GENETIC LINKAGE OF STREPTOMYCIN-DEPENDENCE AND RESISTANCE IN PNEUMOCOCCUS¹

AJIT K. MISHRA² AND ARNOLD W. RAVIN

Department of Biology, The University of Rochester, Rochester, N.Y.

Received January 26, 1966

A spontaneous mutation conferring dependence upon streptomycin for growth was found in a Group A strain of streptococcus, and was successfully transferred into pneumococcus (RAVIN and MISHRA 1965). Subsequently, a dependence-conferring mutation was discovered in a streptomycin-resistant strain of pneumococcus (MISHRA and RAVIN 1966). These discoveries were fortunate for several reasons. First of all, although streptomycin-dependence mutations are not at all rare in wild-type (streptomycin-sensitive) strains of many species of bacteria, they are practically nonexistent in the sensitive strain of pneumococcus (RAVIN and MISHRA 1965). Secondly, the genetic property of streptomycin-dependence lends itself to reciprocal transformations, for sensitive cells can be selected in the presence of a predominantly dependent population, and dependent cells can be selected in the presence of a predominantly sensitive population. Reciprocal transformations, in turn, make possible multi-factor crosses in which the complete variety of recombinant types can be selected. Finally, a number of streptomycin-resistance mutations have already been mapped in the pneumococcus (ROTHEIM and RAVIN 1961, 1964), so that the opportunity exists for determining the position of the dependence-conferring mutations in relation to the resistance-conferring mutations.

The results of a genetic investigation of the two dependence-conferring mutations, *str-d1* and *str-d2*, are presented in this report.

MATERIALS AND METHODS

Strains.—The streptomycin-sensitive (*str-s*) strain of pneumococcus used in these experiments was the capsule-deficient strain SIII-1 (EPHRUSSI-TAYLOR 1951). The streptomycin-resistance mutations used were *str-r1*, *str-r2*, *str-r3*, *str-r42*, *str-r48* and *str-r51*, which are genetically related to each other as shown in Figure 2. Mutations *str-r1*, *str-r2* and *str-r3* arose spontaneously in the SIII-1 strain and confer resistance to streptomycin concentrations up to 6000, 300 and 150 $\mu\text{g/ml}$, respectively (BRYAN 1961). Mutations *str-r51* (conferring resistance to $> 10,000 \mu\text{g/ml}$) and *str-r42* (conferring resistance to 30 $\mu\text{g/ml}$) both originated by secondary mutation in the single-step mutant *str-r41* (which confers resistance to 3000 $\mu\text{g/ml}$; ROTHEIM and RAVIN 1964). Mutation *str-r48* (conferring resistance to 150 $\mu\text{g/ml}$) arose as a secondary replacement of mutation *str-r36*. The streptomycin-dependent strain designated *str-d1* has an optimal requirement for 500–1000 $\mu\text{g/ml}$ streptomycin, and was obtained, as previously mentioned, by transfer of a single-

¹ The research described in this article was supported by Public Health Service Grant AI-00727.

² Present address: Department of Microbiology, University of Texas, Austin; on leave from Bose Institute, Calcutta 9, India.

step mutation that arose spontaneously in a Group A strain of streptococcus isolated from a human patient at the Strong Memorial Hospital. The dependent strain designated *str-d2* has an optimal streptomycin requirement like that of *str-d1*, but the latter strain resists higher concentrations of streptomycin less well than *str-d2*. The *str-d2* mutation arose in a strain already possessing the *str-r3* mutation, from which it was separated by recombination (MISHRA and RAVIN 1966). An erythromycin-resistant marker, *ery-r2* (conferring resistance to 1 $\mu\text{g/ml}$ erythromycin; GREEN 1959; RAVIN and IYER 1961), was introduced into the streptomycin-resistant and dependent strains to be used as donors. The *ery-r2* marker is integrated independently of *str-r* markers (RAVIN 1965).

