THE MAPPING OF GENETIC LOCI AFFECTING STREPTOMYCIN RESISTANCE IN PNEUMOCOCCUS[1]

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THE term "complex locus" implies a series of closely linked genetic determinants all of which produce similar or identical modifications in the phenotype of an organism. Much of the experimental work to be described is concerned with a series of this type conferring streptomycin resistance in Pneumococcus.

BRYAN (1961) has isolated spontaneous streptomycin-resistant mutants of Pneumococcus which show a wide variation in the maximum concentration of streptomycin they are able to resist. A small random sample (four) of these spontaneous mutations was tested for linkage in transformation studies, and they were all found, by the criterion of genetic recombination, to be either allelic (at the same site) or closely linked (at different sites). While this finding could have been due to the small number of mutants tested, it suggested that all streptomycin-resistant mutations occur in the same region (a cistron?) of a DNA molecule. However, analysis of additional markers was necessary to obtain a more complete picture. With this aim in mind the present studies have been undertaken; they have shown that the remainder of the spontaneous streptomycin-resistant mutations isolated by BRYAN (1961), as well as two spontaneous mutations originally isolated by SCHAEFFER (1956), represent either allelic or closely linked markers on the same molecule of transforming DNA. In addition, a nitrous acid-induced mutation to streptomycin resistance has been examined. This mutation appears to belong to a different locus; it does not exhibit the degree of linkage to the spontaneous mutations that the latter exhibit to each other.

MATERIALS AND METHODS

Strains: Two closely related strains of pneumococcus were used in these experiments, SIII-1 and clone 3 of strain R36A. The first was synthesized by EPHRUSSI-

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TAYLOR (1951a) and produces only traces of type III polysaccharide. The second is a clone derived by single-colony isolation from R36A. The exact relationship of strain R36A to strain SIII-1 is described by RAVIN (1959).

Genetic markers: The streptomycin-resistance markers used in these experiments are derived from four sources. The first is a single-step, spontaneous mutation to high level resistance (6000 μg/ml) isolated in strain SIII-1 by RAVIN (1956). This marker will be referred to as *str-r1* (BRYAN 1961). The second group of streptomycin-resistance mutations used in these experiments was obtained as single-step, spontaneous mutations isolated in strain SIII-1 by BRYAN (1961). The designation of these mutations and their approximate levels of resistance in strain SIII-1, as determined by the criteria of BRYAN, are as follows: *str-r2* (300 μg/ml), *str-r3* (150 μg/ml), *str-r4* (300 μg/ml), *str-r5* (300 μg/ml), *str-r9* (2000 μg/ml), *str-r25* (9000 μg/ml), *str-r26* (10–12,000 μg/ml), *str-r27* (6000 μg/ml). The third group consists of two spontaneous mutations to streptomycin resistance isolated in strain R36A by SCHAEFFER (1956). SCHAEFFER has called these two mutations *r2* and *r3*, resistant respectively to 100 μg/ml and 3000 μg/ml streptomycin. According to the system of nomenclature adopted in this laboratory these will be referred to as *str-r28* and *str-r29* respectively. Most of the markers from these three sources were obtained by selection of mutants at streptomycin concentrations greater than 50 μg/ml. A third mutant which can resist, at most, 25 μg streptomycin per ml was obtained by SCHAEFFER (1956) by selecting at an antibiotic concentration of 12 μg/ml. Unfortunately, this mutant, termed *r1* by SCHAEFFER but redesignated *str-r30* in our nomenclature, was not available for study by us.

The fourth source of markers is a number of mutations induced in clone 3 by nitrous acid using the method described by LITMAN and EPHRUSSI-TAYLOR (1959). The authors are grateful to G. BALASSA who originally isolated and supplied us with these mutants. They confer a relatively low level of resistance (ca. 30 μg/ml) in clone 3.

It should be pointed out that there appears to be a difference in the residual genotypes of clone 3 and SIII-1. While both strains have about the same sensitivity to streptomycin (10^7 cells of either strain failing to produce colonies when plated in concentrations of streptomycin higher than 30 μg/ml), the spontaneous mutations confer somewhat higher resistances in clone 3 than in SIII-1. For example, *str-r3*, *str-r2* and *str-r1* confer resistance to 400, 800 and 20,000 μg/ml respectively, when transferred into clone 3. These differences are probably due to the presence, in clone 3, of enhancer genes of the kind described by BRYAN (1961).

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 *ery-r2* marker was introduced in the donor DNA containing a streptomycin-resistance marker to be tested in order to provide a control on the transformability of the recipient strains.

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: In Rochester all strains were maintained and transformed in Medium 3 of EPHRUSSI-TAYLOR (1951b). Medium 1 (EPHRUSSI-TAYLOR 1951b) was used for the growth of large populations from which DNA transforming preparations (TPs) were made. Medium 2 (EPHRUSSI-TAYLOR 1951b) was used for assay of transformants. In Gif, transformations were carried out in a modification of Medium 3, called "NS." This medium is essentially similar to Medium 3, but lacking extract of beef serum.

