9 ml of the donor cells 0.1 ml P1kc phage with a titer of 6-8 x 10^7 pfu/ml was added. After 20 minutes at 37 C, a portion of this adsorption mixture (0.2 ml) was transferred into 2 ml LSA and plated on LA medium. When the soft agar cooled, the plates were inverted and incubated for 6 hours. Then to each plate 5 ml of LB was added and the plates were allowed to stand in a refrigerator overnight. For harvesting the phage particles, the soft agar layer was broken up with a spreader and poured into centrifuge tubes. A small amount of chloroform (1:40) was added to the lysate, and the mixture was centrifuged by using a clinical centrifuge at maximum speed for 10 minutes. The chloroform was then evaporated by shaking the mixture at 37 C for several hours. Then the supernatant was assayed as described above. The phages were usually grown for another cycle on the donor strain before being used for the transduction experiments.

c. Transduction to recipient cells

The recipient strain was grown as above. The phage which had previously been grown on donor strain was diluted to allow for a multiplicity of infection of about 3 when it was added to the recipient cells. After 20 minutes preadsorption time the mixture was diluted and 0.05 ml of each dilution was plated on media selective for recombinants.

After a suitable incubation period recombinant clonies were purified as described previously.

(ii) Dempsey's method

Dempsey's method was applied here with some modification. The phage P1bt was used as the genetic vector for transferring the gene markers from the donor strain to the recipient strain.

a. Preparation of phage

The P1bt phage strain obtained from Dempsey was diluted to 5×10^{8} titer by adding 0.05 ml of the phage stock (1×10^{10} pfu/ml) into 1 ml ammonium acetate (2%).

Donor bacteria were grown from small inocula in LB overnight. The culture (1 ml), along with diluted phages in 1 ml of 2% ammonium acetate, was added to 30 ml of melted SAH at 45 C. This mixture was then poured evenly onto three 150-cm^2 large sterile petri plates which contained fresh LB agar with $CaCl_2$, at concentration of 2×10^{-3} M, and incubated 5 hours.

Harvesting was done according to Helling (37). The lysates were collected by adding 10 ml 2% ammonium acetate to each petri plate, scraping the top layer of soft agar into a centrifuge tube and emulsifying. The emulsified material was centrifuged at low speed (5,900 x g in a Sorvall Refrigerated Centrifuge, Ivan Sorvall, Inc., Norwalk, Connecticut) for 10 minutes. The supernatant was saved and

the precipitate was resuspended in 10 ml of fresh 2% ammonium acetate and centrifuged again. The precipitate was discarded. The supernatants were combined and stored at 4 C overnight. Then these lysates were concentrated and purified by two cycles of alternative low speed and high speed (2 hours at 20,200 x g in a Sorvall Centrifuge) centrifugation. Pellets from the second high speed centrifugation were resuspended in 2% ammonium acetate and again stored overnight at 4 C.

Usually the phages were grown for two cycle on the donor strain before they were used in the transduction experiments. The method of assay for phage titer was similar to that described in the method of Lennox.

b. Transduction to recipient cells

Transduction was done in 2% ammonium acetate solution. All the solutions used in this experiment were prewarmed to 37 C. The recipient cells were inoculated into LB medium and set at 37 C overnight. Four ml of the overnight culture was added to 60 ml of fresh L broth and incubated for 4 hours. The cells were centrifuged by using an International Centrifuge (International Equipment Company, Boston, Massachusetts) at 3,020 x g for 10 minutes. The cell pellets were suspended in 4 ml of 2% ammonium acetate in a sterile centrifuge tube, and then 0.7 ml of 0.1 M $MgCl_2$ was added. Half of this suspension was pipetted into another sterile tube. One of the tubes was used as the control. One-tenth

ml of 2% ammonium acetate was added to one control tube, and the same volume of phage (3×10^9 pfu/ml) which had been grown on donor cells was added into another tube. Then 0.4 ml of 0.5 M $CaCl_2$ was added to each tube. After allowing the mixture to stand for 5 minutes at 37 C, the tubes were centrifuged and the cells were suspended in 1.4 ml Try-Mg solution. Finally, serial dilutions of the cell suspensions were spread on agar media selective for recombinants.

I. Genetic Nomenclature

The rules and symbols of genetic nomenclature proposed by Demerec et al. (21) are followed in this thesis. The symbols try, his, tyr, thr, leu, and met designate the genes which determine the synthesis of tryptophane, histidine, tyrosine, threonine, leucine, and methionine respectively. Plus and minus signs indicate the cell's ability or inability, respectively, to synthesize the amino acid. The gene xyl, gal, and lac determine the utilization of xylose, galactose, lactose respectively. Plus and minus signs indicate the ability and inability to utilize the substrate. The str gene determines the response to streptomycin (s and r indicate sensitivity and resistance, respectively, to this antiobiotic). The gene T1var designates the variable response toward phage T1. Plus and minus signs indicate the gene in active (variable response) or inactive (sensitive to T1) form, respectively. For instance T1var$^+$

indicates that the variable coding region is active giving the variable response, whereas Tlvar$^-$ indicates that the variable region is inactive and gives not the variable response but the sensitive response.

IV. RESULTS

A. Isolation of Escherichia coli Mutants Carrying Various Genetic Markers

The strains used throughout this work were all derivatives of the E. coli B/r strain. Most of these mutants were isolated by using mutagen and penicillin treatments as described in Materials and Methods. Table I gives the data concerning the origin and genotype of each isolated mutant and some other bacterial strains which have been used in this work.

During the isolation process, several approaches had been tried for the isolation of the streptomycin resistant mutants. No success was achieved until it was realized that because of their low mutation rate to streptomycin resistance the cells had to be treated with a mutagen (EMS) and allowed to grow overnight in nutrient broth to allow for phenotypic expression. The xylose negative mutants usually could be isolated easily by spreading a portion of the overnight culture of mutagen treated cells on bromothymol blue-xylose-nutrient agar plates.