Media and transformation: Media were the same as used previously (EPHRUSI-TAYLOR 1951; ROTHEIM and RAVIN 1961). The procedures used for the isolation of DNA and for the transformation of recipient strains are essentially similar to those described by ROTHEIM and RAVIN (1961). For transformation a saturating concentration of the appropriate DNA was added to samples of thawed recipient cells which had been previously made competent and stored at -30°C in a glycerol medium (Fox and HOTCHKISS 1957). The action of DNA was terminated by the addition of Mg^{++} -activated DNase after 10 to 20 minutes of incubation at 30°C . The DNA-treated cells were then allowed to express the acquired donor markers by one of the following procedures: (1) Direct selection—samples of the transforming mixture, after incubation at 37°C , were spread on the surface of selective agar media; (2) Delayed selection—immediately after the addition of DNase, samples from the transforming mixture were added to 10 ml of melted, nonselective agar in plates, mixed thoroughly, and when solidified, incubated at 37°C for a specified time period, and then overlaid with 10 ml of selective agar. Direct selection was the method usually employed, unless otherwise mentioned in the text or tables. After incubation of the plates, colonies were counted to determine the frequency of transformation. Streaks from these colonies were drawn on agar plates of the same kind as those in which the transformants had originally appeared. After the streaks had been permitted to grow, velvet replications (LEDERBERG and LEDERBERG 1952) were made onto a series of plates containing various concentrations of antibiotic.

Allelism tests. (1) *str-d1 as recipient:* The streptomycin-dependent strain was grown under conditions promoting competence and stored by freezing in the usual way. Streptomycin, at a concentration of 500 $\mu\text{g/ml}$, was necessarily in the medium at this time. After the competent cells were thawed, samples were transferred into a transforming medium containing the desired DNA. One sample (A) was transferred into a medium containing an optimal streptomycin concentration for the growth of the *str-d1* strain (500 $\mu\text{g/ml}$); another sample (B) was transferred into medium containing no streptomycin. In the latter, the streptomycin carried over with the competent cell inoculum was diluted below a concentration (10 $\mu\text{g/ml}$) which would allow the prolonged growth of dependent cells. However, this concentration would inhibit growth of sensitive cells (RAVIN and MISHRA 1965). Following a brief exposure of DNA and further incubation at 37°C , direct selection was carried out by plating each of the two cultures in media containing either 500 $\mu\text{g/ml}$ streptomycin and 0.25 $\mu\text{g/ml}$ erythromycin (S + E), or no antibiotic (plain agar or P). The former plating selects *ery-r2* transformants, whereas the latter selects for *str-s* or *str-r* transformants. For measurement of the total population at the same time, platings were also made in media containing 500 $\mu\text{g/ml}$ streptomycin (S). DNA was extracted from a sample of each of the different types of transformants produced in the *str-d1* strain, and tested on competent sensitive (*str-s*) cells to determine the genotypes of these transformants.

(2) *str-d1 or str-d2 as donors:* DNA extracted from *str-d1* cells was used to transform sensitive *str-s* cells and cells having a fairly low level of resistance such as the *str-r3* or *str-r42* strains. DNA from *str-d2* cells was used to transform sensitive and *str-d1* cells. Exposure of the recipients to DNA was carried out in the absence of streptomycin, and transformants were selected, after appropriate periods of phenotypic expression, by the direct or by the delayed selection procedure. Streptomycin-dependent transformants were selected in S. If the donor DNA also contained the *ery-r2* marker, E was used to select for *ery-r2* transformants. Transformants were isolated by the usual procedure, and the DNAs extracted from cultures of these transformants were tested on competent sensitive cells.

RESULTS

Transfer of streptomycin-dependence into the sensitive strain: When the *str-s ery-s* strain was treated with DNA from *str-d1 ery-s* cells, the number of transformants was at a maximum after 2 hours of growth following termination of exposure to DNA (Table 1). This period of phenotypic expression is also observed with several *str-r* markers (Fox 1959; GREEN 1959; ROTHEIM and RAVIN 1961; EPHRUSSI-TAYLOR 1962). The time necessary for phenotypic expression of the *str-d1* mutation was found to be the same either by the direct or delayed selection procedure. However, a drop in the number of *str-d1* transformants was observed after maximal expression was attained. Since expression occurred in the absence of streptomycin, prolongation of incubation prior to the streptomycin challenge would be expected to result in a loss of *str-d1* transformants. As shown in Figure 1, however, *str-d1* cells that have already expressed their dependent phenotype are capable of surviving (indeed, of growing) for at least an hour in the absence of streptomycin.