Preparation of DNA: Most of the DNA transforming preparations (TPs) were made according to the procedure described by EPHRUSSI-TAYLOR (1951b). However, the design of some of the experiments demanded that many preparations be made from a large number of independently-derived transformants; to facilitate such studies the procedure was modified to include only one deproteinization of the alcohol-insoluble fraction of the original crude lysate, according to the methods of SEVAG (1934), followed directly by precipitation of the DNA as fibers by the gradual addition of alcohol. These DNA preparations are contaminated with protein and RNA, but since the genetic purposes to which they are put are not concerned with the purity of the DNA preparations, they are suitable for use in these experiments. The DNA preparations were stored either in physiological saline (0.86% NaCl) or buffered saline (0.15 M NaCl, 0.01 M $PO_4\equiv$) at 5°C.

Other DNA preparations were prepared by lysis with versene. Two hundred ml cultures of desired turbidity in Medium 1 were first centrifuged at 1800 rpm for 30 minutes and then resuspended in 5 ml of 0.15 M NaCl, which was 0.1 M with respect to versene, buffered at *p*H 7.6. The suspension was incubated at 37°C for one hour, and lysis was completed after 24 hours at 0°C.

Transformation procedure: Recipient cells are rendered competent by growth in Medium 3 or "NS" to which bovine serum albumin (Armour Fraction IV) has been added to give a final concentration of 0.2 percent. In general, in experiments involving single *str-r* markers, a transforming procedure was used in which a small inoculum of cells was allowed to become competent in the presence of the transforming DNA, and then plated approximately 10–12 hours after competence and absorption of DNA had occurred (RAVIN 1959). This type of transformation procedure is designated as "long term."

Transformation experiments involving more than one *str-r* marker were carried out by a second method designated as "short term." In this method, competent cells are exposed to transforming DNA for 20 minutes (less than one generation time), and the action of the DNA is terminated by a fiftyfold dilution in medium lacking DNA. The treated cells are allowed to grow at 37°C for two hours in order for the transformed phenotype to be expressed (GREEN 1959; RAVIN and IYER 1961). Both the long and short-term experiments were performed with saturating concentrations of DNA.

Controls were cultures of cells grown under conditions identical to the experimental cultures but without the presence of the transforming DNA; such cultures were used to determine the level of resistance of untransformed recipient cells in allelism tests.

Assay of transformants: In most of the experiments transformants were assayed by spreading an aliquot of the culture over the surface of solidified Medium 2 which had been previously dried for 10–12 hours by storage at 37°C. Several aliquots were tested by spreading on each of a number of agar plates containing different concentrations of antibiotic. After spreading, all plates were incubated at 37° for 24–48 hours before counting the number of colonies that developed. In some experiments, transformants were assayed by pipetting suitable aliquots of the culture into Petri dishes and then mixing with molten agar containing the desired concentration of streptomycin. The colonies developing in the agar appeared after 48 hours of incubation at 37°C, at which time they were counted. Either of these methods of determining the level (or levels) of resistance of a transformant population is referred to in the text as that of "direct plating."

To assay the proportion of erythromycin-resistant cells, erythromycin lactobionate (Abbott) was added to Medium 2 at a concentration of 0.25 μg/ml. The proportion of streptomycin-resistant cells was determined by the addition of streptomycin sulfate (Lilly) to Medium 2, the final concentration varying according to the markers used.

In those experiments in which it was necessary to determine the maximum level of resistance of the transformant class, an additional procedure was used. In order for all of the cells to express the maximum level of resistance conferred by a marker recently acquired by transformation, more time must elapse than that required for expression of a lower level of resistance. Therefore, in the direct plating method streptomycin-resistant transformants are selected by exposure to a concentration of streptomycin that is much lower than the maximum concentration they are genetically capable of resisting. The maximum level of resistance of these transformants is determined in one of the following ways. In the case of transformant colonies growing on the surface of agar, a velvet replica is made (LEDERBERG and LEDERBERG 1952) of the plate containing the lowest concentration of antibiotic (to insure recovery of all transformants) and restamped on a series of plates of increasing concentrations of streptomycin. In this way a growth-limiting concentration of streptomycin is determined. In the case of transformant colonies growing in agar, the colonies are first isolated by means of a platinum needle to a sector of an agar plate devoid of antibiotic. Plates containing such isolates are incubated to permit a sufficient amount of growth, and the growth in each sector is tested either by the velvet-replica technique or by directly streaking a platinum loopful to each of a series of agar plates of increasing concentration of streptomycin.

That concentration of antibiotic above which a significant fraction of a mutant or transformed strain is unable to grow, varies to a certain extent with the physiological state of the culture (BRYAN 1961; RAVIN and IYER 1961). The greatest degree of variation is observed with those mutants capable of resisting the highest concentration of streptomycin. For these reasons, two mutants cannot be phenotypically distinguished if their levels of resistance, although not identical, are very close.

Allelism tests: Two streptomycin-resistance markers will be designated as allelic if they can replace one another but cannot coexist in the same genome, and conversely, they will be designated as nonallelic if they can coexist in the same genome. It has been observed that when nonallelic antibiotic-resistance markers are simultaneously present the level of resistance of the cell is generally increased compared to the level of resistance of the cell when just one marker is present. Furthermore, the new level of resistance is greater than the sum of the levels of resistance conferred by the markers individually (HOTCHKISS and EVANS 1958; RAVIN and IYER 1961; BRYAN 1961). A TP prepared from such a multiply-marked strain will give rise to several classes of transformants. One class is characterized by the same level of resistance as that of the donor strain, due to the sensitive recipient cell acquiring both markers, and the other classes are characterized by lower levels representing acquisition of single markers. The frequency of the different classes is a function of the degree of linkage of the individual markers.

