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Initial Constructions of Double Oc Mutants by Double Episome Matings in Escherichia coli

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INITIAL CONSTRUCTIONS OF DOUBLE O^c MUTANTS BY DOUBLE EPISOME MATINGS IN ESCHERICHIA COLI

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This thesis is recommended for approval by the student's thesis committee.

Chairman

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Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Arts.

Northern Michigan University
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INITIAL CONSTRUCTIONS OF DOUBLE O^C MUTANTS BY DOUBLE EPISOME MATINGS IN *ESCHERICHIA COLI*

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A Thesis

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ABSTRACT

By mutagenizing E. coli with chemical agents, constitutive mutants exhibiting a higher than average level of enzyme production were noted. These double O^C mutants are believed to consist of a two base pair substitution in the lactose operon region of the chromosome, as opposed to a single base pair change in a normal constitutive. Attempts have been made to construct double O^C mutants through a double episome mating of varying combinations of representatives of two single O^C mutant subclasses.

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I also wish to acknowledge the assistance and support of my fellow graduate students, Hope Carlson Langkwel, Robert Briggs and Diana Constance in following this odyssey. Of special importance through all of this, has been the understanding help and support I have received from Eldon Handrich. Having been in both places, he could see the situation more clearly than anyone and gave freely of himself and his own time to do what he could to further communication

among all involved and bring about the completion of this research and thesis. Thank you.

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LIST OF ABBREVIATIONS

IPTG	Isopropyl-β-d-thiogalactoside
PG	phenyl-β-d-galactoside
XG	5-bromo-4-chloro-3-indolyl-β-d-galactoside
t-ONPG	ortho-nitrophenyl-βd- thiogalactoside
e-ONPG	ortho-nitrophenyl-β-d-galactopyranoside
TTC	triphenyl-tetrazolium chloride

INTRODUCTION

The lactose operon of Escherichia coli is an inducible system. The basic operon structure consists of a group of genes working as a functional unit, composed of both regulator and structural genes. The lactose operon controls the metabolism of lactose and is inducible in that it operates only in the presence of lactose or some suitable substrate.

According to the work of Jacob and Monod (1961), the regulator gene, i, produces a repressor protein that can interact with the operator region, o , to prevent the production of enzymes by the structural genes. The three structural genes, z, y, and a, code for β-galactosidase, galactoside permease and thiogalactoside transacetylase respectively. The β-galactosidase catalyzes the breakdown of lactose into galactose and glucose, the galactoside permease can render the cell wall permeable to lactose and the function of the transacetylase is unknown. In addition, Beckwith (1967) has delineated a promoter region, p, which determines the maximal level of the expression of the operon and is the site for the initiation of m-RNA synthesis. The linear mapping order of these genes of the lactose operon is i-o-p-z-y-a.

In the functioning of the lactose operon, the regulator gene produces a repressor, which binds with the operator region, blocking the m-RNA synthesis, in the absence of an inducer. When an inducer is added to the system, either as a by-product of lactose metabolism or an artificial one, such as isopropyl-β-d-thiogalactoside (IPTG),

this binds with the repressor, producing conformational changes that reduce its affinity for the operator. By inactivating the repressor, this allows for transcription of the m-RNA and the maximal synthesis of the enzymes by the z, y and a genes. (Gros, 1965 and Reznikoff, 1972)

In contrast to the inducible wild-type, constitutive mutations are also present in the lactose system, either in the regulator or operator genes. By definition, a constitutive mutation is one that results in the uncontrolled synthesis of a particular enzyme, irrespective of the presence or absence of the inducer to which the wild-type is sensitive (Gros, 1965). The operator constitutive mutations, O^C, results in a structural change, which causes reduced repressor affinity and thereby allows more enzyme production than normal. For example, a partial constitutive may produce 5-15% of the β-galactosidase of a fully induced normal. The O^C mutation is dominant to the wild-type (Jacob and Monod, 1961). O^C mutations also exhibit some promoter-like defects in that the maximal level of the gene expression is appreciably increased or decreased as compared to the wild-type.

As shown by Smith and Sadler (1971), chemically induced mutations in the operator region are generally of the one base pair substitution type. These mutations result in an 8-100 fold reduction in the repressor affinity for different sites. There exist between 12 and 16 sites in the operator where a transition can produce a detectable O^C phenotype. These sites are separated into classes on the basis of their O^C P values, where $P= \frac{(z/b)basal}{(z/b)maximal}$, and into subclasses on the basis of the promoter-like effects. The classes are designated by Roman numerals, with the member of a pair exhibiting the higher basal

value designated subclass a, the lower, subclass b. (Figure 1)

Mapping studies (Sadler and Smith, 1971) indicate that the operator is divided into two modules. There are 5-8 pairs of sites with the members of a pair symetrically on opposite sides of the center. (Figure 2) Mutation to one or either member of a pair can result in the same amount of repressor affinity, but exhibits very different promoter effects. There is one member of the subclass in each operator module.

In the study of the classification of O^c mutants, Smith and Sadler (1971) indicate probable double O^c mutants obtained from their mutation processes and selection of multiple O^c mutants. These are represented by triangles on the chart of Figure 1. The double O^c's are thought to be a two base pair mutation that results in a drastic change in repressor affinity. These doubles then have a higher constitutive level and are selected on this basis. The effect of a secondary mutation is to raise the constitutive level by 10-100 fold in most cases. This closely parallels the effect of single O^c mutations, an 8-100 fold increase in the z/b values, over the wild-type basal level. (Smith and Sadler, 1971).

The purpose of this study is to construct double O^c mutants by recombination of two episomes carrying different known single O^c mutations. By comparing the constitutivity of a double O^c to that of the original two single mutations, some statistical model of the activity relation between the two modules can be determined. From this comparison, additional information can be supplied on the transcriptional and translational nature of the operator, as well as on

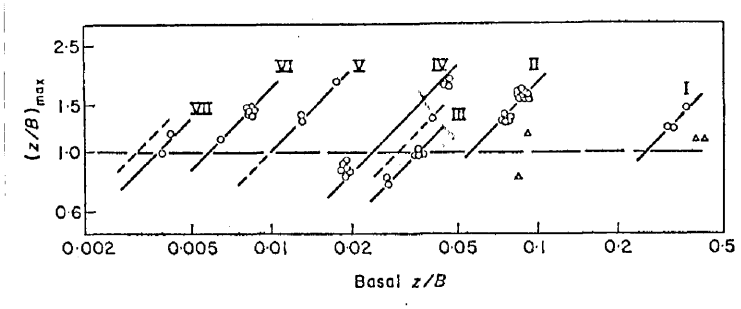


Figure 1. Correlation of constitutive and fully induced (Z/B) values for selected O^C mutants. The abscissa gives the constitutive (Z/B) value and the ordinate the fully induced (Z/B) value. Solid diagonal lines link clusters of mutants with equal or nearly equal P values. Triangles give the coordinates of mutants presumed to be double mutants. (From Smith and Sadler, 1971)

