THE INHERITANCE OF STREPTOMYCIN RESISTANCE AND DEPENDENCE IN CROSSES OF ESCHERICHIA COLI

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ESCHERICHIA COLI mutates by a single step to a high level of streptomycin resistance, and in strain B/r these mutations occur spontaneously with an average frequency of about 1 per 5×10^9 cell divisions (NEWCOMBE and HAWIRKO 1949; SCOTT 1949). Using ultraviolet or gamma irradiation similar changes can be produced in much larger numbers (NEWCOMBE 1949).

Two main categories of mutant have been described: streptomycin resistant (*sr*) and streptomycin dependent (*sd*), the former having the ability to grow either in the presence or in the absence of the drug, the latter growing only in its presence. Within the *sd* category is a range of forms differing with respect to the drug concentration required. The two extremes of this range are represented by mutants requiring as little as one or two units per ml on the one hand and as much as 500 units per ml on the other.

The *sd* mutants are capable of further mutational changes resulting in the restoration of drug independence. Such two-step mutants may be streptomycin sensitive (*sd/ss*), streptomycin resistant (*sd/sr*), or intermediate between the two (NEWCOMBE 1949, NEWCOMBE and NYHOLM 1950).

The bacterial crosses described here were made to determine whether or not there is an allelic series of genes corresponding to the various streptomycin mutants. In addition to this, information concerning the linkage relationships of streptomycin resistance and certain genetic marker characters has been sought.

MATERIALS

Four bacterial lines derived from *E. coli* strain K12 were very kindly supplied by DR. JOSHUA LEDERBERG. These had the following mutant characters:

line Y-40,	M^-, B^-, V_1^r;
line Y-53,	B_1^-, T^-, L^-, Lac^-;
line 58–161,	M^-, B^-;
line W-677,	B_1^-, T^-, L^-, Lac^-, Mal^-, Gal^-, Ara^-, Xyl^-, Mtl^-, V_1^r.

(M^-, B^-, T^-, L^-, and B_1^- indicate a requirement for methionine, biotin, threonine, leucine, and thiamin respectively; Lac^-, Mal^-, Gal^-, Ara^-, Xyl^-, and Mtl^- indicate an inability to ferment lactose, maltose, D-galactose, L-arabinose, D-xylose, and D-mannitol respectively; and V_1^r indicates resistance to phages T1 and T5. The wild type characters are designated + where the mutants are −, and s where the mutant is r. Of the above character differences all except *Mtl* were used in the present studies).

Crosses have been made with these streptomycin sensitive (*ss*) lines, and

with the streptomycin mutants (*sr*, *sd*, *sd/ss*, and *sd/sr*) derived from them in this laboratory.

METHODS

Sr and *sd* mutants were obtained from ultraviolet (2537 Å) irradiated bacterial suspensions using the following procedure: Samples from the suspension were poured with 2 ml of melted soft agar (0.7 percent agar in Difco nutrient broth) over the surface of Difco Nutrient Agar plates, and when solid a second 2 ml layer of agar was added to prevent the developing microcolonies from being disturbed by subsequent treatment. After a preliminary incubation of 3 to 4 hours at 37°C, streptomycin was applied in the form of a third agar layer containing 10,000 units of the drug. The plates were chilled overnight to permit diffusion and were then incubated until colonies appeared.

Sd/ss and *sd/sr* two-step mutants were obtained simply by spreading irradiated suspensions of the *sd* form on the surface of nutrient agar in the absence of streptomycin, and incubating at 37° until colonies developed. Cultures from these colonies were categorized by streaking in duplicate on nutrient agar in the absence and in the presence of streptomycin (1000 units per ml). When growth took place only in the absence of the drug the culture was termed *sd/ss*; when it occurred on both plates the culture was termed *sd/sr*. This classification is arbitrary since the mutants exhibit a more or less continuous range with regard to degree of resistance. The particular *sd/ss* and *sd/sr* forms chosen for crossing were from among the most sensitive and the most resistant.

Crosses between parent strains, requiring respectively biotin and methionine, and threonine, leucine and thiamin, were made by spreading washed suspensions (containing 10^8 to 10^9 cells of each of the two types) on a growth factor deficient medium. This consisted of mineral salts, glucose, and asparagine (the "minimal agar" of TATUM and LEDERBERG 1947), and except where otherwise stated was supplemented with thiamin. On such a medium the parent forms are restricted in their growth while certain sexually derived recombinants multiply to produce colonies. When the five factors are absent, all recombinants that form colonies are growth factor independent. In this respect they resemble the original strain from which the parent forms were derived and are therefore termed "prototrophs" (LEDERBERG and TATUM 1946). When thiamin is added the number of colonies is increased and, since many of them require thiamin and are therefore not true prototrophs, they are known by the more general name of "growth factor recombinants."