Allelism tests to be described are based on this principle. Recipient strains bearing one streptomycin-resistance marker, e.g. *str-r2*, are treated with DNA of another marker, e.g. *str-r1*. The transformants are then screened for a class (presumably *str-r1-r2*) whose resistance is greater than that of either component or as great as *str-r1* (representing a substitution of the *str-r2* marker by the *str-r1* marker), or even for phenotypes having a level of resistance greater than *str-r2* but less than *str-r1*. Such a class might represent the *str-r1-r2* recombinant since it has been observed (RAVIN and IYER 1961) that nonallelic erythromycin-resistance markers can be antagonistic in their effect on the phenotype. DNA preparations are made from all transformants isolated from such "crosses" and their genotype is determined by treating the sensitive strain with these DNAs, and observing the classes of transformants thus obtained.

Isolation of transformants: Transformants are isolated by restreaking, by means of a platinum needle, single colonies arising in direct platings. The term "independent transformants" refers to colonies selected from different experiments, e.g. five independent transformants indicates that the experiment was done five times; from each experiment a single colony was selected to be tested. In this manner, all independent transformants represent the results of different transformation events.

RESULTS

The sites of str-r2, str-r3 *and* str-r1: Strains *str-r2* and *str-r3* were individually treated with TP *ery-r2 str-r1*. In each case transformant colonies were selected on a concentration of streptomycin at which the recipient cells would not be able to grow (500 or 1000 μg/ml). The results of several typical experiments are summarized in Table 1A, B. The plates containing the lowest concentration of streptomycin were replicated to plates containing concentrations of streptomycin greater than 1000 μg/ml, and in all cases it was observed that the transformants exhibited a phenotype indistinguishable from that of cells bearing the marker *str-r1* alone. Independent transformants were isolated, grown in the absence of

TABLE 1

Allelism tests between different str-r *markers*

Cells	TP	Frequency of transformants ($\times 10^{-4}$)* resisting: Erythromycin 0.25 μg/ml	Streptomycin 500 μg/ml	Streptomycin 1000 μg/ml
A. *str-r2 ery-s2*	*str-r1 ery-r2*	30	–†	9.8
	str-r1 ery-r2	5.0	–	1.0
	none	$< 1 \times 10^{-7}$	–	2×10^{-6}
	none	$< 1 \times 10^{-7}$	–	1.7×10^{-6}
B. *str-r3 ery-s2*	*str-r1 ery-r2*	1.2	.08	–
	str-r1 ery-r2	1.0	.4	–
	none	$< 1 \times 10^{-8}$	4×10^{-7}	–
	none	$< 1 \times 10^{-8}$	4×10^{-7}	–
C. *str-r5 ery-s2*	*str-r2 ery-r2*	1.4	–	4×10^{-7}
	none	$< 1 \times 10^{-6}$	–	6×10^{-7}
D. *str-r28 ery-s2*	*str-r2 ery-r2*	4.7	7×10^{-7}	–
	none	$< 1 \times 10^{-7}$	7.2×10^{-7}	–

* Unless otherwise indicated.
† Hyphen indicates not tested by direct plating.
In A and B all transformants selected on the concentration of streptomycin indicated were able to grow on concentrations of streptomycin up to 6000 μg/ml, as determined by velvet-replica method. In A and B duplicate experiments are reported.

streptomycin for a short time, and their level of resistance determined by means of direct plating and velvet-replica technique. The results confirmed the previous observation of a single class of transformants, phenotypically identical to mutants containing the marker *str-r1*. If the recombinant classes *str-r1-r2* or *str-r1-r3* existed among the transformants, they did not have a greater level of streptomycin resistance than the class expected from a substitution of the *str-r2* or *str-r3* markers by the *str-r1* marker. DNA preparations were made from five independent transformants obtained in each "cross." When the sensitive SIII-1 strain (*str-s1-s2-s3 ery-s2*) was treated with each of these TPs, it was observed (Table 2A, B) by direct plating followed by velvet replication of plates containing the lowest concentration of streptomycin, that only one class of transformants could be obtained. In every case, this class is phenotypically identical to *str-r1*. There is no evidence of the simultaneous presence of the *str-r2* or *str-r3* marker with the *str-r1* marker in the cell lines derived by the treatment of *str-r2* or *str-r3* with TP *str-r1*.

This observation excludes the possibility that marker *str-r1* is unlinked to *str-r2* or *str-r3*. Marker *str-r1* is either allelic to *str-r2* and *str-r3*, or if it is possible to obtain the recombinant classes *str-r1-r3* and *str-r1-r2*, these classes occur less frequently than once in about five recombination events in the "crosses" *str-s1-r2* × *str-r1-s2* and *str-s1-r3* × *str-r1-s3*.