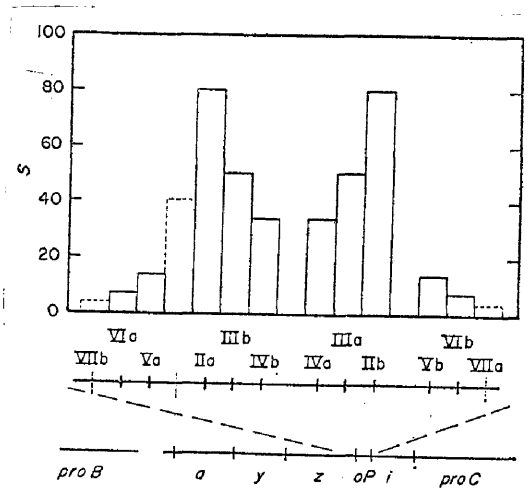


Figure 2. A diagram of the preferred order for the O^C sites on the operator and the related S-value histogram constructed on the basis of the constitutive β-galactosidase activities. S is the ratio of the noninduced or constitutive P values and measures the constitutive effect of an O^C normalized for its promoter effect. (From Smith and Sadler, 1971)

the repressor-operator interaction.

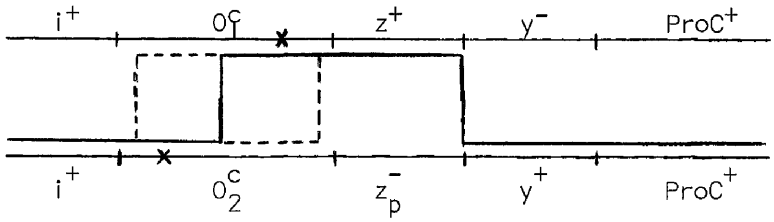
There is some question whether the effect of the secondary mutation acts dependently or independently of that created by the single O^c mutation in the operator. When two corresponding sites in the wild-type operator interact independently with the repressor, the result is a multiplicative in the reduction of affinity. Smith and Sadler (1971) have constructed a double O^c mutant from O^c_{3-4} with a P value of 5.6% and O^c_{3-5} with a P value of 10%. The double mutant exhibited a P value of 17%, indicating an additive effect rather than multiplicative. It appears that in some cases, repressor interactions at two operator sites are independent, while at one pair of sites, the interactions are cooperative. It is theorized that a double mutation within the same module of the operator should show a constitutive level that is the product of the two single values, while a double O^c composed of mutations in the two separate modules have an additive value.

Previous work on this effect has been done with double O^c's obtained through secondary mutation and recombination. In the studies using recombination doubles, it was concluded that the multiplicative hypothesis does not apply to higher valued O^c's at all times and that this method should be tested for the lower classes. (J. Sadler, unpublished).

In E. coli, chromosomal material is transferred by conjugation. One member of a pair of bacteria, designated the male or donor cell since it contains the fertility factor, F, transfers all or a section of the circular chromosome to the female or recipient cell, through cytoplasmic bridges. According to the work of Taylor (1967) it takes approximately 90 minutes for the entire chromosome to pass

linearly to the recipient cell. The lactose operon-pro C interval transfers 10 minutes from the beginning. Recombinant chromosomes are formed as a result of cross-overs between the donor and recipient strands and recombinants can then be selected on differential media. By using known marker genes, the recombining ability, mapping order and the frequency can be determined.

In this study, we attempted to construct a double O^c through the recombination of two single point O^c mutations carried on episomes in two separate lac-proC deletion parents. Schematically, the double O^c recombination is as follows:



This involves a four-factor cross, with two cross-overs, which results in single O^c's of the parent strains as well as doubles containing both mutations. Selection of the double O^c's is done by growth on media that demands high O^c values and the $z^+y^+ProC^+StrR$ characteristics. It is known that there is a minimum distance allowed between cross-overs in recombination. Therefore, the probability of obtaining double O^c's is highest when using two O^c mutations that appear in opposite modules in the operator region. (See Figure 2)

METHODS AND MATERIALS

(a) Bacterial Strains Used and Candidate Selection

All experiments utilized strains of Escherichia coli K 12. O^c mutants were obtained from the stocks of Smith and Sadler, as episomes in P92. The O^c mutant subclasses were plated on t-ONPG for the selection of z^+y^- and z^-_p mutants and these subsequently plated on TTC-Lactose plates for the selection of those with a solid Lactose$^-$ characteristic. Although all of the subclasses were plated and screened in this manner, the best candidates were obtained from subclasses IIa, Va and Vb and these were used in the experiments. Candidates were tested for mating ability by cross-streaking with D509, which carries a z_{ul18} marker and with D703 containing the P_{L8} marker.

Table I lists the characteristics of all bacteria strains used in this experiment. The constitutive P values and β-galactosidase production rates for the candidate subclasses are given in Table 2.

TABLE 1. CHARACTERISTICS OF BACTERIA UTILIZED

P91	F^- lactose-proB deletion, Spc R, StrS, Rec^+
P92	F^- lactose-proB deletion, Spc S, StrR, Rec^+
D509	$F^- i^+ o^+ z^-_{u118}$, $proB^-$, SpcS, StrR
D703	$F^- i^+ p^-_{L8}$ $o^+ z^+ y^+$, $proB^-$, SpcS, StrR
XA7007	F^- lactose-proC deletion, StrR, B^-_1 , SuA^+
XA8001	$F^- i^- p$ deletion $o^+ z^+ y^+ a^+$, thr^-, StrR (L1deletion)
D531P D536P D537P	(Carried as episomes on P92) P92/F lactose-proC, deletion, SpcR, StrS, Rec^+

TABLE 2. RELATIVE -GALACTOSIDASE SYNTHESIS VALUES FOR O^C SUBCLASS MATING CANDIDATES

Class	Episome #	Constitutive P Value in HfrC	(Z/B)max in HfrC	(Z/B)basal in AT2410
IIa	D531P	.056	1.85	.042
Va	D537P	.0098	1.80	.0072
Vb	D536P	.0098	1.33	.0060

(From Smith and Sadler, 1971)