For the isolation of amino acid mutants, the relative chance for isolating each mutant from mutant suspect colonies was quite different. Some mutants could be isolated easily and other with great difficulty. For example,

Table I. Description of *Escherichia coli* B/r Strains

Strain Number	Origin	Mating Type	Relevant Genotype								
			try	his	T1var	tyr	str	xyl	thr	leu	met
AC 2522	from Boyer	HfrB1	+	+	−	+	s	+	+	+	+
AC 2523	from Boyer	HfrB2	+	+	−	+	s	+	+	+	+
AC 2524	from Boyer	HfrB3	+	+	−	+	s	+	+	+	+
CH 1001	from AC 2524	HfrB3	+	+	−	+	s	+	−	+	+
CH 1002	from AC 2522	HfrB1	+	+	−	+	r	+	+	+	+
CH 1003	from CH 1002	HfrB1	+	−	−	+	r	+	+	+	+
CH 1004	from AC 2523	HfrB2	+	+	−	+	s	+	−	+	+
CH 1005	from AC 2523	HfrB2	+	+	−	+	s	+	+	−	+
CH 1006	from CH 1004	HfrB2	+	+	−	+	s	+	−	+	−
CH 2001	B/r/1v	F⁻	+	+	+	+	s	+	+	+	+
CH 2002	from CH 2001	F⁻	−	+	+	+	s	+	+	+	+
CH 2003	from CH 2002	F⁻	−	+	+	+	r	+	+	+	+
CH 2004	from CH 2003	F⁻	−	+	+	+	r	−	+	+	+
CH 2005	from CH 2002	F⁻	−	+	−	+	r	+	+	+	+
CH 2006	from CH 2005	F⁻	−	+	−	+	r	−	+	+	+
CH 2007	from CH 2001	F⁻	+	+	+	+	s	−	+	+	+
CH 2008	from CH 2004	F⁻	−	+	+	−	r	−	+	+	+
CH 2009	from CH 2004	F⁻	−	−	+	+	r	−	+	+	+
CH 2010	from CH 2009	F⁻	−	−	+	−	r	−	+	+	+
CH 2011	from CH 2012	F⁻	+	+	−	−	s	+	+	+	+
CH 2012	B/r	F⁻	+	+	−	+	s	+	+	+	+

in the case of isolating thr$^-$ mutants from strain CH 2007 no mutant could be obtained even after 683 mutant suspects (the small colonies which appeared on low amino acid containing agar plates) had been picked up. While for the isolation of his$^-$ mutants from strain CH 2004, four mutants were obtained from 60 suspects. The frequencies for obtaining the mutants from small suspect colonies are given in Table II.

B. Reversion from the Variable (B/r/1v) to the Sensitive (B/r) Trait

Besides the phenotypic instability described by Carta and Bryson (13), it was also found that the T1-variably resistant mutant (B/r/1v) is also genetically unstable. The revertant rate was calculated by testing the T1-variable character of each colony from a B/r/1v stock at different time intervals. The results are shown in Table III. The data indicate that there is a high reversion rate from the variable trait (B/r/1v) to the T1-sensitive (B/r) trait.

C. Acridine Orange Test

Before attempting to genetically map the T1-variable locus (T1varA), it was necessary to check at first whether or not this locus was associated with cytoplasmic or episomal genes. Acridine orange was used in this experiment for detecting whether a cytoplasmic genetic factor or

Table II. Frequency of mutants arising from mutant suspects

Parental Strains (strain number)	Genotype of Isolated Mutants	Frequency
CH 2001	try$^-$	1 : 88
CH 2007	thr$^-$	<1 : 683
CH 2007	leu$^-$	<1 : 456
CH 2004	lys$^-$	<1 : 86
CH 2004	tyr$^-$	<1 : 27
AC 2523	thr$^-$	2 : 159
AC 2523	leu$^-$	3 : 554
CH 2004	tyr$^-$	2 : 60
CH 2004	his$^-$	4 : 60
CH 2010	tyr$^-$	4 : 84

Table III. Revertant rate of Tl-variably resistant mutant CH 2001 (B/r/lv)

Time of Testing	Revertant Rate
Feb. 1967	Two out of 223 B/r/lv cells reverted back to the Tl-sensitive genotype
Mar. 1968	One out of 154 B/r/lv cells reverted back to the Tl-sensitive genotype
Oct. 1968	One out of 138 B/r/lv cells reverted back to the Tl-sensitive genotype

episome exist that may control the variable response. Ninety colonies were picked up from acridine orange treated B/r/1v cells and none of them lost the variable response to phage T1. This result indicated that the gene controlling the T1-variable response in the B/r/1v cell is probably chromosomally rather than episomally located.

D. Conjugation Experiments

In an attempt to identify and map the gene responsible for the controlling of T1-variably resistant character, a bacterial cross was first carried out between strains AC 2522 (HfrB1 carrying try^+, $T1varA^-$) and CH 2004 (F^- carrying try^-, $T1varA^+$). The strain AC 2522 (HfrB1) was used as the donor cell which can transfer its genetic markers to other F^- strains at high frequencies. In this Hfr donor strain the point of origin for transferring genetic markers is near to the try region. The direction of transfer is near to the try region. The direction of transfer is counter-clockwise. This means that the donor cell transfers its genes starting from the try region toward the gal-lac-thr region. Both the entry point and the direction of gene transfer of this HfrB1 strain are shown in Figure 2 (p.55). The results of crosses between the HfrB1 and the CH 2004 (F^-) are given in Table IV. In this experiment try^+ was used as the selected marker. From the try^+ recombinants population, 182 recombinant colonies were picked up randomly and

Table IV. Frequency transfer of donor unselected markers among recombinants of crosses between AC 2522 x CH 2004

Donor Strain	Recipient Strain	Selected Marker*	Number of Recombinants	Frequency (%) Transfer of Unselected Markers
				T1var$^-$
AC 2522	CH 2004			
HfrB1; str^s, try$^+$, T1varA$^-$	F$^-$; str^r, try$^-$, T1varA$^+$	try$^+$	182	0

*The counter selected marker was str^r.

assayed for the T1-variable character. It was found that none of these 182 recombinants lost the variable response character. Therefore, it appeared that the gene controlling the T1-variable response is not at the try-gal-lac--- region on the chromosome.

For further investigation of the locus of the T1-variable gene, the region on the other side of the chromosome was checked by using the donor strain CH 1001 (HfrB3). The entry point of this HfrB3 is also near the try region, but the direction of gene transfer is opposite to that of HfrB1. This means the genes transfer in a clockwise direction (Figure 2). Several crosses were made between strain CH 1001 (HfrB3) and mutants of CH 2004 (F^-). The frequencies of transferred donor markers are shown in Table V. When try^+ was used as selected marker, it was found that about 10% of the donor's $T1varA^-$ marker could be transferred with the try^+ gene into recipient cells. The cotransferred frequencies of both the str^S and xyl^+ genes were found also to be about 10%. These results indicated that the T1varA gene is located somewhere near the try-his-str-xyl--- region on the chromosome. However, the relative distances of the T1varA gene from other markers still could not be determined from these data because the transfer frequencies of the unselected markers were too low.

For further mapping the T1varA gene, the his marker

Table V. Frequency transfer of donor unselected markers among recombinants of crosses between CH 1001 (HfrB3) and CH 2004 (F^-)

Donor Strain	Recipient Strain	Selected Marker*	Number of Recombinants	Frequency (%) Transfer of Unselected Markers			
				try^+	$T1varA^-$	str^S	xyl^+
CH 1001	CH 2004						
$HfrB3;try^+$,	$F^-;try^-$,	try^+	300	100	10.3	9	12
$T1varA^-,str^S$,	$T1varA^+,str^r$,	try^+	110	100	16.3	11.8	16.3
xyl^+,thr^-	xyl^-,thr^+						

Recipient strain revertant rate control : The 114 colonies from CH 2004 had been picked up before mating and tested for variable response, none of them lost the variable response.

