TRANSFORMATION OF STREPTOMYCIN MARKERS IN ROUGH STRAINS OF *RHIZOBIUM LUPINI*. II. THE RELATION BETWEEN THE DETERMINANT OF STREPTOMYCIN DEPENDENCE AND THOSE FOR STREPTOMYCIN RESISTANCE AND SENSITIVENESS

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RECENTLY, streptomycin dependence and resistance in bacteria have come to be viewed as accommodations requiring or permitting certain alterations by streptomycin of the expression of other genetic traits carried by the organism (e.g., GORINI 1964). For future evaluation of this viewpoint, it would be useful to have information about the relation of genetic factors governing dependence, resistance and sensitivity in a number of different species.

The present article describes some analyses of these factors in a rough mutant of the plant symbiont *Rhizobium lupini.* In these bacteria genetic transformation by DNA has been accomplished (BALASSA 1957), and streptomycin resistance and dependence (BALASSA and GÁBOR, 1961, 1965) were among the characters which have been transferred.

When streptomycin dependent (str-d) strains served as donor (DNA source) to homologous streptomycin sensitive (str-s) recipients, transformation occurred at very low frequencies. All transformants were dependent on streptomycin and most of them at levels similar to that of the donor. A heterologous str-d donor produced still fewer transformants, partly dependent and partly resistant (str-r), at a variety of different streptomycin levels (BALASSA and GÁBOR 1965). These findings suggest a linkage and a possible overlap between the determinants str^d and str^r.

It seemed desirable to investigate further the relation between these markers and that for sensitivity, particularly by studying the back-transformation of a str-d strain and its derivatives. One can ask, for example, whether streptomycin dependent strains carry genetic factors for resistance, and to what extent factors for dependence are or can be suppressed to give lowered resistance or sensitivity.

MATERIALS AND METHODS

Bacterial strains. The strains used were *R. lupini* H-13-3 and related mutants. The original strain H-13-3 will be referred to as wild type or simply as str-s because of its sensitivity to streptomycin. All streptomycin dependent and resistant strains were obtained by single-step selection of spontaneous mutants or of transformants from str-s, and are designated by the abbreviations str-d and str-r, with an associated isolation number indicating separate origins. Some

TABLE 1

Growth of different used strains on nutrient agar containing various concentrations of streptomycin

Strains	Concentrations of streptomycin (μg/ml)						
	0	10	30	50	100	300	1000
Recipients:							
str-d-15*	—	—	—	—	++	+++	++++
str-d-2*	—	—	—	—	++	+++	++++
Donors:							
3str-s (wild)	++++	—	—	—	—	—	—
str-tr-21†	++++	++++	++++	++++	++++	++++	++++
str-r-3b‡	++++	++++	++++	++++	++++	++++	++++
str-r-4b‡	++++	++++	++++	++++	++++	++++	++++
str-r-10§	++++	++++	+	—	—	—	—
str-s-81‖	++++	—	—	—	—	—	—
str-ts-112¶	++++	—	—	—	—	—	—

After 48–72 hours of incubation, growth was scored from — (none) to ++++ (very heavy).
* str-d substrains originating from parent strain H-13-3-str-d (1000).
† str-r strain obtained in transformation of streptomycin resistance.
‡ str-r revertants obtained from same str-d parent strain.
§ spontaneous, low level str-r mutant.
‖ str-s revertant from strain str-d-15.
¶ str-s strain obtained in transformation of streptomycin nondependence with DNA of the wild-type 3str-s added to recipient str-d-15.
The media used and preparation of DNA were as described in BALASSA and GABOR (1965).

streptomycin-sensitive strains were obtained by subsequent isolation of streptomycin nondependent mutants or transformants obtained from str-d by selection on medium lacking streptomycin. The designation of transformant strains includes the symbol t. Responses to streptomycin of the principally used strains are shown in Table 1.

Transformation technique. In general, a 24-hour growing culture of the recipient bacteria was used in a semisynthetic medium (BALASSA and GÁBOR 1965). This medium contained (per liter) K_2HPO_4, 3.6 g; KH_2PO_4, 0.4 g; $MgSO_4 \cdot 7\ H_2O$, 0.05 g; NaCl, 0.5 g; ferric ammonium citrate, 4.0 mg; agar, 20 g. The pH was adjusted to 7.0–7.2. To the synthetic medium were added: glucose to a final concentration of 5 g, casein hydrolysate 1 g, and yeast extract 1 g, per 1000 ml.