(b) Media and Indicators Used

1. T Broth - An enriched Liquid media which is, per liter of distilled water, 8g Bacto-tryptone (Difco), 1g Bacto-yeast extract (Difco) and 5g NaCl.
2. P Broth - This is a rich liquid media of 17g Difco Penassay Broth per liter of distilled water.
3. D Buffer and minimal plates - Davis minimal buffer is a basic phosphate buffer. Per liter of distilled water, the composition is 0.3g KH_2PO_4, 0.7g K_2HPO_4, 0.05g $Na_2SO_4 \cdot 3H_2O$, 0.01g $MgSO_4 \cdot 7H_2O$, 0.1g $(NH_4)_2SO_4$. Plates are made by the addition of 0.02% sugar, either succinate or lactose and 15g purified agar (Difco) per liter of D buffer.
4. R Buffer - This buffer, described in Smith and Sadler (1971) contains 5.3g NaCl, 3.0g KCl, 1.1g NH_4Cl, 0.095g KH_2PO_4, 0.03g Na_2SO_4, 0.21g $MgCl_2 \cdot 6H_2O$, 13.22g Trizma·HCl (Sigma) and 1.94g Trizma base (Sigma), per liter of distilled water.
5. Tri-Phenyl tetrazolium chloride (TTC) - The TTC-Lactose agar media is that used by Horiuchi, Tomizawa and Novick (1962). The composition, per liter of distilled water, is 8g Bacto-tryptone (Difco), 1g Bacto-yeast extract (Difco), 5g NaCl, 15g Difco agar, 2g K_2HPO_4, 10 g lactose and 50 mg TTC (Sigma). On TTC-lactose plates, Lac+ bacteria appear whitish or pink, while Lac$^-$ are red.
6. Phenyl-β-d-galactoside (PG) - This can be converted to an inducer by the action of β-galactosidase. Plates are composed of 0.02% PG (Sigma), 0.02% lactose, 5g KH_2PO_4, 11g purified agar (Difco), per

liter of D buffer. High constitutive mutants are selected on these plates. All levels of constitutives will grow with the addition of 10^{-3}M IPTG to the media.

7. o-Nitrophenyl-β-d-thiogalactoside (t-ONPG) - t-ONPG is a galactoside that selects against permease function (Gilbert and Muller-Hill, 1966). The plate composition used, per liter of D buffer, is 2g sodium succinate, 0.3g t-ONPG (Pierce or Cyclo) and 11g purified agar (Difco). With this indicator, any z+y$^-$ bacteria appear blue, while Lac+ of z^-_py$^?$ appear white.
8. 5-Bromo-4-chloro-3-indolyl-β-d-galactoside (XG) - These indicator plates are based on R buffer. The media contains 25mg XG (Sigma), 2g sodium succinate, 500mg Bacto Casamino acids (Difco) and 11g purified agar (Difco) per liter of R buffer. On these plates, constitutive z+ appear various shades of blue, depending on their level of β-galactosidase.

(c) Mating Procedure - Double Episome Cross

All donor males were grown up overnight in 5 ml of P broth and diluted with fresh broth and incubated for an additional 45 minutes. Recipient females were also grown up overnight in 25 ml of P broth and subjected to violent shaking in the waterbath. The P91 females were then radiated with ultraviolet light for 30 seconds, from a height of approximately 3 feet.

Into each mating tube or flask, there was added 1 ml of P91, the recipient female, plus 1 ml each of two episome-carrying males, for a total volume of 3 ml. Control flasks were prepared with 1 ml of P91 and 1 ml of a male culture, to check the mating ability. The tubes were incubated for 1 hour, followed by the addition of 2 ml of fresh P broth to each tube and then incubated for another hour. Equal volumes of 200 ug/ml of streptinomycin in P broth were then added to each tube to give a final dilution of 100 ug/ml of strep to kill any unmated P91 which is StrS. The tubes were then shaken for 1-3 hours.

Prior to plating, 5 ml of each tube was spun down twice in D buffer for 10 minutes, at approximately 5,000 rpm in a standard Sorvall centrifuge, and resuspended in 5 ml of D buffer. For each mating set, 0.05 ml of a 10^6 dilution in D buffer was plated on phenyl-galactoside plates, with and without 10^{-3}M IPTG, succinate minimal plates and lactose-strep minimal plates.

All of the plates were scored and the phenyl-gal, without IPTG were replica plated into P92 on phenyl-gal or XG plates to have them in a non-radiated background for assay. The three double episome combinations used for the matings are listed in Table 3.

TABLE 3. PRINCIPAL CROSSES USED IN MATINGS

1. Subclass:	IIa	+	Vb	$\rightarrow\!\!\!\!\times$ P91
Candidates:	D531f	+	D536d	$\rightarrow\!\!\!\!\times$ P91
Characteristics:	z^-p	+	z^-p	$\rightarrow\!\!\!\!\times$ P91
2. Subclass:	IIa	+	Va	$\rightarrow\!\!\!\!\times$ P91
Candidates:	D531f	+	D537F	$\rightarrow\!\!\!\!\times$ P91
Characteristics:	z^-p	+	$z+y^-$	$\rightarrow\!\!\!\!\times$ P91
3. Subclass:	Va	+	Vb	$\rightarrow\!\!\!\!\times$ P91
Candidates:	D536d	+	D537F	$\rightarrow\!\!\!\!\times$ P91
Characteristics:	z^-p	+	$z+y^-$	$\rightarrow\!\!\!\!\times$ P91

(d) Beta-galactosidase Enzyme Assay Procedures

Ten candidates from each mating set were selected at random from the P92 replica plates and grown in 1 ml of T broth. A second outgrowth for 16 hours was done by inoculating 0.01 ml of the above cultures into both 1.5 ml of T broth and 1.5 ml of T+IPTG, for uninduced and fully induced comparisons. To obtain a bacterial count or density, 0.05 ml of each set was put into 1.5 ml of D buffer and the optical density read at 350nm on a Beckman model DB spectrophotometer.

For the assay, 0.05 ml of bacteria culture was put into 1 ml of D buffer. The cells were lysed with one drop of toluene and a drop of 5% n-lauroyl sarcosine. The tubes were then vortexed to insure lysis and placed in a 28°C waterbath for at least 20 minutes. After this ti 1.5 ml of o-nitrophenyl-β-d-galactopyranoside (e-ONPG) was added to each tube and this listed as time zero. The activity of the enzyme was quenched with 1.5 ml of 1M Na_2CO_3, whenever a suitable optical density, indicated by a deep yellow color was reached. After a minimum of 10 hours refrigeration, samples were brought to room temperature and read at OD 420 nm.

The (Z/B) was determined by the formula: $\frac{Z}{B} = \frac{\text{Net OD 420}}{\text{Net OD 350 } \Delta t}$ for both the maximum and basal levels. In this equation, Δt is the time from the e-ONPG addition to the Na_2CO_3 shut off.