A total of 144 transformants selected in S were tested for phenotype, and all proved to be identical to *str-d1*. In other experiments, no transformants could be obtained by selecting at a streptomycin concentration (20 μ g/ml) just sufficient to prevent growth of the recipient *str-s* cells. The yield was poor when trans-

TABLE 1

Transfer of dependence into streptomycin-sensitive recipient cells

A. *str-s ery-s* cells $\times$ *str-d1 ery-s* DNA

Time allotted for phenotypic expression (hr)	Average No. transformants per 0.1 ml examined*			
	Direct plating in S		Delayed selection in S	
	DNA	No DNA	DNA	No DNA
0	0	0	0	0
1	120	0	18	0
2	387	0	393	0
3	147	0	353	0
4	46	0	62	0

B. *str-s ery-s* cells $\times$ *str-d1* (or *-d2*) *ery-r2* DNA

(Time allotted for phenotypic expression = 2 hr)

DNA		Average number transformants per 0.1 ml examined			<i>str-d/ery-r2</i>
		Expt. No.	E	S	
<i>str-d1</i>	<i>ery-r2</i>	1	519	146	0.29
		2	652	255	0.39
<i>str-d2</i>	<i>ery-r2</i>	2	933	188	0.20
no DNA		1	0	0	
		2	0	0	

* 144 transformants were tested for phenotype and all were found to be of donor type.

FIGURE 1.—Survival and growth of *str-d1* strain in the presence and absence of streptomycin. 0.05 ml of 1:10 dilution of a fresh turbid culture was added to two tubes containing 5 ml NS medium, one including 500 $\mu\text{g/ml}$ streptomycin and the other devoid of streptomycin. At 0, 1, 2, 3 and 4 hours of incubation at 37°C, samples of the culture were plated on agar media containing 500 $\mu\text{g/ml}$ streptomycin.

formants were selected at concentrations higher than 50 $\mu\text{g/ml}$ but less than 500 $\mu\text{g/ml}$ (S), but all such transformants proved to possess the *str-d1* phenotype. Hence, the *str-d1* mutation is transferred discretely. In experiments treating *str-s ery-s* recipients with DNA from *str-d1 ery-r2* donors, and allotting 2 hours for phenotypic expression (which is also sufficient for erythromycin resistance; GREEN 1959), the frequency of *str-d1* transformants relative to that of *ery-r2* transformants was found to vary between 0.29 and 0.39 (Table 1B).

By similar experiments it was found that the *str-d2* mutation is transferred

TABLE 2

Transformation of str-d1 ery-s cells with str-s ery-r2 DNA

Series	Time allotted for phenotypic expression (hr)	Factor increase of total population in time interval between platings	No. transformants/0.1 ml selected in		Transformants isolated from P		
			S+E	P	No. tested	<i>str-s ery-s</i>	<i>str-s ery-r2</i>
A	0	...	0	19	30	28	2
	1	4.2	415	9	21	21	0
	2	3.3	1,740	5	10	8	2
	3	3.7	7,080	4	8	8	0
	4	2.1	13,400	0	0	0	0
Total					69	65	4
B	0	...	0	102	84	80	4
	1	3.0	56	57	72	71	1
	2	0.9	85	11	24	22	2
	3	0.6	120	6	6	6	0
	4	0.5	110	3	12	2	10
Total					198	181	17

Transformation and phenotypic expression in presence of : 500 $\mu\text{g/ml}$ streptomycin=A series; ca. 10 $\mu\text{g/ml}$ streptomycin=B series. S+E=streptomycin 500 $\mu\text{g/ml}$ + erythromycin 0.25 $\mu\text{g/ml}$ (selects for *ery-r2*); P=no antibiotic (selects for *str-s*). Cells not treated with DNA produced no transformants in any of the above selective media.

discretely into *str-s* cells. Its efficiency of transformation relative to that of the *ery-r2* marker is about the same or only slightly lower than observed in the case of *str-d1* (Table 1B).