*The counter selected marker was thr^+.

was also selected for in recipient cells (CH 2009). Crosses were performed by using CH 1001 (HfrB3) as the donor cells. The transfer frequencies for other unselected markers among the try$^+$ and his$^+$ recombinants are listed on Table VI and VII respectively. The data on Table VI agree with the results from Table V indicating that the T1varA locus is located at the try-his-str-xyl--- region. The data on Table VII show a higher cotransferred frequency (36-40%) for T1varA$^-$ when his$^+$ was used as the selected marker. This result indicates that of the markers T1varA, str, xyl, and try the one closest to his is T1varA.

In order to determine more precisely the relative position of the his and the T1varA loci on the chromosome map, a new marker (tyr) was developed in the recipient cell (CH 2010). Crosses were carried out between CH 1001 (HfrB3, tyr$^+$) and CH 2010 (F$^-$, tyr$^-$). Selection was made for his$^+$, tyr$^+$, or try$^+$ recombinants and the frequencies transfer of unselected markers including T1varA were determined. The results are shown in Table VIII-XI.

Among the his$^+$ recombinants tested (Table VIII), 46 to 48% of the recombinants carried the donor's T1varA$^-$ (T1-sensitive) marker. This result again indicates the close linkage between his and T1varA genes. Also when tyr was used as the selected marker (Table IX), an even closer linkage relationship between T1varA and tyr was indicated

Table VI. Frequency transfer of donor unselected markers among recombinants of crosses between CH 1001 (HfrB3) and CH 2009 (F^-)

Donor Strain	Recipient Strain	Selected Marker*	Number of Recombinants	Frequency (%) Transfer of Unselected Markers				
				try^+	his^+	$TlvarA^-$	str^S	xyl^+
CH 1001	CH 2009							
HfrB3;try^+, $TlvarA^-$,his^+, str^S,xyl^+, thr^-	F^-;try^-, $TlvarA^+$,his^-, str^r,xyl^-, thr^+	try^+	152	100	4.6	9.3	6.6	6.6

Recipient strain revertant rate control : The 55 colonies from CH 2009 had been picked up and tested for variable response, none of them lost the variable response.

*The counter selected marker was thr^+.

Table. VII. Frequency transfer of donor unselected markers among recombinants of crosses between CH 1001 (HfrB3) and CH 2009 (F^-)

Donor Strain	Recipient Strain	Selected Marker*	Number of Recombinants	Frequency (%) Transfer of Unselected Markers				
				try^+	his^+	$T1varA^-$	str^S	xyl^+
CH 1001	CH 2009							
HfrB3;try^+,	F^-;try^-,							
his^+,$T1varA^-$,	his^-,$T1varA^+$,	his^+	81	11.1	100	39.5	18.5	22.2
str^S,xyl^+,	str^r,xyl^-,	his^+	121	11.5	100	36.4	19.0	21.4
thr^-	thr^+							

*The counter selected marker was thr^+.

Table VIII. Frequency transfer of donor unselected markers among recombinants of crosses between CH 1001 (HfrB3) and CH 2010 (F^-)

Donor Strain	Recipient Strain	Selected Marker*	No. of Recombinants	Frequency (%) Transfer of Unselected Markers					
				try$^+$	his$^+$	T1varA$^-$	tyr$^+$	str^S	xyl$^+$
CH 1001	CH 2010								
HfrB3;try$^+$,	F^-;try$^-$,	try$^+$	116	100	12.1	18.9	14.6	14.6	9.5
his$^+$,T1varA$^-$,	his$^-$,T1varA$^+$,	his$^+$	102	15.7	100	46.1	33.3	20.6	24.5
tyr$^+$,str^S,	tyr$^-$,str^r,	his$^+$	76	31.5	100	48.6	31.5	31.5	30.2
xyl$^+$,thr$^-$	xyl$^-$,thr$^+$								

*The counter selected marker was thr$^+$.

Table IX. Frequency transfer of donor unselected markers among recombinants of crosses between CH 1001 (HfrB3) and CH 2010 (F^-)

Donor Strain	Recipient Strain	Selected Marker*	No. of Recombinants	Frequency (%) Transfer of Unselected Markers					
				try$^+$	his$^+$	T1varA$^-$	tyr$^+$	str^S	xyl$^+$
CH 1001	CH 2010								
HfrB3;try$^+$,	F^-;try$^-$,								
his$^+$,T1varA$^-$,	his$^-$,T1varA$^+$,	tyr$^+$	108	13.6	24.1	79.6	100	32.4	31.6
tyr$^+$,str^S,	tyr$^-$,str^r,	tyr$^+$	158	19.0	30.4	80.3	100	37.3	34.1
xyl$^+$,thr$^-$	xyl$^-$,thr$^+$								

*The counter selected marker was thr$^+$.

Table X. Frequency of occurrence of unselected markers in tyr^+ recombinants

Donor Strain	Recipient Strain	S.M.*	N.R.	Unselected Markers			
				his^+ T1varA$^-$	his^+ T1varA$^+$	his-T1varA-	his^-T1varA$^+$
CH 1001	CH 2010			%	%	%	%
HfrB3; try^+,his^+, T1varA$^-$, tyr^+,str^S, xyl$^+$,thr^-	F^-; try^-,his^-, T1varA$^+$, tyr^-,str^r, xyl^-,thr^+	tyr^+	101	22.8	3	60.3	13.9
		tyr^+	145	30.3	2.1	56.6	11.0

S.M. : Selected Marker

N.R. : Number of Recombinants

*The counter selected marker was thr^+.

Table XI. Frequency of occurrence of unselected markers in his^+ recombinants

Donor Strain	Recipient Strain	S.M.*	N.R.	Unselected Markers			
				$tyr^+ T1varA^-$	$tyr^+ T1varA^+$	$tyr^- T1varA^-$	$tyr^- T1varA^+$
CH 1001	CH 2010			%	%	%	%
HfrB3;	F^-;						
try^+, his^+,	try^-, his^-,	his^+	100	31.0	3.0	16.0	50.0
$T1varA^-$,	$T1varA^+$,	his^+	76	26.3	5.2	22.3	46.2
tyr^+, str^s,	tyr^-, str^r,						
xyl^+, thr^-	xyl^-, thr^+						

S.M.:Selected Marker

N.R.:Number of Recombinants

*The counter selected marker was thr^+.

since 80% of the donor trait T1varA$^-$ marker could be transferred together with tyr$^+$ marker to the recipient cells.