All assays were run as duplicates or triplicates. Minimal glucose media was substituted for T broth in the last assay sets. In addition, the parents of each mating type were assayed by the same procedure, taking 0.01 ml of a 12% glycerol stock for growth.

(e) Additional Procedures for Double O^C Verification

1. Suppressor Addition

Double O^C mutants in P92 rec^- were transferred into XA7007 by single mating. Cultures were grown overnight in 1 ml of P broth and freshened with an additional 1 ml of P broth one hour prior to mating. 0.05 ml of the double O^C culture and 0.05 ml of the XA7007 were incubated at 37°C without shaking for 3 hours. Equal volumes of 200 ug/ml of streptinomycin in P broth were added and shaken for one hour. The mating mixture was then streaked on TTC-lactose-Strep plates for selection. Clones were then grown in T and T+IPTG for β-galactosidase assay.

2. Resolving Double O^C mutants

Single matings, as for suppressor addition, were done with a double O^C mated to D509 and a double O^C mated to XA8001. Platings were done on phenyl-β-d-galactoside media and these replica plated into P92 rec^-. Clones were then selected for β-galactosidase assay.

(f) Utilization of Data

The (Z/B), both maximum and basal was computed for each assay set according to the formula: $\frac{Z = \text{Net OD } 420}{B \quad \text{Net OD } 350_{\Delta t}}$, where Δt is the time from the e-ONPG addition to the quenching with Na_2CO_3 for each tube. The P values are obtained by the ratio of the basal to maximum rate for each tube: $P = \frac{(Z/B)\text{basal}}{(Z/B)\text{maximum}}$. Statistical histograms were made of P values to determine the fit when relevant. Correlation plots of (Z/B) maximum vs. basal were constructed to provide a grouping of the resultant values and indicate any promoter effects or misfits.

DATA

TABLE 4. SUMMARY OF SELECTED MATING DATA FOR INITIAL DOUBLE EPISOME MATING

Cross	Class	Mating	Average of 2 sets (z/b)basal	(z/b/)maximal	P
A 7	IIa ⇀↽ IIa	531d + 531f ⇀↽ P91	.0980	2.0030	.0500
B 4	IIa ⇀↽ Va	531d + 537E ⇀↽ P91	.0085	.9885	.0088
E 8	IIa ⇀↽ Va	531f + 537F ⇀↽ P91	.0085	1.5440	.0055
G 4	IIa ⇀↽ Vb	531d + 536d ⇀↽ P91	.0610	.2645	.2310
J 7	Va ⇀↽ Vb	537F + 536d ⇀↽ P91	.0175	4.1600	.0042
J 10	Va ⇀↽ Vb	537f + 536d ⇀↽ P91	.0030	.0805	.0380
F 1	Va ⇀↽ Va	537E + 537F ⇀↽ P91	.0180	1.8630	.0096

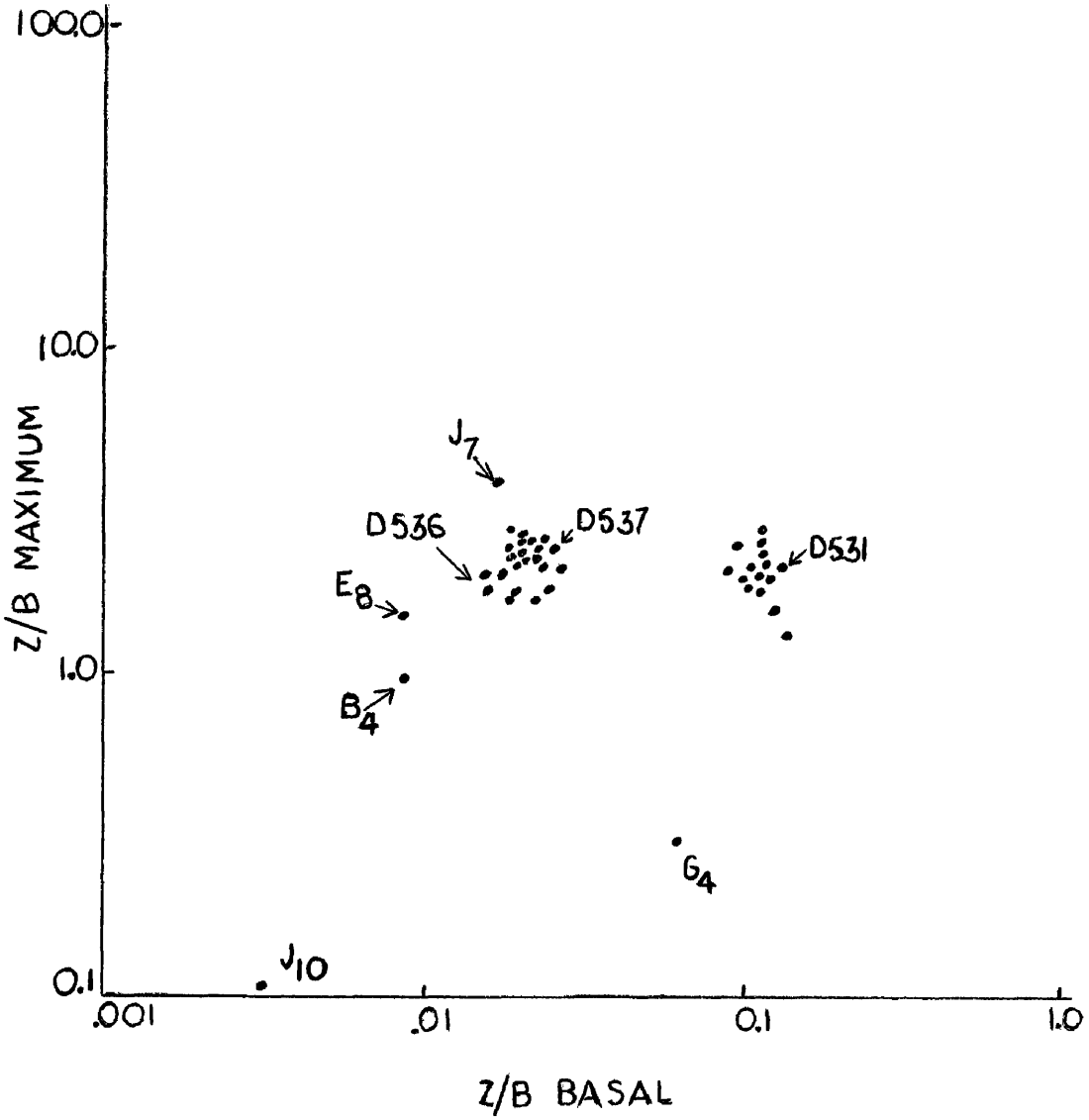


Figure 3. Correlation plot of (Z/B)maximum vs. (Z/B)basal values for the first double episome mating

