A GENETIC FACTOR ON THE DNA MOLECULE CAPABLE OF DEPRESSING THE FREQUENCY OF TRANSFORMATION AT A LINKED SITE[1]

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THE genetic factor responsible for the depression of the frequency of streptomycin-resistance transformations in Pneumococcus was first studied by GREEN (1959). He found that when Rx cells are exposed to a DNA transforming preparation (TP) containing genetic markers for erythromycin resistance and streptomycin resistance, approximately identical frequencies of transformation by both markers are observed. However, if strain Rz is exposed to the same DNA, the frequency of streptomycin-resistance transformations decreases. This difference between the two strains has been termed a "host specificity." The specific depression of transformation of a streptomycin-resistance marker has also been reported by STUY (1961). GREEN hypothesized that this specific depression is due to a genetic factor (depressor or *dep*) which is linked to the streptomycin-resistance marker and is usually transmitted in association with that marker when the latter is successfully integrated into the recipient genome. However, this conclusion was not completely justified since the experimental design permitted several alternative conclusions; it was possible that the depressor was associated with both the erythromycin-resistance marker and the streptomycin-resistance marker, or even with the erythromycin-resistance marker alone.

GREEN suggested that the depressor has its effect at the time the transferred markers are genetically integrated into recipient genomes. He suggested that the depressor factor is possibly one that interferes with the recombination event. Since studies of heterospecific transformation (SCHAEFFER 1956; ALEXANDER, LEIDY and HAHN 1954) have shown that a certain homology between the DNA of the recipient and the transforming DNA is necessary for efficient transformation, GREEN suggested that the depressor is a portion of a DNA molecule, in the TP, which lacks homology with a corresponding region of the same DNA molecule in the recipient Rz strain, but which is homologous to this region in the Rx strain. Consequently, the depressor lowers the frequency of transforma-

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tion of Rz cells by markers in the streptomycin-resistance locus to which it lies adjacent, but has no effect on the frequency of transformation of Rx cells.

According to the hypothesis of GREEN (1959), the following was expected: streptomycin-resistance markers linked to the marker studied by GREEN (*str-r1*) should be linked to the depressor and should be affected by it. Conversely, all markers unlinked to the *str-r1* marker should not be affected by the depressor. The studies to be described here were undertaken to test GREEN's hypothesis in these ways. However, the question as originally phrased could not be directly tested since these studies have shown that the depressor is not present in these strains prior to the occurrence of the streptomycin-resistance mutation; it has probably arisen simultaneously with the mutation to resistance. Nevertheless, it has been established that the depressor is linked specifically to the *str-r* mutation, the genetic integration of which is depressed. Furthermore, these studies have revealed that most of the streptomycin-resistance markers linked to the one studied by GREEN have a depressor associated with them. On the other hand, it has been tentatively concluded that a streptomycin-resistance mutation not linked to the *str-r1* site is not associated with a depressor.

MATERIALS AND METHODS

Three strains of pneumococcus were used in these experiments, Rx, Rz and SIII-1. The first two are capsule-deficient mutant strains of spontaneous origin, obtained at different times from different SIII-N strains. RAVIN (1959) has shown that these strains are genetically different since they are able to reconstitute the fully encapsulated SIII-N condition when reciprocally transformed, although treatment of either strain with autologous DNA produces no encapsulated transformants. Strain SIII-1 was synthesized originally by EPHRUSSI-TAYLOR (1951). It produces only traces of type III polysaccharide. Strain SIII-1 is identical to Rz with respect to action of the depressor.

Genetic markers: The erythromycin-resistance marker (*ery-r2*) used in these experiments was isolated as a spontaneous mutation to low level resistance (1 μg/ml) in strain Rx (GREEN 1959; RAVIN and IYER 1961).

The streptomycin-resistance markers are derived from various sources and have been described previously (ROTHEIM and RAVIN 1961). These markers differ in their levels of resistance; the procedures for the replacement of one streptomycin-resistance marker by another, or for combining two markers in the same genome, can be found in ROTHEIM and RAVIN (1961). The marker most frequently used is *str-r1* which was obtained as a single-step, spontaneous mutation to high level resistance (6000 μg/ml) isolated in strain SIII-1. This is the marker used by GREEN (1959).