These results become more interesting when considered with the observation of BRYAN (1961) that markers *str-r2* and *str-r3* are nonallelic; they exhibit linkage, being located at different sites on the same transforming molecule. When cells *str-r2* were treated with TP *str-r3*, a recombinant class (*str-r2-r3*) was obtained which had a phenotype distinct from that of *str-r2* or *str-r3* alone; namely, it was capable of resisting up to 4000 μg/ml streptomycin. If the sensi-

TABLE 2

Transformation of sensitive SIII-1 strain by TPs derived from transformants obtained from different allelism tests

TP	Frequency of transformants ($\times 10^{-4}$) resisting: Erythromycin (μg/ml) 0.25	50	Streptomycin (μg/ml) 100	500	1000
A. *str-r2*(TP *str-r1*) *ery-r2* isolate no.					
1	5.6	–*	9.1	8.0	7.4
2	2.5	–	3.7	3.5	3.2
3	4.8	–	1.3	–	1.2
4	1.2	–	2.2	2.3	0.8
5	1.4	–	0.91	1.3	1.5
B. *str-r3*(TP *str-r1*) *ery-r2* isolate no.					
1	2.1	5.1	–	4.4	3.8
2	0.81	0.33	–	0.37	0.35
3	0.65	0.21	–	–	0.19
4	11.0	9.6	–	10.0	8.4
5	0.81	0.62	–	–	0.45
C. *str-r2*(TP *str-r25*) *ery-r2*	2.6	–	3.0	3.4	3.2
	2.2	–	2.6	2.7	2.2
	2.4	–	4.2	3.9	3.4
D. *str-r3*(TP *str-r25*) *ery-r2*	0.18	0.09	–	0.09	0.06
E. *str-r2*(TP *str-r26*) *ery-s2*	–	–	16.5	21.8	18.9
F. *str-r3*(TP *str-r26*) *ery-r2*	0.14	0.17	–	0.10	0.10
G. *str-r2*(TP *str-r29*) *ery-r2*	6.2	0.34	–	0.3	0.3
H. *str-r3*(TP *str-r29*) *ery-s2*	–	0.12	–	0.13	0.14

* Hyphen indicates not tested on this concentration.
Nomenclature of TPs indicates the origin of the strain from which each TP was prepared. For example, in A, the strain was obtained by exposing cells *str-r2* to TP *str-r1*. The *ery-r2* marker was then added to the transformant thus obtained, and a TP prepared. Similar notation used for TPs B-H.
All plates containing 50 μg/ml or 100 μg/ml streptomycin were replicated to plates containing from 1000 to 5000 μg streptomycin, and all colonies were observed to be capable of growth at the higher concentration.

tive strain is treated with the DNA from a transformant of the type *str-r2-r3*, it is found that for every transformant of the double type (*str-r2-r3*) there are 40 of the single type (*str-r2* and *str-r3*).

The results suggest, therefore, that the *str-r1* marker represents a multisite mutation, overlapping the sites of the *str-r2* and *str-r3* markers. It must be strongly emphasized that on the basis of these three experiments the possibility cannot be ruled out that *str-r1* lies between the sites of the *str-r2* and *str-r3* markers with the ends of the *str-r1* marker lying close to those of the adjoining two. These alternatives are shown in Figure 1.

A final analysis of the size of the *str-r1* marker requires the mapping of other markers with relation to the *str-r2* and *str-r3* markers. Evidence that the *str-r1* marker could replace a third marker known to be at a site different from those of the *str-r2* and *str-r3* markers would establish that the *str-r1* marker is, indeed, a multisite mutation. Until such evidence is obtained, the conclusion that marker *str-r1* covers at least two sites must be regarded as tentative. However, if marker *str-r1* does lie between the markers *str-r2* and *str-r3*, it must be of sufficient size to

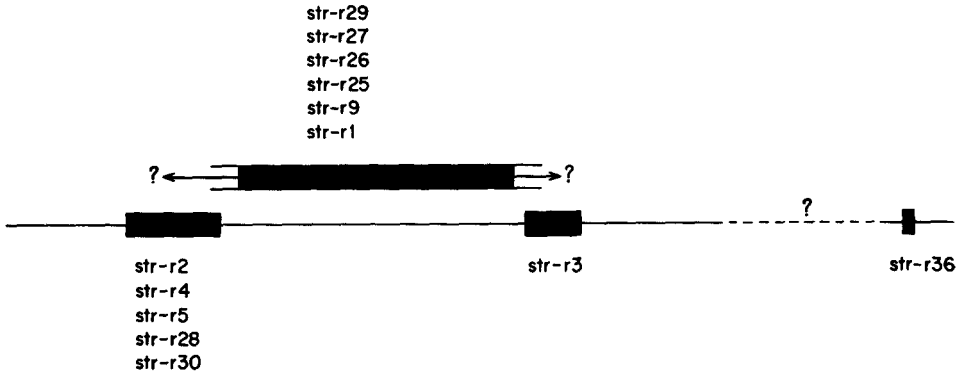

FIGURE 1

MAP OF THE STREPTOMYCIN-RESISTANCE LOCUS

FIGURE 1.—The location of *str-r30* is based on SCHAEFFER (1956). Two possibilities are envisaged for the *str-r1* site. (1) Its ends lie within the segment between the *str-r2* and *str-r3* sites, or (2) its ends overlap one or both of the other sites.

account for the observation that, although *str-r1* must be closely linked to *str-r2* and *str-r3* (since it generally replaces the latter two markers in transformation), markers *str-r2* and *str-r3* exhibit relatively loose linkage.

These data have established at least three sites at which streptomycin-resistance mutations can occur on a single transforming DNA molecule. This suggests that perhaps all streptomycin-resistance markers occur in one region of the bacterial genome and will exhibit linkage to the three sites established above. The following two sections describe the mapping of a random sample of spontaneous mutations to streptomycin resistance.