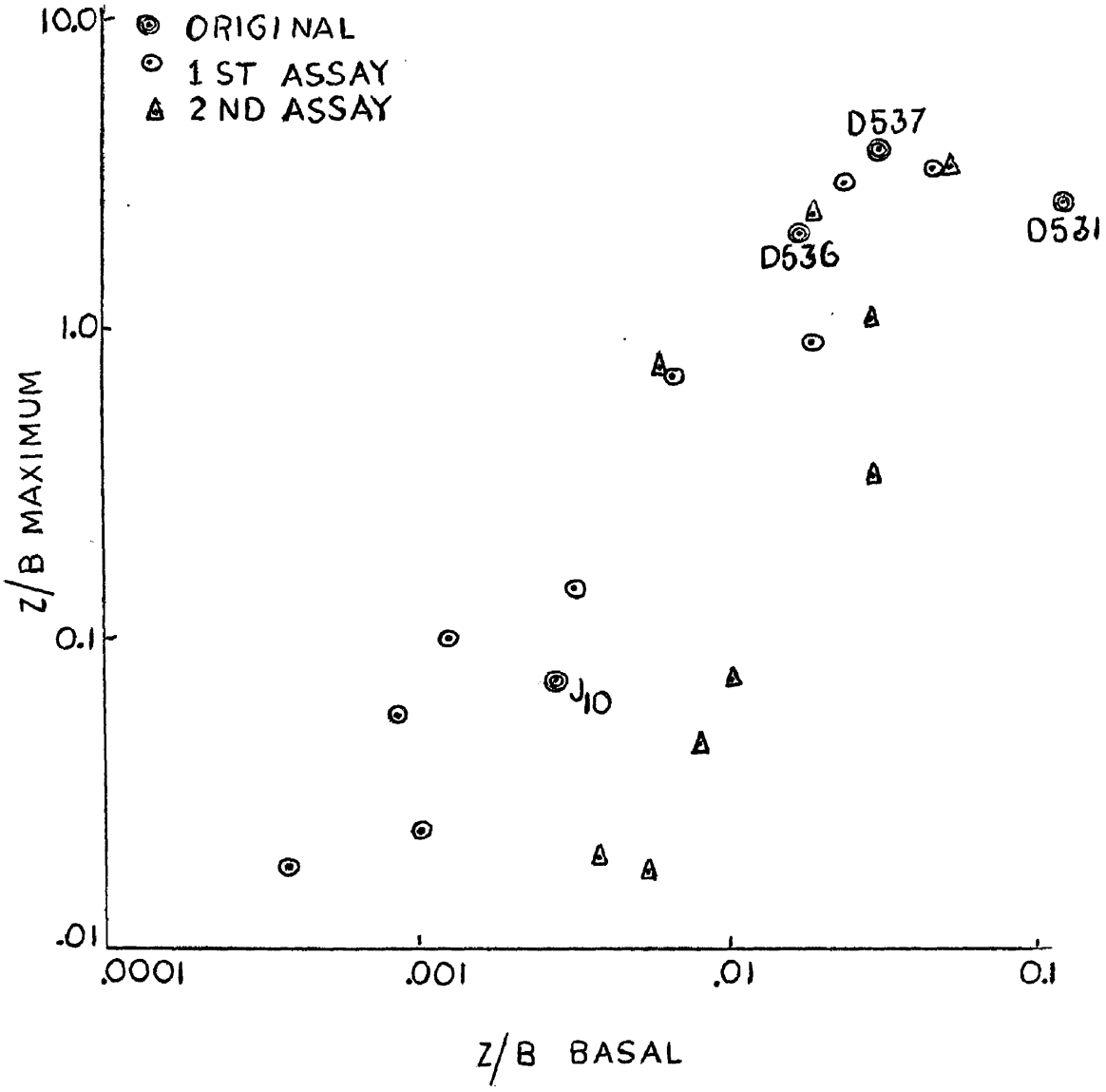


Figure 4. Correlation plot of (Z/B)maximum vs. (Z/B)basal values for assay data of clones of J_{10}