A sensitive strain will be designated as follows: SIII-1 *ery-s2 str-s1*, or *ery-s2 str-s2*, or etc., depending on the streptomycin-resistance marker being discussed.

Media and transformation procedure: In general, the media and transformation procedures have been described in ROTHEIM and RAVIN (1961). However, the "short-term" transformation experiment, which was used primarily in the

synthesis of multiply-marked strains, was carried out somewhat differently. Competent cells were exposed to transforming DNA for a limited time (ca. 20 minutes, or less than one generation time); exposure of cells to DNA was terminated by the addition of Mg^{++}-activated deoxyribonuclease (WORTHINGTON, IX crystallized), in a final concentration of 0.001 μg/ml. Competent cells were obtained by prior growth of the culture in a Medium 3-albumin mixture (RAVIN 1959) and their competent state was maintained, when desired, by freezing in ten percent glycerin (GREEN 1959). All experiments were performed with saturating concentrations of DNA.

RESULTS

Linkage of depressor to str-r *marker:* To determine if the depressor is linked to the *str-r1* marker (as hypothesized by GREEN 1959) or to the *ery-r2* marker, and its degree of linkage, the following two series of experiments were performed. The same two doubly-marked DNAs were used in both series, TP *ery-r2 str-r1* dep^+ and TP *ery-r2 str-r1 dep*. When strain Rz or SIII-1 is treated with the first DNA it is observed that the frequency of streptomycin-resistance transformations is equal to, or in some cases, slightly greater than the frequency of erythromycin-resistance transformations (Table 1A). This TP may be designated as not having the depressor and has been symbolized as dep^+. On the other hand, when strain Rz or SIII-1 is treated with the second TP, it is observed that the frequency of streptomycin-resistance transformations is less than the frequency of erythromycin-resistance transformations (Table 1B). The depressor is assumed to be present in this TP and has been so designated by the symbol *dep*.

In the first set of experiments duplicate cultures containing sensitive Rz cells were treated with TP *ery-r2 str-r1 dep* for 20 minutes (short-term experiment). About three hours later aliquots were plated on agar containing erythromycin. One isolate was chosen from each tube, thus assuring that they were the result of separate transformation events. These transformants were then individually treated, in a second short-term experiment, with the DNA *ery-r2 str-r1* dep^+. In this manner, doubly-marked cells were obtained in which the erythromycin marker came from a DNA containing the depressor and the streptomycin marker came from a DNA in which the depressor was absent. TPs were then made from each of the doubly-marked strains thus synthesized.

The second series of experiments represents the reciprocal of set I. Sensitive Rz cells were also initially treated with TP *ery-r2 str-r1 dep*, but this time streptomycin-resistant transformants were selected by plating aliquots from each of the duplicate cultures on streptomycin-containing agar. The independent streptomycin-resistant transformants were then treated in a second short-term experiment with TP *ery-r2 str-r1* dep^+, but in this second step erythromycin-resistant transformants were isolated. Thus, from the second series independent transformants were synthesized which had derived their *str-r1* marker from a "depressed" TP and their *ery-r2* marker from an "undepressed" TP. DNA preparations were also made from the isolates in this group.

TABLE 1

Transformation of the sensitive SIII-1 strain

TP	Transformation frequency ($\times 10^{-4}$) *ery-r2*	*str-r1*	*ery/str*
A. *ery-r2 str-r1 dep*$^+$	0.36	1.1	0.3
	8.40	11.7	0.7
	16.9	14.7	1.2
	1.5	2.1	0.7
	5.9	5.2	1.1
	2.2	2.7	0.8
B. *ery-r2 str-r1 dep*	0.5	0.27	1.9
	0.8	0.08	10.0
	3.8	1.3	2.9
	0.33	0.13	2.5
	0.14	0.015	9.3
	0.76	0.098	7.8
C. Set I—*ery-r2* derived from TP B, *str-r1* derived from TP A isolate:			
1	0.46	0.97	0.5
2	1.79	2.3	0.8
3	0.37	0.62	0.6
4	0.83	0.75	1.1
5	7.6	7.6	1.0
6	0.99	1.0	1.0
7	2.1	3.0	0.7
8	4.3	5.4	0.8
D. Set II—*ery-r2* derived from TP A, *str-r1* derived from TP B isolate:			
1	4.81	0.66	7.3
2	1.88	0.23	8.2
3	1.28	0.28	4.6
4	2.9	0.72	4.0
5	0.62	0.27	2.3
6	1.33	0.54	2.5
7	0.18	0.04	4.5
8	0.33	0.41	0.8
9	21.7	19.7	1.1
10	0.31	0.41	0.8
11	10.8	11.2	1.0
12	1.88	0.17	11.0
13	3.2	0.44	7.3
14	5.27	1.53	3.5