Alleles of the str-r1 *marker:* The following long-term experiments ("crosses") were performed:

	Cells	TP
1.	*str-r1 ery-s2*	*str-r26 ery-r2*
2.	*str-r1 ery-s2*	*str-r25 ery-r2*
3.	*str-r9 ery-s2*	*str-r1 ery-r2*
4.	*str-r29 ery-s2*	*str-r1 ery-r2*

The two mutations of the last "cross" individually confer approximately the same level of resistance; consequently, transformants were screened for a phenotypic class whose level of resistance would be greater than the level conferred by *str-r1* (>6000 μg/ml streptomycin). Such a class might represent the doubly-marked recombinant type *str-r29-r1*. No phenotypic class of this type could be observed ($<1 \times 10^{-8}$) although the frequency of *ery-r2* transformations (3.5×10^{-3}) indicated that the recipient cells were physiologically competent. Similarly, in the third "cross" no phenotypic class of transformants could be observed with a level of resistance greater than that of *str-r1* ($<1 \times 10^{-8}$) although the frequency of *ery-r2* transformations was 1.6×10^{-4}. In the first two "crosses" trans-

formant classes having the level of resistance of cells containing the markers *str-r26* and *str-r25*, respectively, were obtained. No class having a greater phenotypic level of resistance than that conferred by either *str-r26* or *str-r25* alone was encountered ($<1 \times 10^{-8}$).

Although the phenotypic levels of resistance conferred by the individual markers used in the experiments cited above are not identical, the differences between them are not, in general, great enough to allow a clear phenotypic differentiation between the markers with the methods commonly employed. As has been pointed out previously, given the physiological variation in resistance known to occur in a culture of any mutant strain, complicated by the similarity of the maximum level of resistance of the average cells in the two cultures, identification of the two markers by phenotypic means becomes impracticable. Therefore, should a randomly isolated transformant from any of the experiments described above be genetically a doubly-marked strain, e.g. *str-r9-r1*, a TP prepared from this strain would be of no value since the transformant classes could not be phenotypically distinguished.

Additional evidence for the allelism of these markers is found in the observation that at least three others tested (*str-r25*, *str-r26* and *str-r29*) bear the same relationship to markers *str-r2* and *str-r3*. When strains containing either *str-r2* or *str-r3* were individually treated with either TP *str-r25*, TP *str-r26* or TP *str-r29*, only one class of transformants could be obtained (as determined by direct plating and velvet-replica technique), this class being indistinguishable from cells containing *str-r25*, *str-r26* or *str-r29* alone. When DNA preparations were made from randomly isolated transformants and then tested on the sensitive recipient strain, it was found that genetically as well, only one class was present, this class being identical to that found when the sensitive strain is treated with DNA containing either the marker *str-r26*, *str-r25* or *str-r29* alone (Table 2 C-H). Just as in the case of the *str-r1* marker, there was no evidence of markers *str-r2* or *str-r3* being present simultaneously with either markers *str-r25*, or *str-r26* or *str-r29*.

The simplest explanation is that all these markers (*str-r25*, *str-r26* and *str-r29*) represent different spontaneous mutations at the *str-r1* site. However, it must be pointed out that none of the experimental criteria used offers absolute proof that this is indeed the case. Conclusive evidence must await the mapping of these markers with respect to additional sites of mutation.

BRYAN (1961) has shown by similar analysis that a fifth marker, *str-r27*, occurs at the same site as that of the *str-r1* marker. It is of great interest to note that all markers which apparently fall at this site confer a greater level of resistance than is conferred by markers at the other two sites (*str-r2* and *str-r3*). Furthermore, the double recombinant type *str-r2-r3* has a level of resistance (ca. 4000 μg/ml) almost equal to the level of resistance of *str-r1* (6000 μg/ml). If *str-r1* can be shown unequivocally to be a multisite mutation, then there is a suggestive correlation between the length of the mutation and the level of resistance conferred.

Alleles of str-r2*:* Cells *str-r5 ery-s2* and *str-r28 ery-s2* were individually

treated with TP *str-r2 ery-r2* (Table 1 C, D). It was observed that if the recombinant classes *str-r2-r5* and *str-r28-r2* exist, they do not have a phenotype distinguishable from marker *str-r2* alone. It was therefore concluded that marker *str-r2* replaces both *str-r5* and *str-r28*, and that the three markers represent different spontaneous mutations at the same site. A fourth marker (*str-r4*) has been similarly shown (BRYAN 1961) to occur at this site. Three of the four markers have identical phenotypes (the exception is *str-r28*), although all confer a much lower level of resistance as compared to *str-r1* and its alleles.

All the markers at this site (*str-r2*, *str-r5*, *str-r28* and *str-r4*) interact with TP *str-r3* to produce the recombinant classes *str-r2-r3*, *str-r5-r3*, *str-r28-r3* and *str-r4-r3* respectively. Furthermore, all markers show the same order of magnitude of linkage to the *str-r3* marker. When the sensitive strain is treated with a DNA prepared from one of these recombinant types, there is about one doubly-marked transformant obtained (e.g. *str-r28-r3*) for every 40 singly-marked transformant (*str-r28* or *str-r3*). Since it has been observed that the degree of linkage (frequency of the doubly-marked class obtained when the sensitive strain is treated with the doubly-marked TP) varies in different experiments with a given DNA preparation,[4] it is not possible to make an absolute comparison of the linkage between *str-r3* and each of the markers cited. However, it is observed that the variations found in the degree of linkage between *str-r3* and each of the markers is no greater than the variation found in repeated tests of one preparation (e.g. *str-r3-r28*).