The inoculating culture, when it reached 1 to 5×10^7 bacterial/ml, was freshly diluted 10 or 100 times into 10 ml medium, containing 300 μg/ml streptomycin. The DNA was added in 0.1 ml of fresh medium, to give a final concentration of 9 μg/ml. In the controls, DNase was added at the same time as DNA. In most experiments, contact between donor DNA and recipient cells was not interrupted for 24 hours, during which the mixture was incubated at 30°C.

Method of selecting nondependent transformants. After contact with the transforming DNA, samples of the transformation mixture now containing about 10^7 bacteria/ml were plated on the surface of nutrient agar lacking streptomycin. Three 0.1 ml samples of treated culture were plated directly; the remainder was centrifuged, washed twice with saline, and the recovered cells were plated at both tenfold concentrations (three samples) and at original volume (four samples). The total bacterial population before and after washing with saline was determined on nutrient agar containing 1000 μg/ml streptomycin. The frequency of transformation is calculated in percent of the total number of cells and represents *all* streptomycin nondependent colonies which had appeared at 24 hours in relation to the total number of cells of the recipient population at 24 hours.

The streptomycin nondependent colonies which grew up from the streptomycin-lacking nutrient agar were suspended in 0.5 ml of saline, and loopfuls (10^{-3} ml) were streaked on the nutrient agar with different concentrations of streptomycin (0, 10, 30, 50, 100, 300 and 1000 μg/ml). Evaluation of the growth was made after incubating two or three days at 30°C.

RESULTS

Transformation of streptomycin-dependent recipient strains. When sensitive recipients were treated with DNA from a related str-d donor (BALASSA and GÁBOR 1965) the transforming frequency to dependence was from 4 to 24 per 10^6. The dependent strain is, on the contrary, transformed to independence at a much higher rate. When, for example, the substrain str-d-2 was treated with DNA from the resistant str-tr-21, the yield of str-r was 120 per 10^6. The substrain str-d-15 gave yields still 10 to 30 times higher than this (see Table 2), and it was used in most of the further experiments.

Since transformations with standard technique and standard DNA preparations give reasonably reproducible results, these relative yields would seem to reflect some difference in the processes involved.

When str-d-15 was treated with wild-type DNA, the number of transformants no longer dependent on streptomycin was 475 per 10^6 (Table 3). What is noteworthy is that among a large population none of these was resistant, nor were any dependent at lower concentrations. This, together with the earlier observation that only dependents were recovered from the reciprocal transformation, seems clear evidence that *str*^{d} does not consist of a resistance determinant supplemented by a (separable) modifier or suppressor leading to dependence. The data consistently indicate that, within the limits of the resolution available, this homologous pair of sensitive and dependent strains do not, taken together, contain the information necessary to produce streptomycin resistance.

On the other hand, a possible explanation for the low frequency of *str*^{d} incorporation would be that this determinant results from mutations at several sites within the *str*^{s} determinant; if so, some part of this multisite mutation could be also present in str-r mutants. If this were true, some str-r donors could be expected to give virtually as high rates of transformation to independence as does the str-s, but to produce mainly str-r and few if any str-s transformants.

In the numerous experiments summarized in Table 2, it is found that the average str-r donor transformed the str-d strain to independence as often as the wild-type donor and more often than did certain str-s donors. The smaller series

TABLE 2

The transformation frequency of str-d-15 to str-nondependence with different DNA donors

Donors	Average of transformation frequency in percent	Number of transformations from which the average was calculated
3str-s (wild)	0.13	5
str-tr-21	0.14	10
str-r-4b	0.31	5
str-r-10	0.33	6
str-s-81	0.04	6
str-ts-112	0.05	6
str-d-15 (spontaneous mutants)	0.00052	5

The cells were exposed to DNA during 24 hours growth in streptomycin. Average rates of transformation are given for several experiments which in themselves included 4–12 platings, with estimated reliability of ± 20% for the means.