On Table X and XI, the distribution and the relationship of the unselected markers among his$^+$ and tyr$^+$ recombinants are presented. It is noteworthy that 2-3% of the tyr$^+$ recombinants were his$^+$ T1varA$^+$ whereas 11-14% of the tyr$^+$ recombinants were his$^-$ T1varA$^+$. Similar results were obtained when the selected marker was his (Table XI). Among his$^+$ recombinants, the percentage of tyr$^+$ T1varA$^+$ cells is 3-5 whereas the percentage of tyr$^-$ T1varA$^-$ cells is 16-22. These data indicate strongly that the T1varA gene is located between the his and tyr loci and the order of these three markers is his-T1varA-tyr-str-.

Summarized results of the recombination frequencies for all the above experiments are listed in Table XII.

E. Interrupted Conjugation

Three different approaches (described in Materials and Methods) were tried for the interrupted conjugation experiment. The third approach was patterned after Haan and Gross's method (32). In this method the mating mixture was diluted 400 fold before being spread on the selective medium. Because of the low efficiency of mating between strain CH 1001 (HfrB3) and the T1-variable mutant CH 2010 (F$^-$), no recombinant could be observed. Therefore, the dilution

Table XII. Recombination frequency of various donor and recipient cells

Donor	Recipient	Recombinant Class	Recombination Frequency (%) (recombinants per ml/Hfr cells per ml) x 100
HfrB1	CH 2004	try^+	5.4
HfrB1	CH 2004	try^+	6.9
HfrB3	CH 2004	try^+	0.11
HfrB3	CH 2004	try^+	0.143
HfrB3	CH 2008	tyr^+	0.1265
HfrB3	CH 2009	his^+	0.0242
HfrB3	CH 2009	try^+	0.1804
HfrB3	CH 2010	tyr^+	0.057
HfrB3	CH 2010	try^+	0.198
HfrB3	CH 2010	his^+	0.539

method could not be applied for this mating system.

Different shearing forces were employed to blend the paired cells by using a Virtis blender or an Omni-mixer in the first two approaches. The blended cells were spread on agar plates selective for recombinants and the number of colonies appearing on the agar plates were counted after incubation. The results obtained were confusing and therefore unsatisfactory. No definite entering time for the markers could be observed.

The possible reasons for the anomalous results obtained in these experiments are discussed later.

F. Transduction Experiments

The first transduction experiment was done between strains CH 2004 (carrying T1varA$^+$) and CH 2012 (B/r, carrying T1varA$^-$) with phage P1kc. Lennox's method (49) was followed in this experiment. The phages were grown on donor cells (strain CH 2004) for three cycles (the titer of phage obtained after each cycle usually was very low). The phage stock was then mixed with recipient cells (CH 2012) and spread on medium selective for recombinants. Reciprocal transduction was attempted in the same manner. In these experiments no recombinant could be obtained on the selective medium. This was probably caused by host restriction of the donor cell to phage P1kc and is discussed later.

The next transduction experiments were done using the phage P1bt, which had been propagated on the E. coli B/r strain for a number of cycles. The results of conjugation experiments indicated that the gene which controlled the variable response toward phage T1 in the E. coli B/r/1v cells was situated in the chromosomal region between markers his and tyr. Therefore, attempts were made to map more precisely the location of the T1varA gene by transducing the T1varA, his, and tyr genes with the help of phage P1bt. In this experiment (Table XIII) the markers of donor cells (CH 2012 carrying his^+, tyr^+, and $T1varA^-$) were transduced into recipient cells (CH 2010 carrying his^-, tyr^- and $T1varA^+$) by means of phage P1bt. With tyr^+ as the selected marker, the T1varA gene was cotransduced at a frequency of about 30-45%. This means, according to the data collected by Taylor and Trotter (61), that the T1varA locus should be located 0.5 minute from the tyr locus.

Reciprocal transduction was attempted. However, the titer obtained was very low (10^3 pfu/ml) after the phages had been grown on donor cells (strain CH 2010). Thus no recombinant could be obtained on selective medium after mixing transducing phages with recipient cells (strain CH 2012).

The results obtained from both the conjugation and transduction experiments indicate that the exact position

Table XIII. Cotransduction frequency of donor unselected markers among crosses between strains CH 2012 and CH 2010

Donor Strain	Recipient Strain	Selected Marker	Number of Recombinants	Frequency of Unselected Markers	
				T1varA$^-$	his$^+$
CH 2012	CH 2010			%	%
his$^+$,tyr$^+$,	his$^-$,tyr$^-$,	tyr$^+$	80	45	0
T1varA$^-$	T1var$^+$	tyr$^+$	177	30	0

Recipient strain revertant rate control : The 70 colonies from CH 2010 had been picked and tested for variable response, none of them lost the variable response.

of T1varA marker can be mapped at about 0.5 minute from try on the his side of the genome. This location in relation to other markers is shown in Figure 2.

G. Second Locus Which May Control the T1-variable Response

A very interesting phenomenon was observed during the isolation of the streptomycin resistant strains. It was found that among 93 streptomycin resistant mutants isolated, there were 27 str^r mutants which reverted from the T1-variable genotype to the T1-sensitive genotype. That is, when the cells changed from streptomycin sensitive to streptomycin resistant about 29% of the cells lost the variable response to phage T1 and gave the T1 sensitive response. This result indicated that another variable response locus, besides the one which was mapped by conjugation and transduction experiments, could be located very close to the str locus. That is, deletion mutations occurring at the str gene region (causing the change from streptomycin sensitivity to resistance) might have extended through this apparent second variable locus and inactivated it making the cell sensitive. This second T1-variable gene has been designated the T1varB locus.

The data showing the relationship between the variable response and the level of streptomycin resistance in the streptomycin resistant population is shown on Table XIV.

Figure 2. Partial genetic map of the E. coli chromosome (61). The arrowheads on the circle indicate the origin and direction of transfer of the Hfr strains used. The precise location of TlvarB gene in relation to str locus has not been determined.