In each case cited above the recombinant class *str-r3 r*X (where X represents any one of the markers cited) shows a level of resistance far greater than the level of resistance conferred by either of the individual markers. However, the level of resistance of the doubly-marked recombinant classes is not the same in all cases. The combinations *str-r2-r3*, *str-r5-r3* and *str-r4-r3* confer a tolerance of up to 3–4000 μg/ml streptomycin. On the other hand, the combination *str-r28-r3* confers resistance to only about 500 μg streptomycin per ml. This observation coupled with the slightly different level of resistance conferred by marker *str-r28* alone (as compared to *str-r2*, *-r5* or *-r4*) suggested that marker *str-r28* represents a different mutation at the same site.

BRYAN (1961) has shown that an unlinked modifier can increase tenfold the level of resistance conferred by the *str-r2* marker, although this modifier does not alone confer any resistance to the cell. She has also shown that this enhancer (*en*) can increase the resistance conferred by markers *str-r4*, *str-r27* and *str-r1*. It seemed of interest then to determine whether the enhancer would affect all the alleles at this site. Cells *str-r28* and *str-r5* were treated with TP *en str-r2 ery-r2* and it was observed that the enhancer did increase the resistance of *str-r5* cells but could not modify the level of resistance of the *str-r28* cells (Table 3). Since it was possible that the *en str-r28* combination did not result in as high a level of resistance as the *en str-r2* combination, transformants were selected by

[4] Values of 1/20–1/60 have been obtained for the ratio of doubly-marked/singly-marked transformants, in different experiments with the same DNA preparation.

TABLE 3

Transformation of different str-r *markers by TP* en str-r2 ery-r2

Strain	Frequency of cells capable of resisting:* In presence of TP erythromycin (0.25 μg/ml)	In presence of TP streptomycin (1000 μg/ml)	In absence of TP erythromycin (0.25 μg/ml)	In absence of TP streptomycin (1000 μg/ml)
str-r5 ery-s2	2.5	1.58	$< 1 \times 10^{-9}$	1×10^{-9}
	1.0	1.0	..	5×10^{-8}
str-r2 ery-s2	1.0	1.5	..	1×10^{-7}
	1.0	0.9	..	6×10^{-6}
str-r28 ery-s2	6.15	$< 1 \times 10^{-9}$	..	8×10^{-7}
	1.3	3×10^{-7}	..	7.7×10^{-7}
	2.4	1×10^{-7} †	..	4.6×10^{-7} †
	4.2	1×10^{-7} †	..	3.0×10^{-7} †
			†	1.8×10^{-7} †
			†	4.3×10^{-7} †

* $\times 10^{-4}$ unless otherwise indicated.
† Same experiment—replicate tubes.

direct plating on 300 μg/ml, 500 μg/ml and 1000 μg/ml. The results at these three concentrations are identical to those represented for 1000 μg/ml in Table 3. Since the previous data presented indicate that *str-r28* occupies the same site as that of *str-r2*, *-r4* and *-r5*, then it follows that the enhancer is allele specific. That is to say, it does not indiscriminately modify all the alleles at a given site. Moreover, this observation supports the hypothesis that marker *str-r28* represents a different mutation at the same site as that of the *str-r2* marker. Differentiation by means of the enhancer can then serve as a valuable tool for differentiation between the alleles of this locus.

The site of the str-r36 *mutation:* The nitrous acid-induced mutation, *str-r36*, confers a relatively low level of resistance to streptomycin. At concentrations of streptomycin above 35 μg/ml, a significant fraction of *str-r36* mutants are incapable of forming colonies. This compares with a streptomycin concentration of 5 μg/ml, above which a significant fraction of sensitive wild-type (*str-s*X) cells fail to produce colonies. Transformation of the sensitive strain by TP containing the *str-r36* marker can be detected only when the competence of the recipient strain is high, so that the transformants can be distinguished from a background of spontaneous mutants arising from the feeble growth of an appreciable minority of sensitive cells plated at streptomycin concentrations from 20 to 30 μg/ml.

In addition to being able to demonstrate the transfer of the *str-r36* marker into the sensitive clone 3 strain, it has been possible to raise the level of resistance of a clone 3 strain already bearing the *str-r36* marker by transforming it with TP containing either the *str-r3*, *str-r2* or *str-r1* marker. The transformant classes thus obtained are capable of resisting ca. 1000, ca. 5000 and ca. 20,000 μg/ml streptomycin, respectively. While the latter class has a level of resistance indistinguishable from that conferred to clone 3 by the marker *str-r1* alone, the former two classes possess levels of resistance significantly higher than those con-

ferred respectively by markers *str-r3* (400 μg/ml) and *str-r2* (800 μg/ml) alone. On the basis of these results, it may be presumed that *str-r2* and *str-r3* can recombine with *str-r36* to produce, respectively, doubly-mutant *str-r2-r36* and *str-r3-r36* transformants.

Determination of the genetic constitutions of the transformants enhanced in their resistance in this way was accomplished by extracting DNA from them, and testing the DNA preparations on the sensitive clone 3 strain. The results are recorded in Table 4. Transformants were generally selected at three concentrations of streptomycin, and, in addition, colonies produced at the previous concentrations were isolated and tested for their maximum level of resistance.