TABLE 3

Recovery of different types of transformants, using str-d-15 as recipient

Donors	Number of experiments	Number of experiments analyzed	Trans-formed colonies in 0.1 ml	Number of colonies tested	Number of colonies of indicated type*				
					Sensitive	Resistant high degree	Resistant low degree	Dependent high degree	Dependent low degree
3str-s (wild)	4	2	475	2900	2900	0	0	0	0
str-tr-21	4	2	437	218	0	213	0	1	2
str-r-4b	3	2	260	105	0	91	0	13	0
str-r-10	3	1	392	41	0	41	0	0	0
str-s-81	5	3	245	276	204	0	63	0	1
str-ts-112	3	2	212	144	71	0	61	4	4
Controls: (spontaneous mutants)†									
str-d-15	4	2	8	52	1	3	39	2	7
without DNA	11	5	6	122	1	9	63	8	41

Number of plates used for calculation of the average: 4–10. Number of total bacteria in 0.1 ml plated: 10^6. For description of strains see Table 1.
Levels of streptomycin used: 0, 10, 30, 50, 100, 300, 1000 μg/ml.
Resistant classes do not include dependent ones. Transformants to "dependent, high degree" first grew in total absence of streptomycin, then later showed dependence which although high was demonstrable over a wider or narrower range of streptomycin concentrations than that of the recipient strain.
* Number of colonies reported in the various classes from transformations are corrected by deducting the average number of spontaneous mutants expected from the samples tested.
† Note that the number of colonies tested represents 12-20 times larger volume of control than transformed culture.

of experiments in Table 3 do not show significant differences but present the overall analysis of the transformants produced. The three resistant donors gave mainly resistant transformants, and in no case were sensitives produced. It thus appears that the str^d and str^r determinants together do not contain all the information necessary for sensitivity. The wild-type str^s, the str^d and the str^r determinants mutually displace each other as if they were overlapping alleles, at least within the revolving power of the units introduced by transformation.

The str^d and str^r regions, if overlapping, need not necessarily share identical mutations (e.g., base changes) in the common region. Some suggestion that they may in part do so comes from the third resistance transformation (donor str-r-10) listed in Table 3. This donor, having low resistance only, donates a resistance factor to the high level dependent recipient and a highly resistant transformant is produced, in relatively high yield. This suggests that here only a dependence factor has been displaced (by a resistance determinant) while an additional factor governing the ability to withstand high streptomycin concentration has remained, or been reconstituted by the donor DNA. The separate or separable nature of determinants for resistance itself and others affecting concentration level seems to be indicated. This would presumably also apply to the two highly resistant donors (str-tr-21 and str-r-4b) which are resistant at the same high streptomycin concentrations at which the recipient is dependent.

Analysis of streptomycin-sensitive revertant and transformants. Evidence that the region governing str-d results from a multisite mutation of considerable extent is found in the fact that among several experiments with the object of obtaining spontaneous str-s revertants, only one revertant was found among

approximately 10^9 cells examined, indicating at the same time that a deletion is not involved. It would be reasonable then to expect sensitive revertants and transformants coming from the str-d strain to retain portions of the mutant region. One way to test for such mutant sites would be to examine these strains for their ability to transmit a str^d or str^r genotype.

Rows 5 and 6 of Table 2 present results with sensitive donors which were obtained by reversion and transformation respectively from str-d-15. Transformants are produced by these donors less than half as frequently as by wild-type str-s. This is consistent with the possibility that these donors differ from wild-type and may retain some mutant sites which reduce their overall probability of altering the str^d gene sufficiently to produce a str-s phenotype.

Examination of the phenotypes of the transformants coming from the sensitive revertant and the sensitive transformant reveals that half or more of them are sensitive, and the remainder are low resistants similar in type to, but much more numerous (per ml) than, spontaneous revertants coming from the controls (rows 7 and 8, Table 3). There is, then, some evidence that the phenotypically sensitive revertants still contain mutant sites which, recombined with sites present in the str^d genome, frequently give rise to resistant phenotypes similar to those which are among the mutational possibilities of the str-d. The fact that the sensitive genotype, applied a second time, as it were, when the previously transformed sensitive str-ts-112 is used as donor, also gives rise to these resistant types, may suggest that certain of the mutant sites act as quantitative factors or modifiers.

Search for suppressors or modifiers of dependence. Some of the results already described would be consistent with another possibility; that the sensitive phenotype is sometimes produced by the addition to the str^d genotype of a suppressor factor at another site. Such suppressors could be present in revertants and transformants produced by the DNA of revertants, and might or might not be present in wild type. Several such sensitive strains produced by reversion and transformation were treated with wild-type str-s DNA to see whether their "suppressors" could be removed, unmasking a dependence or resistance (Table 4). It can be seen that dependent colonies were never recovered in numbers greater than from the untreated controls. In the cases of str-ts-112 and perhaps str-s-81 the recovery of resistant colonies seemed to be slightly higher than from controls, but it is doubtful whether these are significant frequencies.