Transfer of streptomycin sensitivity into the str-d1 strain: The results are shown in Table 2. The greatest number of *str-s* transformants is observed when dependent cells are transformed in the presence of a suboptimal concentration of streptomycin and selection carried out immediately after exposure to DNA. The drop in the number of *str-s* transformants as a function of time of incubation following DNA exposure and prior to plating may be due to a killing effect of the small amount of streptomycin in the transforming medium.

In the dependent strain the expression of *ery-r2* transformants is at a maximum after one hour of growth in the presence of an optimal concentration of streptomycin, for at this time the *ery-r2* transformants begin to increase at the same rate as the population as a whole (quadrupling every hour). Even in the presence of a suboptimal concentration of streptomycin, however, *ery-r2* transformants are expressed. Under these conditions the maximum number of *ery-r2* transformants eventually detected is similar to the maximum number of *str-s* transformants found by selecting immediately after DNA treatment. This finding suggests, while not proving, that the *str-s* transformants arising in the dependent strain are fully integrated after a brief exposure to transforming DNA and that the maximum yield of such transformants is obtainable by selection at this time.

DNA extracted from a sensitive transformant was found to behave like DNA of wild-type *str-s* cells (see bottom line, Table 7); no trace of the streptomycin-dependence mutation, or of a streptomycin-resistance marker, was detected in it.

Transformation of the str-d1 strain by DNA from str-r mutants: Using DNA *str-r51 ery-r2* on competent *str-d1 ery-s* cells (Table 3), selection for *str-s* or *str-r51* transformants was carried out by plating in antibiotic-free agar (P). Agar containing S + E selected for *ery-r2* transformants; agar containing E alone selected for double transformants which had acquired the *ery-r2* marker and either the *str-r* or *str-s* marker. The vast majority of the transformants selected in the absence of antibiotic proved to be *str-r51* phenotypically. In the four experiments recorded in Table 3, a total of 604 transformants were isolated from antibiotic-free agar and tested phenotypically; 594 were identical to *str-r51*. In only one of these experiments, some sensitive cells were obtained (10 of 92 colonies tested). These sensitive cells may have arisen through back mutation at the *str-d1* site or by mutational acquisition of a modifier. As will be discussed below, sensitive cells may arise in *str-d1* cultures in either of these ways.

The maximal frequency of transformants selectable in P is obtained by plating immediately after exposure to DNA. Since this is the optimal condition for the recovery of *str-s* transformants in the *str-d1* strain, the rarity of the *str-s* type of transformant when *str-r51* DNA is used indicates that the site of the *str-r51* mutation is either very close to, or overlaps (i.e., is allelic to) the site of the *str-d1* mutation.

Another result of this experiment worth noting is that the frequency of *ery-r2*

TABLE 3

Transformation of str-d1 ery-s cells with str-r51 ery-r2 DNA

Expt. No.	Series	Time allotted for phenotypic expression (hr)	Factor increase of total population in time interval between platings	No. of transformants/0.1 ml selected in			Phenotypic characterization of transformants isolated from P			
				S+E	P	E	No. tested	<i>str-r51</i> <i>ery-s</i>	<i>str-r51</i> <i>ery-r2</i>	<i>str-s</i> <i>ery-s</i>
I	A	2	..	15.5	63.3	5.5	23	21	2	0
	B	2	..	1.5	4.5	0	9	8	1	0
II	A	0	..	0	260	..	72	61	11	0
		1	2.0	154	516	..	72	69	3	0
		2	2.3	367	842	..	72	68*	4	0
	B	0	..	0	76	..	72	54†	18	0
		1	2.0	26	188	..	72	64	8	0
		2	0.8	52	201	..	72	63	9	0
III	A	3	..	360	537	100	48	44	4	0
	B	3	..	6.5	51	0	44	28	6	10
IV	B	2	..	15	115	..	48	47	1	0

Series A and B as in Table 2. S+E=streptomycin 500 µg/ml + erythromycin 0.25 µg/ml (selects for *ery-r2*); P=no antibiotic (selects for *str-r* or *str-s*); E=erythromycin 0.25 µg/ml (selects for *str-r* [or *str-s*] *ery-r2*). Total population growth was determined by plating in S (500 µg/ml streptomycin). Cells not treated with DNA produced no transformants in any of the above selective media. DNAs were isolated from two of the transformants indicated by * and from one of the transformants indicated by †; these DNAs were tested with sensitive recipients; only *str-r51* and no *str-d1* transformants were produced.

transformants is always less than that of *str-r51* transformants, under conditions of maximal recovery of the two markers, respectively. In view of the findings described in the previous two sections, this would mean that the *str-r51* marker is not less efficient in replacing the *str-d1* mutation than is the wild-type homologue of the *str-d1* mutation. This result supports the conclusion that *str-r51* is allelic to the *str-d1* mutation.