Average E/S for experiments cited in A$=0.8\pm0.3$.
Average E/S for experiments cited in B$=5.7\pm3.7$.
This is the variation obtained in using different batches of competent cells.

Upon the appropriate recipient strain the DNAs prepared from the doubly-marked cells of set I proved to lack the depressor. Eight independent isolates were tested and none showed the depressor (Table 1,C). On the other hand, 14 TPs were tested from the independent isolates obtained in set II and ten possessed the depressor (Table 1,D). Statistical analysis (chi-square method) indicates that the difference between the two sets of data is significant ($.01 > P > .001$). It is correct to conclude therefore, that the depressor shows linkage only to the streptomycin-resistance marker *str-r1* and not to the erythromycin-resistance marker, *ery-r2*. Furthermore, the two markers (*str-r1* and *dep*) are integrated together about 70 percent of the time.

Origin of the depressor: The evidence indicates that the depressor associated with *str-r1* does not exist in any of the strains prior to the occurrence of the streptomycin-resistance mutation. It has been observed in experiments described above with Rz (and in other unreported experiments with strains SIII-1 and Rx) that exposure of sensitive cells (Rx, Rz or SIII-1) to DNA *str-r1 dep* can result in the recombinant transformant class *str-r1* dep^+. It would be impossible to obtain this genetic class if the depressor were simultaneously present in the strain and in the DNA; it can only occur through a recombination event between the DNA containing the *dep* marker, and the strain containing the dep^+ marker. The possibilities are, therefore, that the depressor either arose at the same time the streptomycin-resistance mutation occurred, or else was the result of a second, independent mutational event which occurred after the mutation to resistance. The evidence to be presented now suggests that the former is correct.

Genetic analysis (ROTHEIM and RAVIN 1961) has shown that all the spontaneous streptomycin-resistance mutations thus far studied represent either identical or closely linked mutations; on the other hand, the single nitrous acid-induced mutation studied (*str-r36*) was shown to be unlinked to the spontaneous mutation group (RAVIN and EPHRUSSI-TAYLOR 1961). The genetic map constructed by this analysis is shown in Figure 1 of ROTHEIM and RAVIN (1961). When sensitive strain SIII-1 is treated with a DNA containing any one of these markers and the *ery-r2* marker, it can be observed (Table 2) that eight of the ten spontaneous mutations (exceptions are *str-r26* and *str-r27*) show a depression of streptomycin-resistance transformations. If Rx cells are exposed to the same doubly-marked DNA preparations there is little evidence for any depression. In the case of every mutant DNA which produced a depression in strain SIII-1, the effect was observed immediately after isolation of the streptomycin-resistant mutant and addition of the *ery-r2* marker. Therefore, it seems unlikely that in each case there has been two simultaneous, independent mutation events to account for the genome *str-r dep*. The occurrence of two spontaneous mutations without the depressor indicates that while the two appear to occur simultaneously in the majority of mutational events at this locus, it is possible to separate the two phenomena. Since the depressor has no effect on the level of resistance of a cell and can be separated by recombination from the *str-r* mutation, this finding is not unexpected. It cannot be assumed, however, that the same depressor arises with each mutation; in fact, there is evidence that more than one depressor exists.