By the reverse transformations (Table 5), it seems equally clear that these phenotypically sensitive donors do not contain a resistance or dependence marker transferable separately to the wild-type str-s recipient.

DISCUSSION

No evidence was found of determinants suppressing, rather than displacing, the str^d marker, for dependence was never recovered from any spontaneous mutant or transformant that had become sensitive. To conclude that this is because the wild type as well as all of these strains contain an identical "suppressor"

TABLE 4

Action of wild-type DNA on different sensitive recipients derived by reversion or transformation of streptomycin-dependent strains: str-s (revertant or transformant) × DNA str-s (wild type)

Recipients	DNA-treated cultures: Transformed population examined	DNA-treated cultures: Colonies recovered str-d	DNA-treated cultures: Colonies recovered str-r	Controls without DNA: Population examined	Controls without DNA: Colonies recovered str-d	Controls without DNA: Colonies recovered str-r
str-s-81*	6.6×10^7	5	49	3.8×10^7	3	17
str-ts-60†	4.9×10^7	2	1	3.2×10^7	0	1
str-ts-1†	5.8×10^7	3	9	2.8×10^7	1	6
str-ts-1a‡	3.9×10^7	1	10	2.0×10^7	0	4
str-ts-25‡	4.3×10^7	0	7	2.6×10^7	1	5
str-ts-112‡	3.3×10^7	1	15	1.8×10^7	3	0
str-ts-110§	4.0×10^7	2	6	5.0×10^7	2	9

The data indicate the total number of str-d and str-r colonies on plates containing 300 μg/ml streptomycin in three independent experiments. All colonies were further characterized on nutrient medium containing 0, 30, 300 μg/ml streptomycin.
* Spontaneous str-s revertant of the str-d-15.
† str-s transformants obtained by exposing cells str-d-15 to DNA of str-s-81.
‡ str-s transformants obtained by exposing cells str-d-15 to DNA of str-s (wild).
§ str-s transformant obtained by exposing cells str-d-15 to DNA of str-ts-112.

would merely imply an arbitrary redefinition of the mutations leading to dependence.

str^s and str^d seem rather to displace each other in both transformation directions. Because the str^d is transmitted less often (like larger markers), it might be a multisite mutant region. This would mean that some revertants and transformants with sensitive phenotype would be expected to retain portions of the multisite str^d locus. The existence of such residual sites is mainly inferred from the ease of displacement of dependence determinants compared with their low rate of introduction into wild type. Also, when str^d was introduced into wild-type

TABLE 5

Action of DNA from different sensitive revertants and transformants derived from streptomycin-dependent strains upon wild-type sensitive cells: str-s (wild type) × DNA str-s (revertant or transformant)

DNA from donor	Total population examined	Dependent or resistant colonies recovered: str-d	Dependent or resistant colonies recovered: str-r
str-s-81*	3.9×10^7	3	16
str-ts-60†	4.3×10^7	0	28
str-ts-1†	3.3×10^7	1	15
str-ts 1a‡	6.6×10^7	1	13
str-ts-112‡	5.8×10^7	0	22
str-ts-110§	4.9×10^7	1	11
Controls: (spontaneous mutants)			
3str-s (wild)	4.0×10^7	0	10
without DNA‖	3.0×10^7	1	22

Data from three independent experiments. Details and notes as in legend of Table 4.
‖ Control values represent averages from seven sets of three experiments each.

str-s (BALASSA and GÁBOR 1965) there was some evidence that could be interpreted as incomplete transfer. About 43% of the transformants appeared to be dependent at lower concentrations of streptomycin than were the donors. If such dependent clones represent intermediates between the original str^d and str^s genotypes, they are different from the revertants and transformants described here, which were selected for independence and proved nearly all to be sensitive or resistant, and only occasionally dependent in low degree. Sensitive revertants and transformants have not yet been used as recipients to each other or to str-d donors.