Colonies appeared after two to three days incubation, and were categorized for streptomycin response and lactose fermentation (a useful marker character) by growing in a liquid minimal medium plus thiamin, diluting (1/100), and streaking on Levine's eosin methylene blue-lactose agar (known as EMB-lactose). In the latter medium sugar fermentation is indicated by a deep blue coloration of the streak. Duplicate plates were used, one of which had been cross-streaked previously with streptomycin (one loopful of a

solution containing 10,000 units per ml) as described by DEMEREC and FANO (1945) for bacteriophage. In certain experiments additional tests were made in a similar manner for fermentation of other sugars (substituting these for the lactose in the EMB medium) and for phage T1 resistance.

When strains from which *sd* segregants might arise are to be crossed, streptomycin must be present so that these can be recovered. Provided that both parents are capable of growth in the presence of the drug, this can be incorporated in the medium in a concentration of 1000 units per ml. However, where one of the parents is sensitive, crossing must be allowed to take place in the absence of streptomycin (16 to 18 hours at 37°C is sufficient), the drug then being applied as a spray (in these experiments approximately 0.2 to 0.3 ml of a solution containing 100,000 units per ml was used for each plate). In order that no *ss* or *sd* segregants should be missed, all of the crosses involving an *sd* parent (or a parent derived by further mutation from the *sd* form) were made both with and without streptomycin.

A peculiarity in the streptomycin response of the *sd* mutants should be noted. When these were cross-streaked with streptomycin on nutrient agar, growth was, as would be expected, normal in the presence of the drug and thin or invisible where the drug was absent. However, in similar tests on minimal agar supplemented with the five factors, growth was not stimulated by streptomycin and was equally thin in its presence and in its absence. Thus the *sd* parent forms seemed to have requirements in addition to the stated ones.

The *sd* growth factor recombinants derived by crossing such parents did not exhibit this fastidiousness but showed a normal response to streptomycin on both the synthetic and the complete medium. This difference between *sd* parent and *sd* growth factor recombinants is assumed to be due to the segregation of a modifier gene (or genes).

TABLE 1

Segregation of streptomycin resistance, lactose fermentation, and phage T1 resistance, when streptomycin resistance is introduced in alternative parents.

	RECOMBINATION CLASSES			CROSS A (Y-40×Y-53/*sr*)		CROSS B (Y-40/*sr*×Y-53)	
	Str.	*Lac*$_1$	T1	COLONIES	%	COLONIES	%
1	r	+	r	86	34	7	3
2	r	+	s	11	4	1	0.4
3	r	−	r	84	33	8	3
4	r	−	s	51	20	3	1
5	s	+	r	11	4	73	30
6	s	+	s	0	0	4	2
7	s	−	r	8	3	88	36
8	s	−	s	5	2	59	24
Total colonies tested				256	—	244	—
% *sr*				—	91	—	8

sr CROSSES

The segregation of streptomycin resistance, lactose fermentation, and phage resistance is shown in table 1. Two crosses were made (using the lines Y-40 and Y-53) in which streptomycin resistance was introduced in alternative parents. Where a mutant character is studied in this manner the term "reversed crosses" will be used.

It will be noted that somewhat more than 90 percent of the descendent growth factor recombinants ($M^+ B^+ T^+ L^+$) resemble the Y-53 ($M^+ B^+ T^- L^-$ parent with regard to streptomycin response, a fact which would suggest linkage between *sr* and the *M B* loci. However, since the $M^- B^-$ descendents from the crosses cannot be examined there is no certainty of such linkage, and the situation is best described by saying that the *sr* locus from the $B^- M^-$ parent tends not to recombine with $B^+ M^+$. This statement leaves open the possibility of loss at some stage between the first steps of zygote formation and the development of the haploid descendents. That there is loss, and that it occurs early in this process is indicated by the behaviour of diploid heterozygotes from ($B^- M^-$) $ss \times (B^+ M^+)$ *sr* crosses. A number of these have been examined by DOUDOROFF and LEDERBERG (personal communications, and LEDERBERG 1950) and in all cases they have given rise only to *sr* forms when permitted to segregate on a complete medium. This could be due to the gene for streptomycin resistance being present in the diploids either in the hemizygous or the homozygous state. Both of these states have in fact been demonstrated for other loci, certain lactose negative diploids being homozygous Lac^- and certain maltose negative diploids being hemizygous Mal^- (LEDERBERG, personal communication).