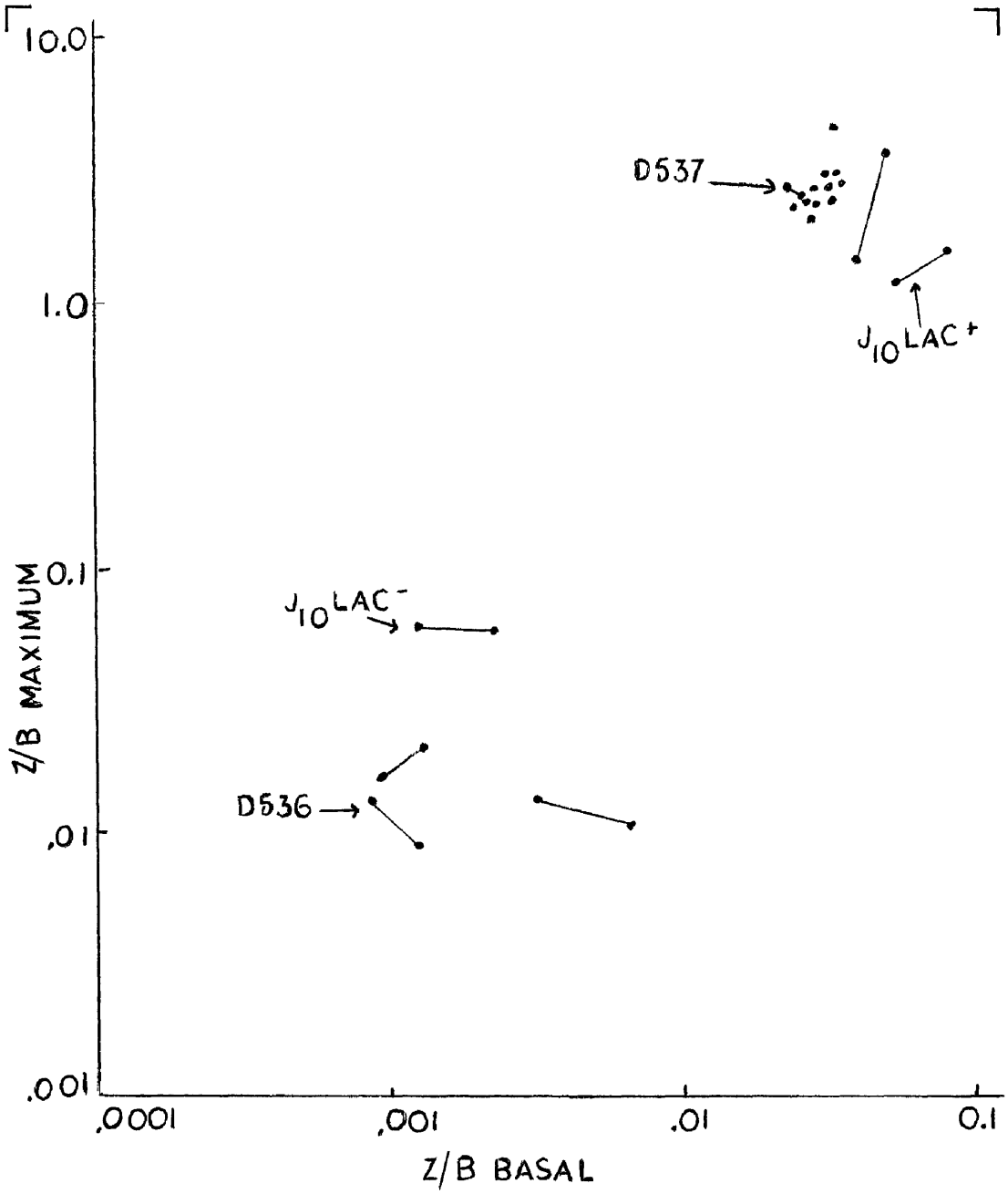


Figure 5. Correlation plot of (Z/B)maximum vs. (Z/B)basal values for duplicate assay sets of nine clones of J_{10}

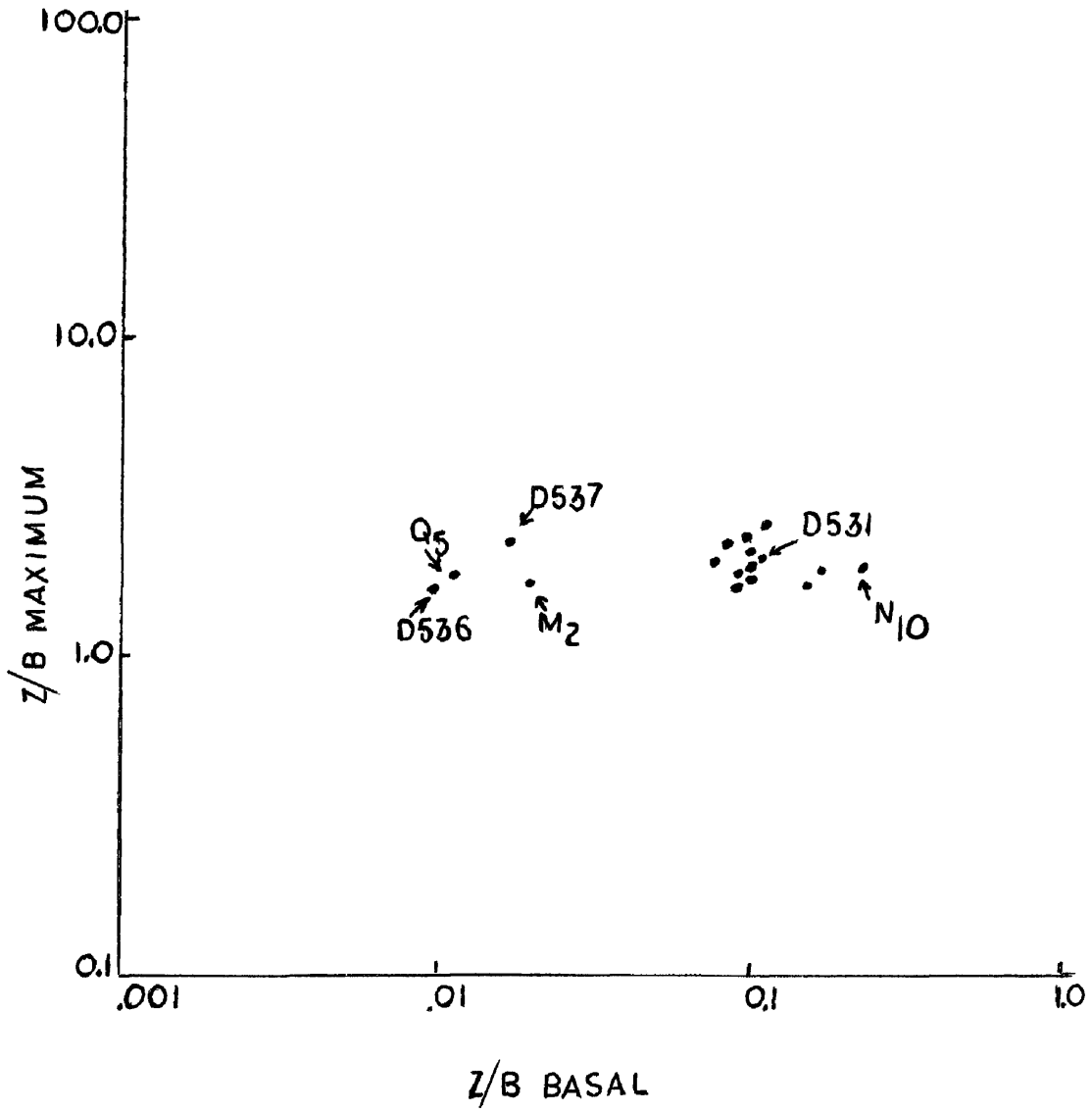


Figure 6. Correlation plot of (Z/B)maximum vs. (Z/B)basal values for reassay of the significant values of the second double episome mating