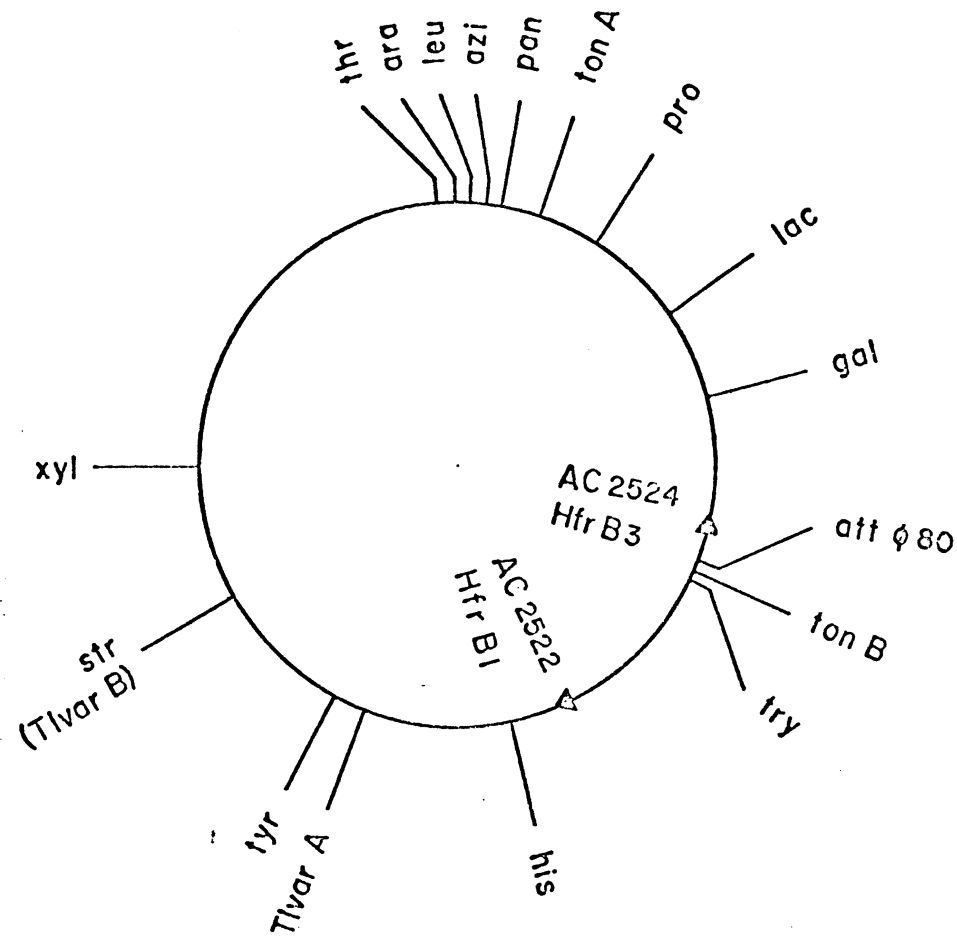

thr
ara
leu
azi
pan
ton A
pro
lac
gal
att φ80
ton B
try
his
tyr
Tlvar A
str
(Tlvar B)
xyl
AC 2524
Hfr B3
AC 2522
Hfr B1

Table XIV. The level of streptomycin resistance and T1-variable response in the streptomycin resistant population

Number of Mutants	T1-variable Response	Maximum Level of Streptomycin/ml Which Cells Grow on					
		600 γ	800 γ	1000 γ	2000 γ	3000 γ	4000 γ
1	S	+	−	−	−	−	−
1	S	+	+	−	−	−	−
1	S	+	+	+	−	−	−
6	S	+	+	+	+	−	−
8	S	+	+	+	+	+	−
10	S	+	+	+	+	+	+
2	V	+	+	+	−	−	−
6	V	+	+	+	+	−	−
28	V	+	+	+	+	+	−
30	V	+	+	+	+	+	+

: grow on streptomycin medium
: do not grow on streptomycin medium
S : T1-sensitive response
V : T1-variable response

It was found that among the 66 streptomycin resistant mutants which retained the variable trait, there were 8 mutants failing to grow on 3,000 γ/ml streptomycin medium and among the 27 streptomycin resistant mutants which lost the variable trait and became sensitive, nine mutants failed to grow on medium containing 3,000 γ/ml streptomycin. When the streptomycin was raised to 4,000 γ/ml, the proportion was 36/66 for T1-variable population and was 17/27 for the T1-sensitive population.

V. DISCUSSION

The variably resistant mutant gives rise to populations that are phenotypically unstable in their response to bacteriophage T1. This means that the variable cells can exist either as phenotypically resistant cells or phenotypically sensitive cells depending upon their growth stage. The rate of change from phenotypically resistant to phenotypically sensitive cells and vice versa was found to be very high (13). Besides these phenotypically unstable cells and some rare stable resistant mutants the results on reversions (p.35) show that the variable populations consist of cells which can revert back to genetically stable sensitive cells at a frequency of 1×10^{-2} to 1×10^{-3}. This is much higher than the spontaneous mutation rate from sensitivity to resistance or from variable to resistance. The actual reason for this high rate of reversion is not known. It could be a true reversion in which the original mutation to T1var^{+} is converted to T1var^{-} (giving a sensitive phenotype) by a second mutation in the T1var region. Or, it also could be a reversion which was the result of a suppressor mutation. A suppressor mutation can be defined as a mutation that reverses the parental phenotype by virtue of mutational alteration at a different position in the

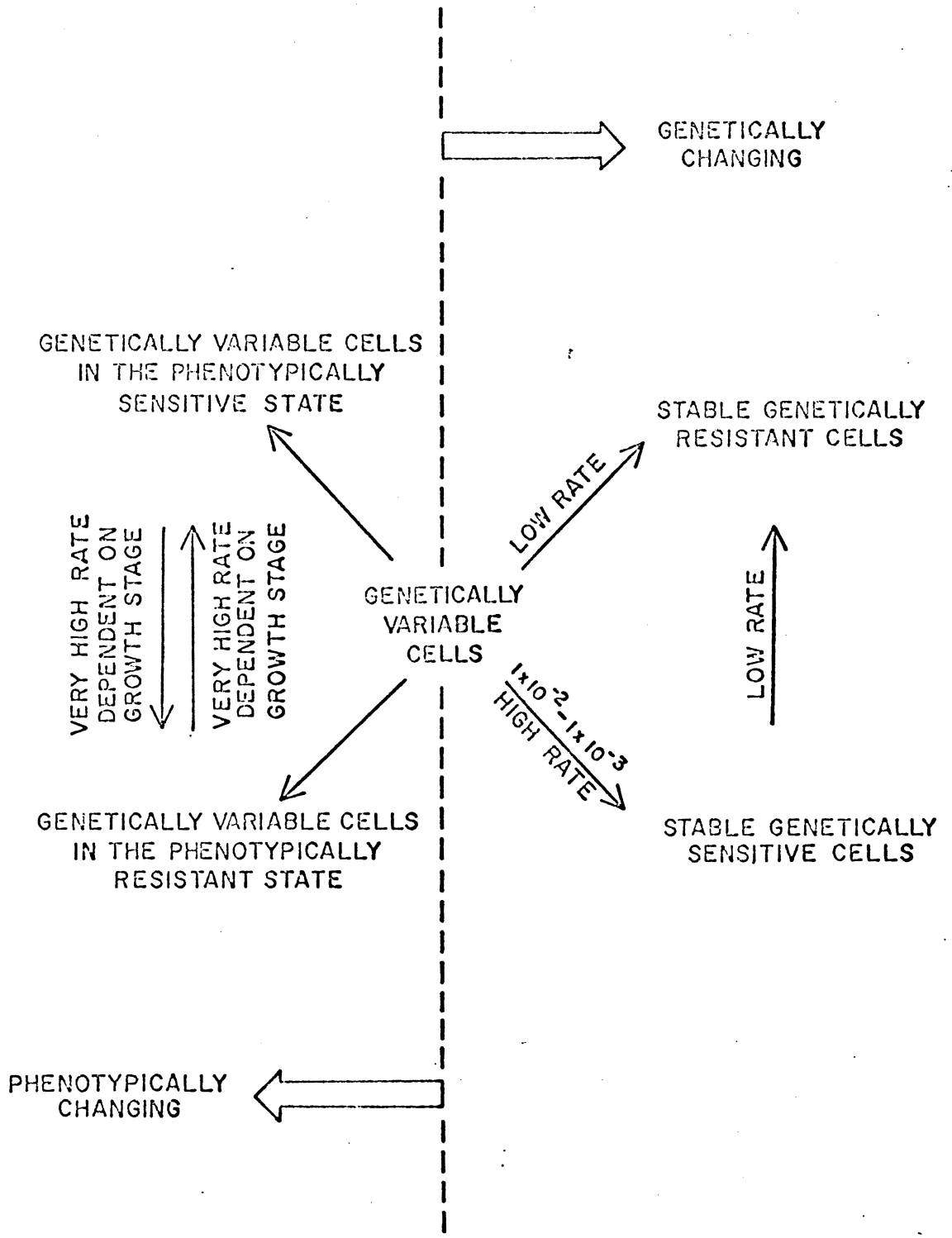
genome. In this case a suppressor mutation would not allow the T1var^{+} gene to express itself and the cells phenotype would be sensitive. Further investigation on the genetic controlling factor is necessary for a better understanding of this unusual type of high reversion rate.