When *str-d1 ery-s* cells were transformed with DNA containing the *ery-r2*

TABLE 4

Transformation of str-d1 ery-s cells with the DNAs from strains bearing an str-r marker plus the ery-r2 marker

Donor of DNA	P/E*	No. of transformants isolated from P†				<i>str</i> marker in transformants‡ belonging to phenotypic class	
		Total tested	Resistant like donor (a)	Dependent (b)	Sensitive (c)	(a)	(c)
<i>str-r51 ery-r2</i> §	1.5-4.1	144	144	0	0	<i>str-r51</i>	
<i>str-r1 ery-r2</i>	1.5	106	106	0	0	<i>str-r1</i>	
<i>str-r2 ery-r2</i>	3.7-7.7	120	120	0	0	<i>str-r2</i>	
<i>str-r3 ery-r2</i>	1.3-2.5	96	96	0	0	<i>str-r3</i>	
<i>str-r42 ery-r2</i>	<0.008	..	..	..	..		
<i>str-r48 ery-r2</i>	0.013	40	1	0	39	<i>str-r48</i>	<i>str-s</i>

* Ratio of number transformants selected in P to number of transformants selected in E, calculated as described in text. Variation indicates extremes of range observed in separate experiments.

† Selected by plating in P immediately after reaction with DNA. Since transformants obtained in the A and B series gave similar results, the data are summed in the table. In the case of DNA *str-r43 ery-r2*, transformants selected 2 hr after DNA treatment are included.

‡ Determined by extraction of DNAs from two or more representatives of each phenotypic class and test of DNAs on sensitive cells, as shown in Table 5.

§ Included in Table 3.

marker plus some *str-r* marker other than *str-r51*, selection was carried out as described above. The principal results are summarized in Table 4. The number of transformants capable of growing in the absence of streptomycin relative to the number of *ery-r2* transformants varied according to the *str-r* marker involved. Like *str-r51*, the *str-r1*, *str-r2* and *str-r3* markers produce streptomycin-independent transformants relatively efficiently. Moreover, none of these transformants proved to have the streptomycin-sensitivity characteristic of the wild-type strain. Rather they were streptomycin-resistant, and DNAs obtained from several such transformants were found to possess the *str-r* marker of the original donor employed. DNAs bearing the *str-r42* and *str-r48* markers, on the other hand, are inefficient producers of streptomycin-independent transformants. Indeed, no such transformants were obtained with DNA bearing the *str-r42* marker. Of the few (40) such transformants obtained with the *str-r48* marker, most (39) proved to be wild-type in their sensitivity to streptomycin.

Some of the streptomycin-resistant transformants produced from *str-d1* recipients were not phenotypically identical to the donor strain. This was observed when the donor marker was *str-r2* or *str-r3*. Some of these transformants resisted streptomycin less well than the donor strain, others resisted streptomycin somewhat better. DNAs were extracted from each of these transformant types, including the type phenotypically identical to the donor, and they were used to transform sensitive cells. Typical results obtained with DNAs from transformants produced by the *str-r2* donor are given in Table 5, although similar results were obtained in the case of the *str-r3* donor. The results showed that in each of the transformants the donor *str-r* marker was present and the recipient *str-d1* marker was absent. In transformants less resistant than the donor, a modifier (*su*), which lowers the level of resistance conferred by the *str-r2* mutation, is postulated to be

TABLE 5

Transformation of sensitive recipients by DNAs derived from transformants obtained by treating str-d1 ery-s cells with str-r2 ery-r2 DNA