Table 2 shows no indication of linkage between streptomycin response and either lactose fermentation or phage T1 resistance. Similarly the streptomycin and thiamin loci appear to segregate independently (data not included in the table).

Linkage has been observed between *sr* and the sugar fermentations other

TABLE 2

Correlation between the inheritance of streptomycin resistance and ability to ferment galactose, arabinose, xylose, and maltose, in the cross: 58-161/sr×W-677.

SUGAR FERMENTATION	NUMBERS OF COLONIES OBSERVED		PERCENT		PROPORTION OF FERMENTERS IN *sr*/PROPORTION IN *ss* COLONIES
	ss	*sr*	*ss*	*sr*	
Gal^+	49	18	3	23	7
Ara^+	118	14	8	18	2
Xyl^+	25	14	2	18	11
Mal^+	22	19	1	25	18
Total colonies tested	1530	77			

Note: Lac^+ segregants constitute 34 percent of the *ss* colonies and 35 percent of the *sr* colonies. Lactose fermentation thus appears to be inherited independently of streptomycin resistance.

than lactose (i.e. galactose, arabinose, xylose, and maltose) using lines W-677 and 58-161 (see table 2). It is not however, such as to enable the loci for these characters to be placed in a linear order (data to be published separately). Similar observations concerning the anomalous inheritance of these particular sugar fermentations have already been made by LEDERBERG and by CAVALLI (personal communications) and the possible significance of this extraordinary phenomenon will not be considered here. It is of interest, however, that this anomalous inheritance is shared by the gene for streptomycin resistance.

To determine whether more than one locus may mutate to produce streptomycin resistance, *sr*×*sr* crosses have been carried out using nine *sr* mutants of independent origin. A total of between 700 and 800 prototroph colonies from these were examined for streptomycin response. Of the crosses none gave rise to *ss* (or *sd*) prototrophs, from which it may be assumed that most of the mutations to the *sr* form, and possibly all of them, take place at the same locus.

sd CROSSES

Reversed crosses of *ss*×*sd* have been made using lines Y-40 and Y-53 (see table 3). The bulk of the recombinants from these, as in the *ss*×*sr* crosses, resembled the B^+ M^+ (Y-53) parent with respect to streptomycin response. However, no *sd* segregants were recovered when the *sd* parent was derived from the B^- M^- line. This dissimilarity in the crossing behavior of *sd* and *sr* forms could be due to differences either in the segregation of *sd* and *sr*, or in the recovery of the products of segregation. Since the parental *sd* forms have growth factor requirements in addition to stated ones, the second of these interpretations is quite possible.

It should be noted also that *sd*×*sd* crosses have been made, using four independently obtained mutant lines. These crosses have yielded recombinant colonies only when streptomycin was present, and all the colonies tested were *sd*.

sr × *sd* CROSSES

If mutations to *sr* and to *sd* involve different gene loci, then *sr*×*sd* should give rise to a proportion of *ss* colonies. Tests of this nature have already been used to distinguish between allelic and non-allelic changes affecting phage resistance, lactose fermentation, and maltose fermentation (E. LEDERBERG

TABLE 3

Segregation of streptomycin dependence.

CROSS	PARENT STRAINS		STREPTOMYCIN ABSENT				STREPTOMYCIN PRESENT			
	Y-40	Y-53	COLONIES TESTED	PERCENT			COLONIES TESTED	PERCENT		
				ss	*sr*	*sd*		*ss*	*sr*	*sd*
A	*ss*	× *sd*	22	100	0	0	565	0	0	100
B	*sd*	× *ss*	505	100	0	0	0	0	0	0

1948, and J. LEDERBERG 1947, 1948, and 1949) and have yielded what appear to be valid conclusions.

For the present test, reversed *sr* × *sd* crosses were made using mutant forms derived from lines Y-40 and Y-53. To eliminate any *ss* cells which might arise by further mutation the parents were grown in broth containing 1000 units of streptomycin per ml, and were then crossed by plating in the usual manner. Table 4 shows the types of descendent colony obtained.