TABLE 2

A representative sample of transformation of sensitive SIII-1 strain by DNA preparations containing different streptomycin-resistance markers

TP	Frequency ($\times 10^{-4}$) of cells resisting: erythromycin	streptomycin	*ery/str*
A. *str-r2 ery-r2*	3.04	0.47	6.5
	1.17	0.13	9.1
B. *str-r3 ery-r2*	1.28	0.21	6.1
	3.8	0.46	8.3
	4.0	1.26	3.2
C. *str-r4 ery-r2*	1.04	0.21	5.0
D. *str-r5 ery-r2*	8.0	2.4	3.3
E. *str-r9 ery-r2*	0.049	0.008	6.1
	0.86	0.10	8.6
F. *str-r25 ery-r2*	0.8	0.31	2.6
G. *str-r28 ery-r2*	8.14	2.2	3.7
H. *str-r29 ery-r2*	4.71	0.24	19.6
I. *str-r26 ery-r2*	3.44	3.41	1.0
	4.02	4.94	0.8
	5.98	5.43	1.1
J. *str-r27 ery-r2**	0.18	0.15	1.2
	0.28	0.25	1.1

* Data from BRYAN 1961.

In one experiment (ROTHEIM and RAVIN 1961) cells of genotype *str-r2 dep-2* were treated with TP *str-r25 dep-25*. A transformant was isolated in which the *str-r2* marker was replaced by the *str-r25* marker. The *ery-r2* marker was added to this isolate and a TP prepared. The average E/S ratio for this TP was 0.8 as compared to the ratio of 2.6 for TP *ery-r2 str-r25 dep* (Table 2F). The results indicate that the depressor associated with marker *str-r25* differs (either physically or physiologically) from the depressor associated with *str-r2*. Additional experiments of similar type are necessary before the number of possible depressor mutations can be determined.

In one culture of SIII-1 *ery-r2 str-r2 dep-2* a reversion to streptomycin sensitivity had occurred; this reversion did not affect the erythromycin resistance. BRYAN (1961) has shown that this reversion is due to a "back mutation" at the site of the original *str-r2* mutation. The reverted strain was treated with TP *str-r1 dep*$^+$, independent streptomycin-resistant transformants selected, and a TP prepared from each of 21 such isolates. When these DNA preparations were tested on the appropriate strain no depression was observed (Table 3). From previous experiments it is expected that at least 70 percent of the time the *dep*$^+$ marker will be simultaneously integrated with the *str-r1* marker into the reverted

TABLE 3

Transformation of the sensitive SIII-1 strain by DNA preparations from streptomycin-resistant clones independently derived from the transformation of the rev-str-r2 ery-r2 *strain by TP* ery-r2 str-r1 dep$^+$

Clone	Frequency ($\times 10^{-4}$) of transformants resisting: erythromycin	streptomycin	*ery/str*
1	1.1	2.2	.5
2	1.7	5.0	.3
3	1.9	3.2	.6
4	5.8	9.7	.6
5	7.0	16.0	.4
6	0.22	0.18	1.2
7	1.2	0.96	1.3
8	2.46	5.0	.5
9	4.5	9.8	.5
10	10.0	13.0	.8
11	3.3	6.5	.5
12	1.4	2.2	.6
13	5.4	9.3	.6
14	2.6	5.2	.5
15	.81	.84	1.0
16	2.6	3.4	.8
17	3.4	6.9	.5
18	.78	.86	.9
19	7.3	9.3	.8
20	9.2	11.1	.8
21	12.4	10.7	1.2
TP *ery-r2 str-r2 dep*	3.0	.46	6.5
	2.1	.42	5.0
	.67	.032	20.9

strain; if the depressor is still present in the reverted strain, approximately 30 percent of the time there will be a recombination resulting in a transformant having the genotype *str-r1 dep*. The fact that 21/21 independent isolates were of the genotype *str-r1 dep*$^+$ would mean, in effect, that 21/21 times the *dep*$^+$ marker was integrated simultaneously with the *str-r1* marker. When these data are compared statistically (chi-square method) with the previous linkage data, it is observed that the difference is significant ($.05 > P > .02$). If it is assumed that the depressor associated with the *str-r1* marker is the same as the depressor associated with the *str-r2* marker, then the conclusion can be drawn that the depressor has reverted with the reversion of the *str-r2* marker. The identity of the two depressors can only be established by making the "cross" *str-r2 dep-2* $\times$ TP *str-r1 dep-1* and observing if it is possible to obtain the transformant of type *str-r1 dep*$^+$ which would indicate the two depressors are different. This experiment has not yet been conducted on a sufficiently large scale. However, there is other evidence that suggests the two depressors are identical. In allelism experiments (ROTHEIM and RAVIN 1961) cells of genotype *str-r2 dep-2* were treated with TP *str-r1 dep*$^+$. A transformant was isolated in which the *str-r1* marker