Donor of DNA	Resistance level of donor* ($\mu\text{g/ml}$ streptomycin)	No. transformants/ 0.1 ml selected in $\mu\text{g/ml}$ streptomycin		No. transformants isolated from 20 $\mu\text{g/ml}$ streptomycin capable of growing in $\mu\text{g/ml}$ streptomycin					Presumptive genotype of donor‡
				300 but not 500 (<i>str-r2</i> type)			500 but not 0 (<i>str-d1</i> type)		
		20	500	Tested	35 but not higher	500			
No. 22	35	1160	0	96	0	96	0	0	<i>str-r2-su</i>
No. 44	500	1190	52†	96	0	92	4	0	<i>str-r2-en</i>
No. 122	300	1230	0	96	0	96	0	0	<i>str-r2</i>
Control:									
<i>str-r2 ery-r2</i>	300	2670	0	48	0	47	1	0	<i>str-r2</i>
No DNA	...	0	0	...	...	...	...	...	

* Maxima in all cases, except No. 44, the maximum level of resistance of which lies somewhere between 500 and 2000 $\mu\text{g/ml}$ streptomycin.

† 96 of these transformants were isolated, and all were found to be capable of growing in the absence of streptomycin and of resisting at least 500 $\mu\text{g/ml}$ streptomycin.

‡ *su* = suppressing modifier; *en* = enhancing modifier.

present also. In transformants more resistant than the donor, an enhancing modifier (*en*) is postulated to be present.

The source of the modifiers is indicated by the control experiment, which is the transformation of sensitive cells by the same preparation of *str-r2 ery-r2* DNA as used originally on *str-d1* cells. The vast majority of the streptomycin-resistant transformants produced were like *str-r2*. The data indicate, therefore, that the source of the modifiers is in the *str-d1* population.

Transformation of str-r mutants by DNA from str-d1 cells: When *str-r3 ery-s* cells were transformed using *str-d1 ery-r2* DNA, three types of transformants were obtained depending upon the medium in which selection was carried out (Table 6). All transformants selected in S (58/58) proved to be dependent like *str-d1*. A few (5/58) were double transformants (*str-d1 ery-r2*), like all of those selected in S + E. All of those selected in E were *str-r3* cells that had integrated only the *ery-r2* marker. The efficiency of replacement of the *str-r3* marker by the *str-d1* mutation, relative to the integration of the *ery-r2* marker (i.e., *str-d1/ery-r2*), can be calculated from this experiment to be equal to 0.30. The genotypes of the transformants were confirmed by extracting DNA from a number of them, and using the DNA to transform sensitive cells.

Using *str-r42 ery-s* cells as recipients and *str-d1 ery-r2* DNA, very few transformants arose in S compared to transformants arising in E. The transformants arising in S, when isolated and tested, proved to be of the *str-d1* type. DNAs were extracted from two such transformants and, when tested on sensitive cells, produced only the *str-d1* class of transformants, even though transformants were selected at a streptomycin concentration (10 µg/ml) at which *str-r42* cells can be recovered. Most of the transformants arising in E were *str-r42 ery-r2* phenotypically and genotypically, although a few streptomycin-sensitive transformants were found among the *ery-r2* transformants. These findings indicate that the *str-d1* marker replaces the *str-r42* mutation, albeit with a very low frequency (*str-d1/ery-r2* = 0.0014 to 0.0035), and more rarely recombinations occur between the two mutations to produce *str-s* cells.

Additional crosses: DNA from *str-d2 ery-r2* cells was used to transform *str-d1 ery-s* cells. Transformants selected in P were fewer than transformants selected in S + E. The transformants arising in the absence of streptomycin, however, proved to be streptomycin-sensitive.

Reversions of the streptomycin-dependent strain: Different types of reversions were found in the *str-d1* strain, depending on the manner in which they were selected. In one method of selection, *str-d1* cells were grown in an optimal concentration of streptomycin and then samples were plated in media lacking streptomycin. The streptomycin carried over from the culture medium into the plates was diluted so that the final concentration of streptomycin was no greater than 5 to 10 µg/ml. By this method (I), about 0.2 to 2.0×10^{-4} of the plated cells were capable of giving rise to colonies, which were heterogeneous in size at the time of counting. The other procedure (II) consisted in growing *str-d1* cells in an optimal concentration of streptomycin, centrifuging and washing the cells, and resuspending them in medium devoid of streptomycin. Samples of these cells were