Figure 3 indicates the various genotypic and phenotypic fates of a variable (B/r/1v) population.

It is known that acridine orange dye can cure cytoplasmic DNA which is not associated with the chromosome (38,39). Therefore, treatment of cells with this dye would eliminate the cytoplasmic or episomal genes. The results of acridine orange tests (p.35) show that all the cells retained their variable trait after the dye treatment. This implies that the variable response gene is probably not located on cytoplasmic DNA, but instead, on chromosomal DNA.

Conjugation mapping techniques were employed for finding the approximate location of the T1varA gene on the chromosome. Since the exact location of the genes tonA and tonB (the genes controlling resistance/ sensitivity to phage T1) are already known (3 and 25 minutes respectively, Fig. 2), it seemed advisable to check first whether the T1varA locus is associated with tonA or tonB or whether it represented a distinct allelic form

Figure 3. Diagrammatic representation of the various genotypic and phenotypic fates of variable cells.


GENETICALLY CHANGING
GENETICALLY VARIABLE CELLS IN THE PHENOTYPICALLY SENSITIVE STATE
STABLE GENETICALLY RESISTANT CELLS
VERY HIGH RATE DEPENDENT ON GROWTH STAGE
VERY HIGH RATE DEPENDENT ON GROWTH STAGE
LOW RATE
GENETICALLY VARIABLE CELLS
LOW RATE
1x10 -2 - 1x10 -3 HIGH RATE
GENETICALLY VARIABLE CELLS IN THE PHENOTYPICALLY RESISTANT STATE
STABLE GENETICALLY SENSITIVE CELLS
PHENOTYPICALLY CHANGING

of these loci. Therefore, initial conjugation experiments were done using crosses between HfrB1 and strain CH 2004. The tonB and tonA markers are located proximally to the point of origin of this Hfr donor strain (Fig. 2). Therefore, during the mating process, the tonB-tonA region should be easily transferred from the donor cells into the recipient cells. This was the case and the results (Table IV) show that all the try$^+$ recombinants retained their variable trait after the conjugation experiment. This indicated that the T1varA gene is not located on the try-tonB-tonA--- region.

The other half of chromosome region was checked by several other conjugation experiments between donor strain HfrB3 and mutants of strain CH 2004. The results on Table IX indicate the close association of the T1varA locus with both the tyr and his markers. The order of these markers was further established (Table X and XI) as his-T1varA-tyr- by determining the linkage relationship between unselected markers in conjugation experiments.

The cotransducible character of T1varA with tyr (Table XIII) further confirmed the close linkage of these two loci. The precise location of the T1varA locus (Fig.2) was established by the transduction experiments as being about 0.5 minute from the tyr marker on the same side with the his marker.

The unsuccessful results in interruptive conjugation mapping were probably due to the low recombination frequency between donor and recipient cells. The frequencies of recombination given in Table XII are 10 to 100 times lower than the recombination values indicated by Jacob and Wollman (43) in their conjugation experiments of E. coli K-12. Because of this low recombinantion frequency, high concentrations of both donor and recipient cells had to be screened for obtaining recombinants, and for the same reason the blended cell mixture could not be diluted before spreading on media selective for recombinants. Because of the high concentration of donor and recipient cells present on the selective media, conjugation probably occurred again on this media and made quantitation impossible. It is probably this conjugation after initial interruption of mating which led to the unsatisfactory results of the first two interruptive conjugation experiments. In the last interruptive conjugation experiment the blended cells were diluted before spreading on plates. No recombinants formed because of the low recombination frequency.

Reeve and Suttis (56) reported the same difficulty on interruptive conjugation experiments used for the mapping of a locus causing chloramphenicol resistance in E. coli K-12. No satisfactory results could be obtained in

experiments involving the interrupted mating of a resistant male to a sensitive female because only a small number of resistant recombinants formed on selective agar.

The low frequency of recombination could be explained by the restriction and modification processes which could have taken place in the recipient cell following chromosome transfer (41). The low conjugation frequency between T1-sensitive and T1-variable cells in these experiment is compatible with the results obtained by Boyer (11). He reported that bacterial crosses with E. coli K-12 as donor and B/r as recipient were found to be different from crosses between K-12 donors and K-12 recipients in the following ways: (1) the frequency of recombination was reduced; (2) the recombinants did not appear at discrete time intervals but did appear simultaneously approximately 30 minutes after matings were initiated; (3) the linkage of unselected markers to selected markers was reduced.

Some aspects of the processes of modification and restriction have been clarified by experiments on E. coli system (3,5,15,16,26,28,29,54). Arber and Dussoix (5) and Dussoix and Arber (26) proposed a model for the host controlled modification and restriction of the bacteriophage lambda. They demonstrated that the DNA of the bacteriophage lambda, which was prepared on one strain of E. coli, was hydrolyzed when it infected another strain of E. coli. This restriction is a process which can occur when foreign

DNA enters a cell, either by conjugation or by transduction.

Modification is a process which acts directly on DNA and probably takes the form of specifically altering certain base sequences, perhaps by methylation (4,45). Any DNA synthesized in a particular strain therefore bears a characteristic modification pattern. If the foreign DNA does not bear a modification pattern which is compatible with the recipient cell, it will be rapidly degraded into small molecular weight components. These two mechanisms have been termed host-controlled modification and restriction.