had replaced the *str-r2* marker; when the *ery-r2* marker was added, and a TP prepared and tested on strain SIII-1, it was observed that the frequency of erythromycin-resistance transformations was greater than that of streptomycin-resistance transformations. In other words, the genotype of the transformant was *ery-r2 str-r1 dep*. In this case, the depressor could only have originated in the recipient *str-r2* cells, and so it is obvious that the *dep-2* marker has an effect on the *str-r1* marker. This suggests that the *dep-1* and *dep-2* markers are similar and lends additional support to the conclusion that when the *str-r2* marker reverted, the depressor reverted simultaneously.

DISCUSSION

The experiments described here concern the analysis of a genetic factor which by itself confers no streptomycin resistance but which apparently has an effect on the genetic integration of streptomycin-resistance markers. This factor, depressor, does not even indirectly modify the level of resistance of the cell since cells of genotype *str-r1 dep*$^+$ are identical in their level of resistance to cells of type *str-r1 dep*. These results do not offer an unequivocal interpretation concerning the nature of the depressor, and it must be emphasized that any scheme concerning the nature of the depressor must be speculative. However, the observations reported here are consistent with the following hypothesis.

It is proposed that when the streptomycin-resistance mutation occurred, there occurred a simultaneous rearrangement (inversion?) of the genetic material in the region including (or adjacent to) the mutated site .This may be schematized as follows:

original genome SIII-1 ABCDEFGHI
↓ ←mutation and rearrangement
AEDCB′FGHI

where B′ confers streptomycin resistance. The different streptomycin-resistance mutations can be symbolized by A′, E′, etc. Transformation of a sensitive strain in which the depressor does or does not follow the *str-r1* marker is shown in Figure 1. It is the presence of the inverted region which affects pairing of the recipient and donor genome and results in the decrease of streptomycin transformations. Thus, while there is no reason to suppose that these hypothetical inversions

A. Simultaneous incorporation of *dep* and *str-r* markers

recipient strain	*ABCDEFGHI*		
+		----→	*AEDCB′FGHI*
DNA *str-r dep*	*AEDCB′FGHI*		resistant transformant with depressor

B. Incorporation of the *str-r* marker only

recipient strain	*ABCDEFGHI*		
+		----→	*AB′CDEFGHI*
DNA *str-r dep*	*AEDCB′FGHI*		resistant transformant without depressor

FIGURE 1.—Scheme for transformation of sensitive strain by DNA *str-r dep*.

at the intramolecular level (i.e., possible inversions in nucleotide sequences in the DNA chain) are structurally identical with the inversions known to exist at the chromosomal level, the final effect of the intramolecular inversion is assumed here to be identical to that of the chromosomal inversion, namely, a reduction in recombination frequency due to pairing difficulties.

Evidence was presented previously indicating that there is more than one depressor (i.e. more than one segment of genetic material which can become inverted). However, this does not necessarily mean that there is a different depressor associated with each marker. It is possible that the same depressor will occur with different markers. Indeed, this would seem to be so in the case of the *str-r1* and *str-r2* markers.

It was observed in (Table 2) that two mutations did not involve the simultaneous occurrence of the depressor. One of these was studied by BRYAN (1961). This mutation (*str-r27*) was a secondary mutation in strain *str-r2* which completely replaced the *str-r2* marker. This finding suggests that when the *str-r2* marker was removed from the genome there was a simultaneous removal of the depressor, just as has been observed in the results reported here. Alternatively, since the data reported by BRYAN are those obtained with DNA prepared from a transformant of marker *str-r27*, it may be that the depressor was present in the original strain but was not integrated simultaneously with the *str-r27* marker into the particular transformant from which the DNA was prepared. The second marker that did not show the depressor effect was marker *str-r26*. No data are available to determine if this marker showed the depressor effect immediately after isolation, so it is not possible to determine if a reversion of the depressor has occurred, without a simultaneous reversion of the streptomycin-resistance marker. However, assuming that this did not happen, then the occurrence of *str-r26 dep*$^+$ indicates that, although the depressor does in general occur with the majority of the streptomycin-resistance mutations in this region, it is not obligatory that the two events occur simultaneously. It is, unfortunately, technically unfeasible to insert the depressor into the genome of the strain carrying marker *str-r26* and determine if the depressor is capable of affecting the *str-r26* marker.