Where the crosses were made in the presence of streptomycin, and growth factor recombinants of either parental type with respect to streptomycin

TABLE 4

Test of allelism of the genes for streptomycin resistance and streptomycin dependence.

CROSS	PARENT STRAINS		STREPTOMYCIN ABSENT				STREPTOMYCIN PRESENT			
	Y-40	Y-53	COLONIES TESTED	PERCENT			COLONIES TESTED	PERCENT		
				ss	*sr*	*sd*		*ss*	*sr*	*sd*
A	*sr*	*sd*	417	1.2	98.8	0	300	0	6.3	93.7
B	*sd*	*sr*	160	0	100	0	158	0	100	0

response were thus enabled to grow, segregation of the *sr* and *sd* mutant characters was similar to that observed in the *sr*×*ss* and *sd*×*ss* crosses shown in tables 1 and 3, thus: (1) with Y-40 as the *sr* parent approximately 6 percent of the colonies were *sr*, and (2) with Y-40 as the *sd* parent *sd* colonies were absent.

Where crosses were made in the absence of the drug the bulk of the growth factor recombinants were *sr*, although a few *ss* colonies (5 out of a total of 417 in cross A of table 4) were found. In a subsequent set of experiments involving similar numbers of tests no *ss* colonies were obtained. The *ss* colonies, however, represent a valid observation despite this irregularity in their appearance and it is with their interpretation that we are primarily concerned. Had the proportion been larger it would have indicated that *sr* and *sd* were mutants of separate gene loci. But in view of the small number of *ss* colonies they could have been the result either of rare recombinations between the *sr* locus and a closely linked *sd* locus, or of mutation from *sd* to *sd/ss*.

Mutations of this kind are known to occur during growth and the precaution of growing the parent lines in high concentrations of streptomycin prior to plating would not prevent them from taking place on the plates. In all crosses the surface of the agar was covered with a confluent bacterial growth which could be seen under a high power dry objective, and in some experiments was visible to the naked eye. The extent of this growth was such that appreciable mutation from *sd* to *sd/ss* would be expected, and the products of these mutations would be at a selective advantage when streptomycin was absent. Thus, although the possibility of rare recombination between *sr* and *sd* cannot be entirely eliminated, it is more probable that *sr* and *sd* are alleles of a single locus and that the *ss* colonies arose by mutation.

sd/ss AND *sd/sr* CROSSES

Mutation from dependence to sensitivity and to resistance (*sd* to *sd/ss* and to *sd/sr*) might occur either by further change of the *sd* gene, or by mutation at some other locus. If the latter is the case, a proportion of *sd* segregants would be expected on crossing the double mutant with a sensitive or a resistant line. This test has been applied to four such forms (Y-40/*sd/ss*, Y-40/*sd/sr*, Y-53/*sd/ss*, and Y-53/*sd/sr*), each of them being crossed with *ss* and with *sr* lines of the appropriate kinds, both in the absence and in the presence of streptomycin.

Of the four double mutants tested, three gave no *sd* segregants (data not shown) indicating that the second mutation took place in the same or in a closely linked locus. From the remaining one, a Y-53/*sd/sr* mutant, an appreciable proportion of *sd* (or to be more precise, potentially *sd*—see below) growth factor recombinants was recovered (see table 5). Segregation in the latter case was exceptional, however, and will be described in detail.

TABLE 5

Crosses with an sd/sr mutant from which potentially sd segregants have been obtained

CROSS	PARENT STRAINS		STREPTOMYCIN ABSENT					STREPTOMYCIN PRESENT				
	Y-40	Y-53	COLONIES TESTED	PERCENT				COLONIES TESTED	PERCENT			
				ss	*sr*	*sd*	POTENTIAL *sd*		*ss*	*sr*	*sd*	POTENTIAL *sd*
A	*ss* ×	*sd/sr*	214	15	0	0	85	86	0	0	0	100
B	*sr* ×	*sd/sr*	218	0	13	0	87	152	0	80	0	20

With all previous crosses the streptomycin response of the majority of the descendents has been similar to that of the B^+ M^+ parent, but with the crosses shown in table 5 the response of the Y-53 parent (i.e. resistance) was not transmitted as such. Instead there appeared among the recombinant colonies a class which was sensitive to high concentrations of streptomycin, but which gave rise to normal *sd* cultures if incubated for two or three days in a synthetic medium containing streptomycin. Growth of this potentially dependent form was inhibited on complete media such as E.M.B. both in the absence and in the presence of streptomycin, although the *sd*'s derived from it behaved normally.