The biochemical nature of these phenomena was mostly unknown, but it is generally believed that the restrictive process must be a result of the action of a nuclease. This enzyme should be able to recognize foreign DNA but will not attack the DNA of the cell in which it was synthesized. Since, it is known that DNA synthesis is not a prerequisite for host-controlled modification (5). Boyer postulated that there are probably two cell products involved in these phenomena, namely, an enzyme capable of recognizing and degrading certain DNA elements (53), and an enzyme capable of modifying all DNA elements within the cell.

Thus restriction may have occurred during the sexual crosses between HfrB3 and T1-variable cultures (B/r/1v)

of E. coli and can be visualized as follows: The introduction of small fragment of the chromosome of donor HfrB3 into the recipient (B/r/1v) cell resulted in the degradation of the fragment before integration could occur. This degradation resulted from the action of a specific nuclease in the recipient cell, which recognized the donor chromosome as foreign DNA. However, the introduction of larger fragment could have saturated the nuclease (degradation process) so that integration occurred before complete hydrolysis. Recombination of the donor exogenote and the recipient endogenote would genetically rescue the donor DNA from further degradation (5). Thus, the appearance of recombinants in restricted crosses can be pictured as a race between hydrolysis of the exogenote and its modification by the recipient cell. This explanation might account for the reduced frequencies of recombination and consequently the unsatisfactory result in interruptive conjugation.

The low phage titer (10^3 pfu/ml) obtained from the transduction experiments between strain CH 2004 and CH 2012 (p.51) is also very probably due to host-controlled restriction. The phage P1kc which had been previously grown on and modified by strain B/r was used for this particular experiment. When these phages gained enterce to the B/r/1v cell, it is possible that they were

recognized as foreign DNA. Then, the degradation process started and the DNA of phage was hydrolized by certain nuclease in the B/r/1v cell, which resulted in the low titer of progeny. The phage titer of this transduction experiment agrees with the data obtained by Arber and Dussoix (5). They noticed that the probability of lambda phage, which had been adapted to E. coli C strain, when grown on E. coli K-12 was about 10^{-4}. The same explanation can be applied to the unsuccessful transduction experiment between strains CH 2010 (donor) and CH 2012 (recipient) with phage P1bt.

Since both the phages strains P1kc and P1bt had already been adapted to E. coli B/r, restriction of these phages by E. coli B/r/1v would probably indicate that the two E. coli strains possess different modification mechanisms.

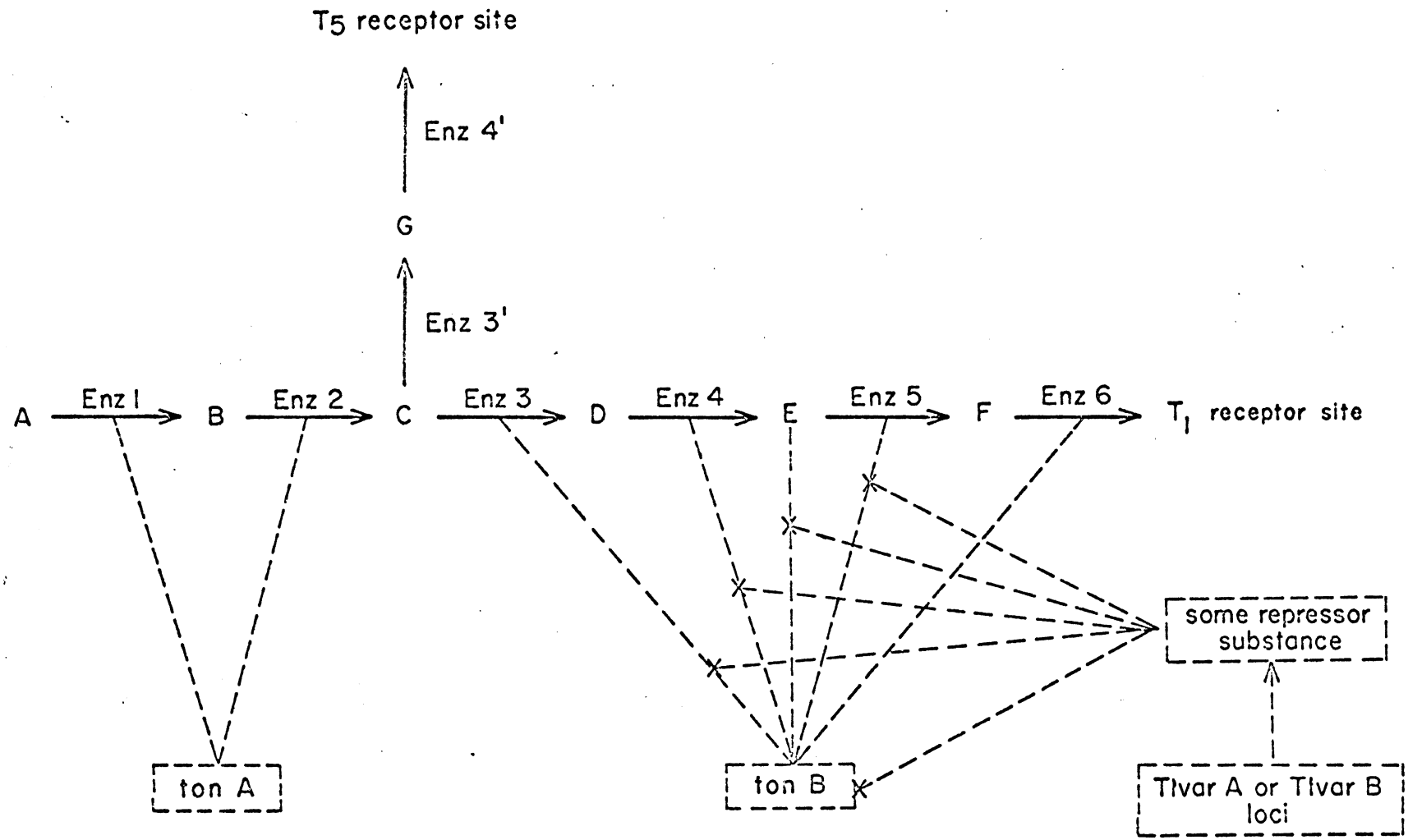
The second locus controlling T1-variable response (T1varB) was discovered during the isolation of the streptomycin resistant mutants from streptomycin sensitive population which were also T1-variable. The fact that 29% of the streptomycin resistant mutants lost the T1-variable response indicated that deletion mutations occurred at str region, which caused a simultaneous mutation at T1varB region. This result implied a close linkage relationship between the T1varB and str loci.