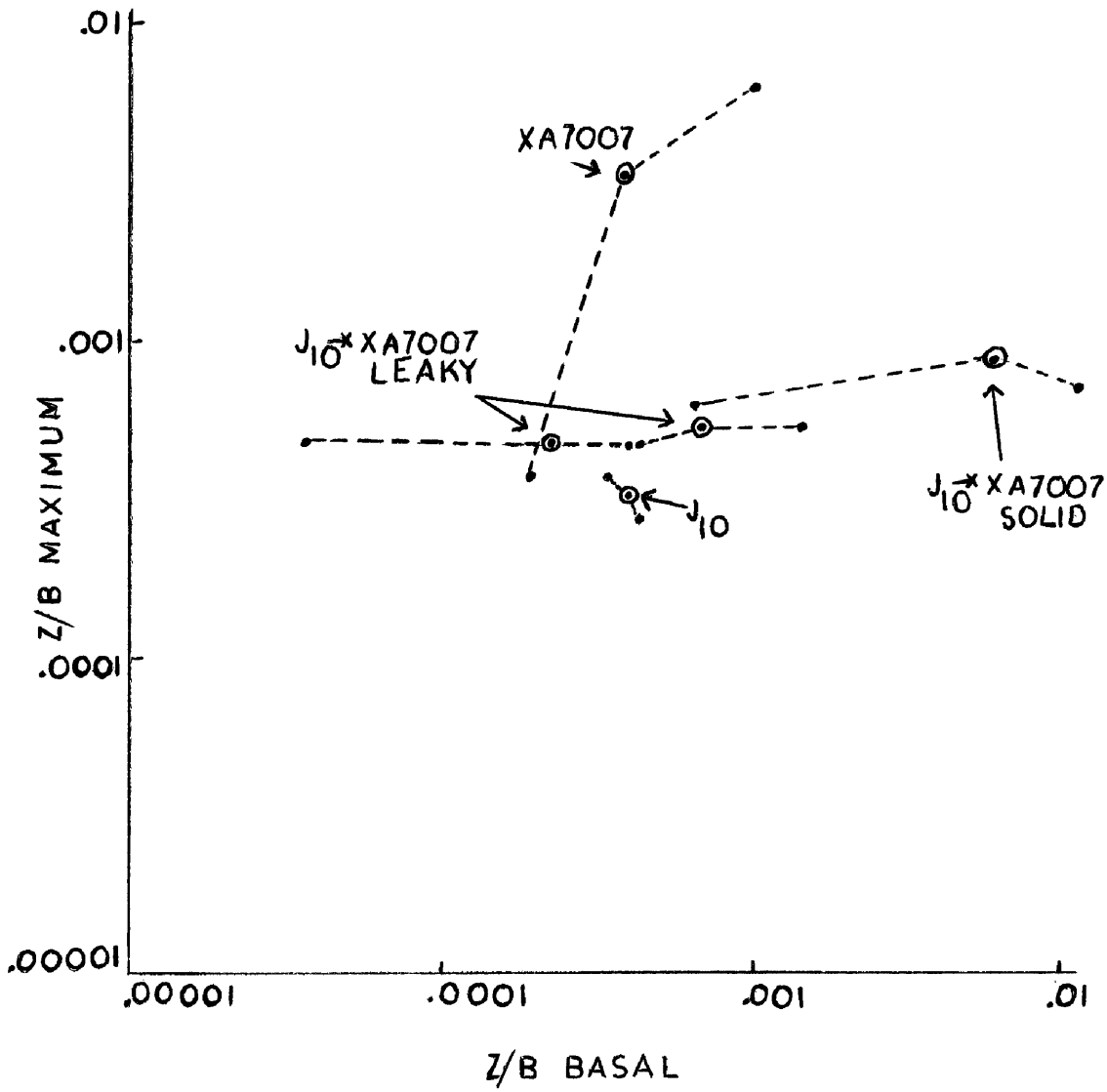


Figure 7. Correlation plot of (Z/B)maximum vs. (Z/B)basal values for the duplicate assay of results of the J_{10} X XA7007 Mating

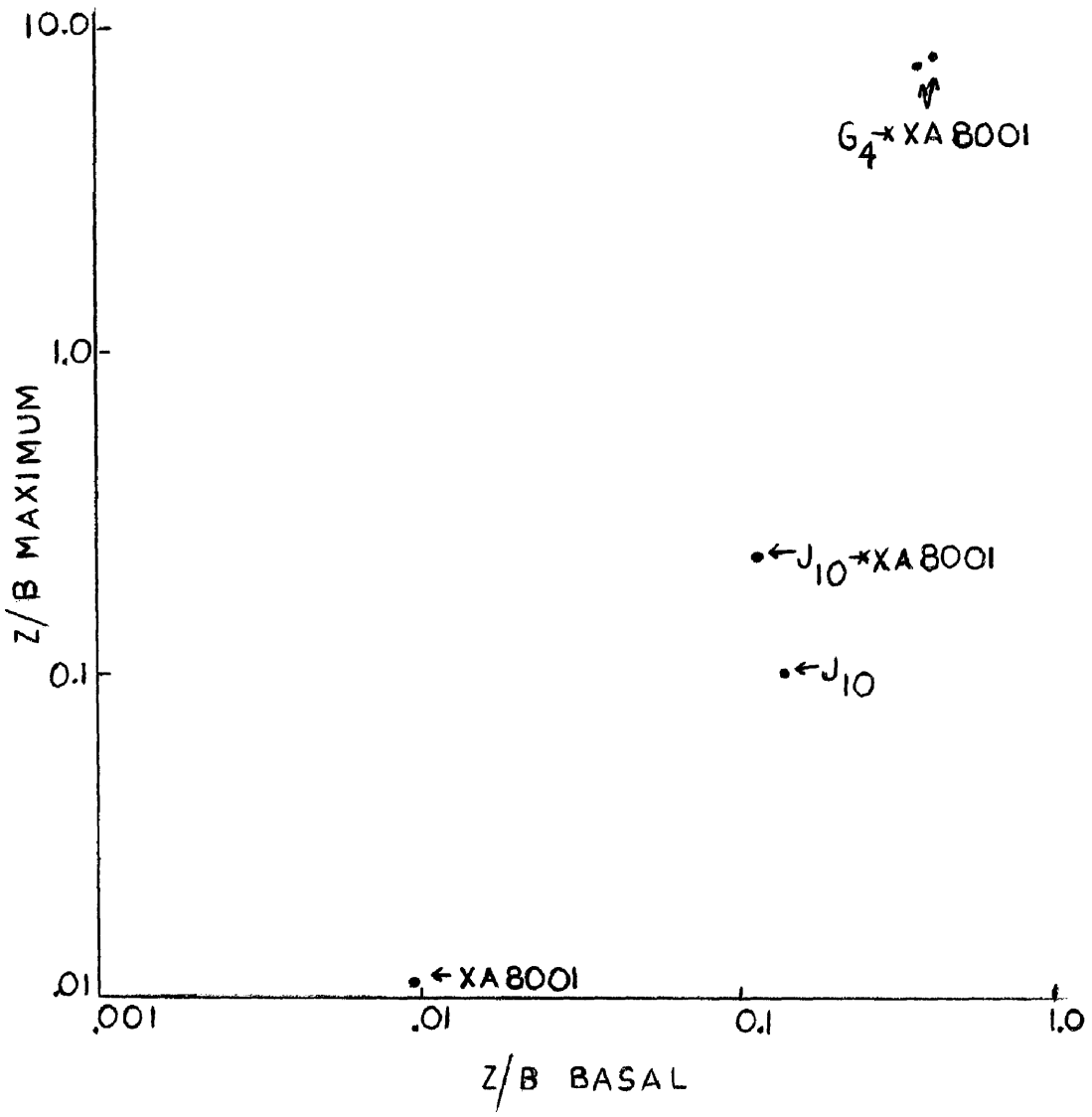


Figure 8. Correlation plot of the average (Z/B)maximum vs. (Z/B)basal values for the duplicate assay data of J_{10} and G_4*XA8001 matings