The significantly greater number of colonies appearing at 25 μg streptomycin per ml than at higher streptomycin concentrations was presumptive evidence that more than one class of transformant was being induced in the sensitive strain. Further evidence came from the marked difference in size of the colonies appearing at 25 μg streptomycin per ml. Many of the colonies that finally developed were not visible at 24 hours of incubation, but were seen as small colonies at 48 hours. These late-appearing or small colonies generally prove to be incapable of resisting 100 μg/ml or higher concentrations of streptomycin; they

TABLE 4

A. *Treatment of sensitive strain with TPs obtained from* str-r36-r*X recombinant types*

TP‡	Expt. no.	Transformants ($\times 10^{-4}$)* capable of forming colonies in agar containing a streptomycin concentration of 25 μg/ml†	100 μg/ml	500 μg/ml	1000 μg/ml
str-r36 (TP *str-r3*)	1	1.55	0.83	$< 10^{-7}$	–
	2	4.7	1.85	3.0×10^{-6}	–
str-r36 (TP *str-r2*)	1	1.62	0.49	–	3.0×10^{-7}
	3	0.15	0.05	–	–
	4	3.65	0.40	–	$< 10^{-6}$
str-r36 (TP *str-r1*)	2	1.23	–	1.0	–
	4	1.68	–	0.33	–

B. *Recovery of distinct classes of transformants*

TP‡	Expt. no.	Colonies selected at streptomycin conc. (μg/ml) of	Total no. isolates tested§	No. of isolates capable of growing at a max. strep. conc. (μg/ml) of 25	100	1000	5000
str-r36 (TP *str-r3*)	1	25	8	6	2	0	0
		100	4	0	4	0	0
	2	500	7	0	0	7	0
str-r36 (TP *str-r2*)	1	25	8	6	2	0	0
		100	4	0	4	0	0
		1000	5	0	1	0	4
	4	25	12 large	2	10	0	0
			6 small	6	0	0	0
str-r36 (TP *str-r1*)	2	25	21 large	0	0	0	21
			80 small	79	0	0	1

* Unless otherwise indicated.
† In case of 25 μg streptomycin/ml, the figures shown are corrected, where necessary, for the small number of colonies appearing in the control plate (no TP).
‡ Nomenclature of TPs as in Table 2.
§ Unless otherwise indicated, isolates are randomly picked from agar containing the concentration of streptomycin indicated. In two of the experiments shown, a separate selection of large and small colonies was made.

correspond, in other words, to a class bearing the *str-r36* marker. The early-appearing or large colonies, on the other hand, generally correspond to a class possessing the other *str-r* marker in the DNA preparation.

From the results obtained by testing individually-isolated colonies, it is clear that the DNA extracted from a transformant presumably *str-r36-r3* in genotype does induce three distinct classes of recombinants in the sensitive strain; transformants corresponding to *str-r36*, *str-r3* and *str-r36-r3*. Similarly, three classes of recombinants, corresponding to *str-r36*, *str-r2* and *str-r36-r2*, are produced in the sensitive strain by DNA extract from the presumed *str-r36-r2* transformant. Finally, two classes of recombinants are produced in the sensitive strain by DNA extracted from the presumed *str-r36-r1* transformant, the two classes corresponding to *str-r36* and to *str-r1*, or *str-r36-r1*, since the phenotype of the latter two types cannot be distinguished. One may conclude therefore that *str-r36* is not allelic with either *str-r2*, *-r3* or *-r1*.

In the case of DNA *str-r36-r3* and DNA *str-r36-r2*, the production in the sensitive strain (*str-s36-s*X) of the doubly-transformed type (*str-r36-r*X) is a relatively rare event. It has never occurred with a frequency greater than one per 100 of the single type (*str-r36* + *str-r*X). This fact, and also the finding that the relative frequency of the doubly-transformed type has varied somewhat with different batches of recipient cells, suggests that the genetic integration of *str-r36* occurs independently of that of either *str-r3* or *str-r2*. In any case, it is evident that *str-r36* is certainly not as strongly linked to *str-r3* and *str-r2* as the latter two markers are to each other.

DISCUSSION

This analysis of 11 randomly isolated, spontaneous mutations has clearly demonstrated that the sites of spontaneous mutation are not randomly distributed in the DNA of pneumococcus. The results of the allelism tests can be summarized by a genetic map (Figure 1) in which the individual mutations have been represented as solid blocks. In addition, the probable location of a twelfth spontaneous mutation *str-r30*, as described by SCHAEFFER (1956), is included for the sake of completeness. For diagrammatic purposes it has arbitrarily been assumed that a mutation conferring a higher level of resistance to streptomycin is larger than one conferring less resistance.

Examination of the map leads to several conclusions. First, it is immediately obvious that there are more mutations than sites. Second, the distribution among the sites can be observed to follow a pattern. All the high level mutations (conferring resistance to 2000 μg/ml streptomycin or more) are found at one site (the site of the *str-r1* marker). At a second site (the site of the *str-r2* marker) there is a group the majority of which confer resistance to 300 μg/ml streptomycin, while the single spontaneous mutation found at the third site confers resistance to only 150 μg/ml. A fourth site, occupied by the only induced mutation examined, confers resistance to only 30 μg/ml streptomycin per ml. The distribution of 12 spontaneous mutations among only three sites begins to suggest a similarity to the finding of frequently mutable sites in bacteriophage

(BENZER 1955) and Salmonella (HARTMAN, LOPER and SERMAN 1960) but which have not previously been reported in pneumococcus. A series of such nonrecombinable (allelic) mutations having similar effects on the phenotype have been designated as a set (HARTMAN *et al.* 1960).