Where crosses were made in the absence of streptomycin the majority of the prototrophs were of the potentially dependent type, the remainder resembling the Y-40 parent. These latter included an abnormally high proportion of lactose positive colonies (63 percent as against the usual 36), whereas the potential *sd*'s were normal in this respect. The difference is statistically significant ($\chi^2=35.1$ for a 2×2 comparison) and represents the only instance of an apparent linkage between streptomycin response and lactose fermentation in any of our crosses.

The anomalies associated with this cross (the development of potential *sd*'s, the non-recovery of Y-53 resembling forms, and the altered Lac^+:Lac^- ratio in the Y-40 resembling descendents) suggest that the *sd/sr* parent con-

tained a number of modifier mutations which adversely affected the survival of some of the recombination types. As the original *sd/sr* mutant strain grew poorly on nutrient agar slants, and gave rise to numerous large colony variants, the accumulation of a number of modifier mutations would not be surprising.

However, for present purposes it is more important that *sd* forms were obtained, even if indirectly, from crosses with an *sd/sr* parent. The fact that recombinants from this cross could revert to *sd*, although the parent *sd/sr* could not, indicates that mutation from *sd* to *sd/sr* must have occurred at a modifier gene locus. (A similar case of modifier mutation has been noted by HOULAHAN and MITCHELL 1947, in Neurospora.)

DISCUSSION

Of the nine *sr* and the four *sd* lines studied, all appear to have arisen by change at a single gene locus. These numbers by no means exclude the possibility of mutations at other loci giving rise to a proportion of the resistant or dependent forms. Nor is it entirely certain that *sr* and *sd* are not "pseudoalleles," and that crossing over between them is too rare to be detected with certainty by the present methods. (For discussions of pseudoalleles see MCCLINTOCK 1944, STEVENS 1948, and GREEN and GREEN 1949.) However, it seems most likely that *sr* and *sd* are alleles of a single locus and, since a considerable range of *sd* forms has been observed, that there is a multiple allelic series at this locus the precise extent of which has not as yet been determined.

That this series is in all probability very extensive is suggested by a number of considerations. One is that the dependent forms differ among themselves, not only in the degree of their dependence, but also with respect to the compounds other than streptomycin which will supply their requirements (DEMEREC 1950). A second is that the double mutants derived from the dependent forms (*sd/ss* and *sd/sr*), an appreciable proportion of which appear to arise through a second mutation at the original locus, form a more or less continuous series with respect to degree of resistance. In addition, two broad categories of mutation (from sensitivity to the lower grades of resistance, and from dependence to the lower grades of dependence coupled with incomplete resistance) have not so far been studied in detail. However, preliminary work in this laboratory indicates that the partially resistant forms of *E. coli* strain *B/r* (those capable of growing in streptomycin concentrations of 16, 32, 64, and 128 units per ml) mutate to the fully resistant type at about 10 to 20 times the normal rate. This would suggest that the same locus is involved in both types of change.

It therefore appears that the streptomycin locus can mutate to a number of different categories of allele, and that each category may contain a range of mutant types. Thus the mutation pattern would seem to have what could be termed a "coarse" and a "fine" structure, and to be as complex as any yet studied.

SUMMARY

1. Streptomycin resistant (*sr*) and dependent (*sd*) mutants derived from the

sexually fertile *E. coli* strain K12 have been crossed with the sensitive (*ss*) form and with each other.

2. The streptomycin response of the bulk of the progeny resembled that of the biotin and methionine independent parent, resistance and dependence being inherited similarly in this respect. Streptomycin resistance is linked with the fermentations of maltose, xylose, D-galactose, and L-arabinose, but not in a manner implying linear arrangement of the genes concerned. No linkage was observed between streptomycin resistance and lactose fermentation, phage T1 resistance, or thiamin requirement.

3. Resistance and dependence are inherited as if controlled by allelic forms of the same gene locus.

4. Of four double mutants derived from the dependent form by further change to sensitivity (*sd/ss*) and to resistance (*sd/sr*), three (two sensitive and one resistant) behaved on crossing as if both mutations had occurred at the same locus. The remaining (resistant) double mutant behaved as if the second of the two changes had been the result of mutation at a modifier locus.

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