But further work still needs to be done for mapping the precise location of the T1varB gene. The level of streptomycin resistance and the T1-response of the isolated streptomycin resistant mutants are listed on Table XIV. It seems from the result that there is no relationship between the level of streptomycin resistance and the T1-response.

It is known that the adsorption of phage is due to the attachment of phage to a bacterial cell wall phage receptor site. Therefore, the presence or the nature of the receptor site usually determine the response of a cell to a particular type of phage. That is, sensitive cells have phage receptor sites whereas resistant cells do not have these receptor sites (1,69). Like the other cell wall components, it could be assumed that receptor sites are synthesized by means of a biochemical pathway. This pathway could be subject to genetic control. The variable response of a B/r/1v cell might be a result of genetic regulation of the biochemical receptor site synthesis. One hypothetical model of regulation of T1varA, T1varB, tonA, and tonB loci is proposed in Figure 4.

In this model the T1varA or T1varB loci could be considered as structural genes which control certain steps in the pathway for T1 receptor site synthesis. This pathway is set up as indicated for the following

Figure 4. Hypothetical model for the interrelationship of the biochemical pathway for receptor site synthesis, tonA, tonB, T1varA, and T1varB loci.


T5 receptor site
Enz 4'
G
Enz 3'
A
Enz 1
B
Enz 2
C
Enz 3
D
Enz 4
E
Enz 5
F
Enz 6
T1 receptor site
some repressor substance
ton A
ton B
Tlvar A or Tlvar B loci

reasons:

(1) It is known that mutations in the tonA region alter simultaneously the bacterial response to T1 and T5 (17,23,51), therefore, the diagram indicates that the T1 and T5 pathway have two shared precursor steps. Alterations in coding regions for these steps then lead to simultaneous alteration in the bacterial response to T1 and T5.

(2) The tonB locus only controls the bacterial response to T1 (31,58,71), therefore any alteration of coding regions controlling Enz3 $\longrightarrow$ Enz6 (enzyme 3 to enzyme 6) would only effect the bacterial response to phage T1.

It is possible that during the variable cells log phase, a high level of some repressor substance is formed (under the control of the T1varA or T1varB loci). This repressor substance could either act at the transcription level to block the action of one or more structural genes or act at the translation level to interfere the formation of some enzymes in the receptor synthesis. As result of this type of repressor action most of the cells respond as phenotypically phage resistant. During lag and stationary phases none or much lower level of the repressor substances is formed thus most of the cells respond as phenotypically sensitive.

For explaining the fact that a variable cell can revert to the T1 sensitive genotype, it could be assumed that some mutation occur at T1varA or T1varB loci which alter ability to make repressor for blocking the T1 receptor sites synthesis therefore the cell gives a sensitive response.

It was felt that T1varA and T1varB might be suppressor mutation reversing the phenotype of a resistant cell (35). The activity of the suppressor might be considered growth stage dependent, giving rise to the variable trait. However, this is probably not likely since the loss of the variable trait leads to a sensitive rather than a resistant cell.

Also, it could be considered that T1varA and T1varB are coding regions for part of the pathway controlling the T1-response and that mutations in these regions are leaky (35). The degree of leakiness would be growth stage dependent and would account for the phenotypic instability of a B/r/1v cell.

VI. SUMMARY

1. Results of acridine orange tests indicated that the genetic determinants controlling the T1-variable response of a radiation resistant strain of Escherichia coli (B/r/1v) is chromosomally rather than episomally located.
2. Eighteen mutants carrying various genetic markers were isolated from E. coli B, B/r, or B/r/1v strains by using penicillin and mutagen treatments. These mutants were used in the genetic analysis of the T1-variable response.
3. Studies on reversion rates showed that the B/r/1v strain can revert back to genetically stable phage T1 sensitive cells at a rate of 1×10^{-2} to 1×10^{-3} which is much higher than the spontaneous reversion rate.
4. Data obtained from conjugation and transduction mapping experiments indicated that the position of the variable response controlling gene (T1varA) is located 0.5 minute from try on the his side of the genome.
5. A second gene which may control the T1-variable response in a B/r/1v cell was discovered and has been

designated the T1varB locus. The location of this locus was found to be very close to the str locus.

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ANALYSIS OF GENETIC DETERMINANTS CONTROLLING THE VARIABLE RESPONSE OF ESCHERICHIA COLI TO BACTERIOPHAGE T1

Chao-yun Ting Shih

Abstract

These studies were undertaken in an attempt to characterize and map the genetic determinants controlling the T1-variable response. This T1-variable response refers to an anomalous type of resistance to bacteriophage T1 that is exhibited by mutants of radiation resistant strains of Escherichia coli B. Variable mutants give rise to populations that are phenotypically unstable in their response to bacteriophage T1. That is, growth stage dependent transitions occur in these populations so that at mid log phase the populations are predominantly in the phenotypically T1-resistant state, whereas during lag and stationary phases the populations are predominantly in the phenotypically T1-sensitive state. These variable mutants have been designated B/r/1v.

Acridine orange was used to determine whether the locus controlling the T1-variable response was cytoplasmically or chromosomally located. Results showed that all cells tested retained their variable trait after the dye treatment which indicated that the gene

controlling the T1-variable response in the B/r/1v cell is chromosomally rather than cytoplasmically located.

Several mutants carrying various genetic markers were isolated from E. coli B/r/1v strain by using penicillin and mutagen treatments. These mutants were used either as donors or recipients in both conjugations and transduction mapping.

The try-tonB-tonA-- region was first transferred from T1varA$^-$ donor to T1varA$^+$ recipient cells by means of conjugation techniques. No recombinant received the T1varA$^-$ marker from the donor which indicated that the T1-variable marker is not located in the try-tonB-tonA--- region.

Other chromosome regions were checked by several conjugation experiments between a his$^+$, tyr$^+$, and T1varA$^-$ donor strain and a his$^-$, tyr$^-$, and T1varA$^+$ recipient strain. The result showed that 80% of the donor T1varA$^-$ marker could be transferred together with the tyr$^+$ marker to recipient cells. This result indicated a close linkage between the tyr and T1varA genes. The order of these markers was further established as his-T1varA-tyr-- by determining the percentage transfer of unselected markers in conjugation experiments.

Transduction experiments were done between a tyr$^+$, T1varA$^-$ donor strain and tyr$^-$, and T1varA$^+$ recipient strain with phage P1bt. The cotransducibility of T1varA$^-$

with the tyr^+ marker further confirmed the close linkage relationship of these two loci. The location of the T1varA locus was established as being 0.5 minute apart from the tyr marker and on the same side with the his marker.

A second locus controlling the T1-variable response (T1varB) was discovered during the isolation of streptomycin resistant mutants that lost the T1-variable trait from streptomycin sensitive T1-variable populations. The precise location of this gene has not been mapped. These data indicated that the second T1-variable gene is very close to the str marker.