The exact size of the *str-r1* marker and its alleles has not been determined by these studies. It has been shown that there are two main possibilities. Either the ends of these markers overlap the other two sites, or else the ends of these markers lie very close to the other two sites. It is obvious that the two ends of any given marker can be any combination of these possibilities. Since there are only a limited number of linked sites available for mapping purposes, the exact size cannot be determined at the present time. Similarly, it cannot be ascertained if all the members of the set at the *str-r1* site are really identical in length. The absence of recombination between them is merely indicative of the fact they have in common certain regions; in reality, they might represent a series of overlapping multisite mutations of the type observed by LACKS and HOTCHKISS (1960). Preliminary evidence (ROTHEIM, unpublished) suggests that this is so; marker *str-r9* appears to be smaller than the other members of the set. Since this is the marker conferring the lowest level of resistance (among the members of this set), this again seems to confirm the observation that there is a correlation between the level of resistance conferred and the size of the mutation.

Because of the lack of information available concerning the relation of these markers to the actual steps in achieving resistance, it is impossible to talk in terms of functional units. However, the spontaneous mutations can be considered to be part of the streptomycin-resistance locus. This grouping of phenotypically similar mutations into one locus is an observation that has been made repeatedly in many other genetic systems (Salmonella, DEMEREC 1956; HARTMAN *et al.* 1960; bacteriophage T4, BENZER 1957; Neurospora, CASE and GILES 1960). Likewise, the differences observed between different mutations at this locus (differences in the level of resistance) parallel the secondary differences observed between mutations affecting the same function in Salmonella (YURA 1956; CLOWES, cited in DEMEREC 1956), bacteriophage (BENZER 1955; STREISINGER, MARTINELLO and WASSERMAN 1957), and Neurospora (GILES 1959; DE SERRES 1958). The primary function we can assign to the mutations in the case reported here is streptomycin resistance. Finally, there is a differentiation observed between the members of a set. There are five markers located at the site of the *str-r2* marker. Three of these (*str-r2*, *-r4* and *-r5*) confer approximately the same level of resistance (300 μg/ml streptomycin) and in combination with *str-r3* all produce a double recombinant type (e.g. *str-r2-r3*) which has a level of resistance of about 3–4000 μg/ml streptomycin. Furthermore, these three markers are all affected by an enhancer gene (BRYAN 1961) which increases the level of resistance tenfold. Marker *str-r30*, which we have not studied, appears to differ in at least one respect: it confers a very low level of resistance (25 μg/ml). Marker *str-r28*, however, differs in all three respects. First, it has a lower level of resistance. Second, the combination *str-r28-r3* gives a much lower level of resistance (ca. 500 μg streptomycin per ml). Third, the

enhancer gene has no effect on marker *str-r28*. These findings indicate that even at one site different mutational changes can occur, and the enhancer mutation can provide a useful tool for differentiating between the members of a locus. Indeed, BRYAN (1961) has shown that the enhancer affects at least two markers at the *str-r1* site (*str-r1* and *str-r27*). It is interesting to note that, should *str-r1* prove to be a multisite mutation, the specificity of the enhancer is such as not to affect all the markers "covered" by the multisite mutation while affecting the multisite mutation itself.

The site of the nitrous acid-induced mutation, *str-r36*, is apparently distant from the three sites of spontaneous mutation that have been discovered. It would be premature to conclude, however, a specificity in the action of this mutagen. An insufficient number of spontaneous mutations selected at low streptomycin concentrations have been analyzed to be sure that the sites of spontaneous mutation are separate from the sites of induced mutation. Only two facts bearing on this question are available. First, in our own experiments, as well as in those of SCHAEFFER (1956), we have found spontaneous mutations that confer resistance to only 20–30 μg streptomycin per ml. Secondly, the only spontaneous mutation of this type to have been studied, *str-r30*, was found by SCHAEFFER to be allelic with *str-r28* and *str-r29*, which we in turn have found to be members of the locus just described (Figure 1). While suggesting that spontaneous mutations conferring low levels of resistance occur within the same locus as spontaneous mutations conferring high levels of resistance, the evidence is insufficient to generalize.

While it is possible to state that the site of *str-r36* is not strongly linked to the sites of spontaneous mutation that have been described, it will require further investigation to determine whether the site of the induced mutation is transferred independently of the sites of spontaneous mutation during pneumococcal transformation. It is even possible that two mutations transferred independently of each other (i.e. *genetically* "unlinked") may be borne on the same molecule of DNA (i.e. *physically* "linked"). Physical and genetic experiments to elucidate this point are now being attempted.

SUMMARY

1. Eleven independently-derived spontaneous mutations to streptomycin resistance in Pneumococcus were found to be distributed among three linked sites of a transforming DNA molecule.

2. The distribution was observed to be non random in that there is a definite tendency for mutations conferring similar resistance to be found at the same site.

3. At any one site, more than one type of mutation has been shown to occur, and it has been possible to differentiate between the mutations by means of a modifier gene.

4. The site of a mutation induced by nitrous acid, which confers only a very low level of resistance to streptomycin, is not strongly linked to the sites of the spontaneous mutations.

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