The results reported here raise the following question: is the occurrence of the depressor limited to the locus of the spontaneous mutations? Some evidence bearing on this question can be obtained from a study of the relative frequencies of transformation of markers *ery-r2* and the nitrous acid-induced *str-r36* using a TP containing both markers. When sensitive SIII-1 cells are tested with such a DNA, one obtains *ery-r2/str-r36* ratios of comparable value to those obtained when the same cells are exposed to DNA *ery-r2 str-r1 dep*$^+$. Although this is suggestive evidence that there is no depressor associated with marker *str-r36*, conclusive proof lies only in the observation that the *ery-r2/str-r36* ratios are equal when sensitive SIII-1 or Rx are treated with the same DNA, irrespective of the numerical value of this ratio. The experiment using Rx as recipient has, however, proven technically difficult for the following reasons. Transformants of genotype *str-r36* must be selected at low concentrations of streptomycin (10–15 μg/ml). Consequently, at this low concentration one obtains some background

growth of the sensitive recipient cells (resistant to ca. 5 μg/ml streptomycin). In the case of strain SIII-1, the number of colonies arising from a plating of the control culture (no TP) has been low enough, compared with the number of colonies produced from a culture treated with TP, that a simple small correction could be made for this background growth. However, in repeated experiments with Rx the background growth was of such a magnitude as to obscure the transformants and make a single correction impossible. Therefore, no final conclusion has been reached at this time as to whether marker *str-r36* has a depressor associated with it, although it is tentatively concluded (on the basis of the experiments with strain SIII-1) that it does not. Accepting this assumption, one might consider what its implications are.

The most general conclusion that could be made is that the depressor is associated solely with the locus of spontaneous mutations. However, it must be remembered that mutation *str-r36* was induced by nitrous acid treatment and it is conceivable that the absence of a depressor is due to a difference between the mechanisms of origin of spontaneous and nitrous acid-induced mutations to streptomycin resistance. It has been suggested (ROTHEIM and RAVIN 1961) that the level of resistance conferred by a given mutation is correlated with its size. If this is correct, then the low level of resistance of marker *str-r36* implies that it is the smallest mutation so far studied and could suggest that the depressor is only associated with more structurally complex mutations. It is hoped that in the near future it will be possible to study unlinked mutations conferring slightly higher resistance (35–50 μg/ml) so that the technical difficulties inherent in working with marker *str-r36* can be overcome.

One last point to consider is the nature of the host specificity of the depressor. Interpretation of this point must also be speculative, but the data cannot be denied. If there were only one depressor site, it would be entirely possible that the Rx strain, which is of different origin than the SIII-1 and Rz strains, has this inversion in common with the mutants. In that case there would be no pairing difficulty and no depression would be observed. On the other hand, since there are apparently at least two depressor sites, and possibly more, it seems improbable that the genome of the Rx strain could contain all these inversions. Therefore, it seems more reasonable to assume that there is a physiological difference between Rx and Rz, such as a longer period of time in which the recombination event can occur in the Rx recipient. This longer period (possibly a longer period of synapsis between host and donor DNA) could compensate for the depressor. Some support for a physiological mechanism is found in the observable variations in E/S ratios in different experiments with the same DNA when both Rx and Rz are used as recipient strains.

SUMMARY

1. A genetic factor (depressor) which has no effect on the level of resistance but which apparently decreases the probability of integration of a streptomycin-resistance marker (*str-r1*) has been shown to be linked to the latter.

2. It was observed that the integration of most of the linked streptomycin-

resistance markers was also affected by the action of a depressor, and that there are at least two different depressors.

3. The depressor was shown to have reverted simultaneously with the reversion of one of the streptomycin-resistance markers (*str-r2*).

4. It was shown to be unlikely that a depressor existed in the strains prior to the occurrence of the streptomycin-resistance mutations and, therefore, probably arose at the same time.

5. A genetic mechanism for the nature and action of the depressor is suggested.

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