Thus, there is no barrier to considering str^s and the str^d dervied from it to be alleles in a region which is probably of multisite nature. The str^d and str^s taken together do not seem to contain the information necessary to build the resistance marker, str^r. It seems also that the str^r and str^d pair do not contain the information necessary to make a str^s recombinant. Within the limits of the resolution attained, therefore, it appears that str^d and str^r are both mutant in the same region or regions of the locus governing sensitivity in the wild type. This is true for several str-r mutants and transformants, but the series of crosses and the numbers of isolates tested were perhaps not extensive enough to separate the markers if they are very closely linked sites as claimed for *Escherichia coli* by HASHIMOTO (1960). This author and also RAVIN and MISHRA (personal communication, 1964) working with pneumococcus, regard the str^d mutations as single-site in nature, but very close to the site of str^r mutations, which are also considered to be at single sites, with the exception of one multisite high-resistance mutation (ROTHEIM and RAVIN 1962). On the other hand, GOLDSCHMIDT, MATNEY and BAUSUM (1962) concluded that for *Salmonella typhimurium*, str^d and str^r mutations were probably multisite and allelic as is proposed for *Rhizobium lupini*.

The several str^r markers described here displace the str^d marker by transformation, introducing resistance but producing no sensitive transformants among 364 examined. It should be noted that str^r is transferred at a high rate, both when transforming wild-type recipients (BALASSA and GÁBOR 1961), and two str-d recipients (this paper). This suggests that the str^r markers may be considerably smaller than the str^d mutant region, which is introduced into wild type only at 1% of this frequency (BALASSA and GÁBOR 1965). It is likely that marker size is only one factor governing transformation frequency (HOTCHKISS and EVANS 1958); marker configuration (RAVIN and IYER 1962) and intrinsic marker efficiency probably related to size and other factors (EPHRUSSI-TAYLOR, SICARD and KAMEN 1965) have also been shown to affect the rates of incorporation.

If str^d is multisite in nature, there is a possibility that str^r and str^d markers possess some shared mutations in their region of overlap. There were indications of this when a low resistant donor transformed a high dependent recipient, giving a transformant resistant at high streptomycin levels, like the recipient. This seems to suggest the existence of a determinant effective in either resistant or dependent strains, which affects the concentration level at which normal growth can, or in the case of dependence must, be maintained. The failure to obtain low resistant transformants in the case of highly resistant donors is a further indica-

tion that the str^r and str^d determinants permitting high streptomycin levels are similar or closely related.

GORINI and KATAJA (1964) have presented evidence that streptomycin dependence can in certain cases be the result of a requirement for a correction, which streptomycin can make, in the expression of a mutant genotype which would otherwise lead to a growth deficiency. This and other work indicates that the site of streptomycin action may be the protein-synthesizing ribosomes (suggested by SPOTTS and STANIER 1961); the factor suggested here governing the level of streptomycin resistance in Rhizobium may also operate at this site. If so, high resistance would presumably be attributed to a *lowered* affinity of the ribosomes for streptomycin. Such a lowered affinity or susceptibility would be expected to lead to high dependence on streptomycin, whenever this drug is needed to restore a growth function lost by mutation. The possibility remains, of course, that altered ability of the cell to bind or transport streptomycin may actually involve cell sites other than ribosomes.

The "affinity region" is not sufficient to determine streptomycin resistance—for str-d donors, assumed to carry it, do not produce str-r transformants from the wild-type recipients. It is reasonable to conclude that an additional "resistance-factor" mutation must be present to create the resistance phenotype, the sum of these constituting the multisite mutation generally transferred, and perhaps only observable, as a total unit. The equivalent "dependence-factor" mutation, combined with an "affinity" mutation, would produce the large str^d multisite mutant region. This formulation suggests a number of future experiments which can serve to test the hypothesis.

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SUMMARY

Streptomycin dependent (str-d) mutants were transformed by DNA of streptomycin resistant (str-r) and sensitive wild-type (str-s) strains. The markers seem to displace each other as alleles; str^d and str^s were not observed to give str^r, and str^d and str^r did not recombine to give str^s. The rates of transformation are similar to those for related markers and 100-fold higher than that of introduction of str^d into str-s by transformation.—These findings suggest that str^d results from a multisite mutation, which may be only partially displaced during spontaneous mutation or transformation to str^s or str^r.—Suppressor genes modifying dependence were not demonstrable among several str-r and str-s mutants and transformants derived from str-d. Crosses with str-r suggest that str-d strains contain a factor determining the concentration of streptomycin at which either resistance or dependence are manifested. This factor, which may control affinity for streptomycin at a cell site, when combined with a resistance or dependence factor would then give rise to the str-r or str-d phenotypes.

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