DISCUSSION

The data in this report is only taken from the matings that have given probable double O^C's. Data from preliminary testing for candidates, numerous test matings and unproductive data from previous double episome matings and assays are not included for reasons of space and sanity. Assay data, as well as correlation plots are shown for the experiments. For simplicity, the crosses were assigned a letter and any single clones from the crosses are subscripted numerically. (Table 4)

(a) Formation of Double O^C Mutants

Two probable double O^C mutants were obtained from matings of IIa + Vb $\rightarrow\!\!\!\!\times$ P9I and Va + Vb $\rightarrow\!\!\!\!\times$ P9I. These are G_4 and J_{10} respectively. Experiments were directed to verify their characteristics by repeating the crosses to obtain more double O^C's and also by resolving G_4 and J_{10} to attempt to separate two O^C's if they were present.

In the initial construction mating, the percentage of recombination, or the $\frac{Lac^+Pro^+}{Pro^+}$ ratio, obtained from growth on minimal lactose and succinate plates is 50% for the J mating group. This value for the G group is only .02% indicating very little recombination has taken place. Both G_4 and J_{10} exhibit slightly leaky Lac^- characteristics on TTC-lactose plates. This may be due to the impurity of the two cultures.

G_4 and J_{10} exhibited significantly different P values from most other clones in their mating groups. (Table 4) The remainder of the group members have P values that cluster around the two parent O^c values. A statistical histogram of the P values for all clones assayed shows two main peaks at the parental values, with few others differing. In addition to J_{10} and G_4 , the clones J_7 , A_8 , E_8 and B_4 also reveal non-parental P values. These four may also have been double O^c's with less promoter effects than G_4 and J_{10} , however, no further study was done on these.

The correlation plot (Figure 3) indicates two clusters around the parent values, with D536 and D537 very close. G_4 and J_{10} show P values of .035 and .22, while the parental value for D536 and for D537 is .008 and D531 is .05. In analysis, it is noted that the product of the parental P values are proportional to the measured J_{10} and G_4 P values. The IIa and Va subclasses are located in the same module, while Vb is in the other. Therefore, these doubles produced from O^c mutations in two different modules exhibit a multiplicative level of constitutivity. It had been expected that the effect of the mutations in the two separate modules should be additive.

Two additional assays of nine clones of J_{10} , to confirm the first double O^c values, resulted in some of the clones with close parental values and the remainder grouped near the original J_{10} value. (Figure 4) Inaccuracies in the assay methods contributed to the scattering. In an effort to minimize these effects, duplicate and triplicate assays were performed on additional clones of J_{10} . The results of the duplicate assay, with clones grown in 0.5% glycerol,

± IPTG, are shown in Figure 5. This indicates the clustering of six clones around the parent D537. The D536 is repressed, and has two of the test clones within its range.

Concurrently, attempts were made to construct additional double O^c's by double episome matings of the subclasses IIa, Va and Vb. In the first of these new mating experiments, the percentage of recombinations ranged from 0.5% to 7.5% for the two matings IIa + Va $\times$ P9I and IIa + Vb $\times$ P9I. The correlation plot (Figure 6) for the (Z/B) values indicates no probable double O^c's. The tested clones cluster around the two parent groupings, with the exception of N_{10}. This exhibits a higher basal rate than either of the parents, but is not affected by a promoter change. Additional mating experiments also did not yield probable double O^c's.

In an attempt to relieve any promoter effects, G_4 and J_{10} were mated with XA7007, which carries a suppressor mutation. Only four clones were obtained, all from the J_{10} mating, after both three and ten hours mating time. Recombinants should have been Lac+, however, one solid Lac$^-$ and three leaky Lac$^-$ were obtained. These would have been XA7007 or some combination thereof, since they were strep resistant. The assay of these three clones, plus the parents, (Figure 7) indicate relative closeness of all clones, eventhough there is a wide separation of duplicate assay values for each one. There is a slight decrease in the promoter effect of the supposed recombinants in comparison with the J_{10} parent assayed at the same time.

(b) Resolving of the Double O^c Mutants

Matings of G_4 and J_{10} with D509 did not produce sufficient recombinants for accurate assay results. In the mating with XA8001, two

clones grew from the G_4 mating and one from the J_{10} mating. These few were assayed (Figure 8) but produced no conclusive evidence for obtaining the separate parents, since there were not enough clones available.

CONCLUSION

The nature of the cooptivity between the two modules of the Lac operator can be measured by comparing the constitutivity of a known double O^c to that of the original two single mutations. By means of varying combinations of mutants from the same modules, as well as in each of the two modules, we had hoped to compare the amount of cooptivity in the probable double mutants. These doubles, G_4 and J_{10}, with the IIa and Va mutation sites in one module and the Vb in the other have both indicated a multiplicative level of constitutivity as compared with their single mutant parents. Since these base substitutions occur in separate modules, it was theorized that they should have an additive effect.

Data from this experiment does show that it is possible to derive probable double O^c mutants by a double episome mating of single mutant O^c's. Therefore, it should be quite possible to generate double O^c's from additional class combinations to yield further comparable information on the nature of the base substitution effects and the repressor-operator interaction with these present.

REFERENCES

Beckwith, J. (1967) Regulation of the Lac Operon. Science 156: 597.

Gilbert, W. and Muller-Hill, B. (1966) Isolation of the Lac Repressor. Proc. of Nat. Acad. Sci. 56:1891.

Gros, F. (1965) The Cell Machinery. In Molecular Biophysics, Ed. B. Pullman and M. Weissbluth. Academic Press, New York. p. 1-80.

Horiuchi, T., Tomizawa, J. and Novick, A. (1962) Isolation and Properties of Bacteria Capable of High Rates of b-Galactosidase Synthesis. Biochem. Biophys. Acta. 55:152.

Jacob, F. and Monod, J. (1961) Genetic Regulatory Mechanisms in the Synthesis of Proteins. J. Mol. Biol. 3:318-356.

Reznikoff, W. (1972) The Operon Revisited. Ann. Rev. of Gen. 6:133.

Sadler, J. R. and Smith, T. F. (1971) Mapping of the Lactose Operator. J. Mol. Biol. 62:139-169.

Smith, T. F. and Sadler, J. R. (1971) The Nature of Lactose Operator Constitutive Mutations. J. Mol. Biol. 59:273-305.

Taylor, A. L. and Trotter, C. D. (1967) Revised Linkage Map of Escherichia coli. Bact. Rev. 31:332-353.