TABLE 6
Transformation of str-r ery-s cells with str-d1 ery-r2 DNA

Recipient	Expt. No.	No. transformants/ 0.1 ml selected in			Phenotypes of transformants selected in			Genotypes of transformants selected in*		
		E	S	S+E	E	S	S+E	E	S	S+E
<i>str-r3 ery-s</i>		740	222	33	60/60 = <i>str-r3</i> <i>ery-r2</i>	53/58 = <i>str-d1</i> <i>ery-s</i> ; 5/58 = <i>str-d1</i> <i>ery-r2</i>	60/60 = <i>str-d1</i> <i>ery-r2</i>	<i>str-r3</i> <i>ery-r2</i>	<i>str-d1</i> <i>ery-s</i> ; <i>str-d1</i> <i>ery-r2</i>	<i>str-d1</i> <i>ery-r2</i>
<i>str-r42 ery-s</i>	1	363	0.5	0	not checked	2/2 = <i>str-d1</i> <i>ery-s</i>		not checked	<i>str-d1</i> <i>ery-s</i>	
	2	7630	29	not checked	462/468 = <i>str-r42</i> <i>ery-r2</i> ; 6/468 = <i>str-s</i> <i>ery-r2</i>	12/12 = <i>str-d1</i> <i>ery-s</i>		<i>str-r42</i> <i>ery-r2</i> ; <i>str-s</i> <i>ery-r2</i>	not checked	

* Genotypes determined by extracting DNA from two or more representatives of each phenotypic class and testing the DNAs on sensitive recipient cells.

TABLE 7
The phenotypes and genotypes of different kinds of revertants arising in the str-d1 strain

Revertant (or control) strain	Method of selecting revertant*	Range of streptomycin concentrations permitting 100% plating efficiency ($\mu\text{g/ml}$)	Doubling time (min) of strain in presence of $\mu\text{g/ml}$ streptomycin		Selectable transformant types produced by exposure of	Genotype of strain
			0	500	<i>str-s</i> cells to DNA of strain indicated	
Revertants						
PR 12	I	30-500	...	..	predominantly <i>str-d1</i> ; 2% PR 12†	<i>str-d1-su_a</i>
PR 14	I	5-100	...	..	predominantly <i>str-d1</i> ; 6% PR 12†	<i>str-d1-su_a-su_b</i>
FR 1	II	0-1	31	∞	no <i>str-d1</i> or <i>str-r</i>	<i>str-s</i>
FR 2	II	0-5	32	∞	<i>str-d1</i>	<i>str-d1-su_c</i>
Controls						
<i>str-d1</i>	..	500-1000	∞	31	<i>str-d1</i> , <1% PR 12	<i>str-d1</i>
<i>str-s</i>	..	0-1	28-32	∞	no <i>str-d1</i> or <i>str-r</i>	<i>str-s</i>

* Procedure I consists of plating in presence of 5-10 $\mu\text{g/ml}$ streptomycin; procedure II of plating in absence of streptomycin.

† Probability of production of *str-d1* transformants relative to that of *ery-r2* transformants cannot be greater than 10^{-4} .

‡ DNAs of some of these rare transformants were extracted and found to behave like DNA of PR 12 on *str-s* cells.

then plated in medium lacking streptomycin. By this method, about 10^{-8} of the plated cells were capable of giving rise to visible colonies.

Colonies obtained by each of the two procedures were isolated and subjected to further phenotypic and genotypic analysis. The results are summarized in Table 7. At least four kinds of revertants have been distinguished. Two types are partial revertants (PR); these are represented by PR 12 and PR 14, the optimal streptomycin concentrations for which are distinguishable from each other and less than that of *str-d1*. These two types each contain the original *str-d1* marker plus at least one suppressing modifier, *su*. The data are consistent with the interpretation that PR 14 and PR 12 share a common suppressor, *su_a*, and that PR 14 contains a second modifier, *su_b*. The other two kinds of revertants are full revertants (FR) in the sense that both can grow at a wild-type (*str-s*) rate in the absence of streptomycin. However, FR 2 has a very slight resistance to streptomycin, while FR 1 is apparently identical to the *str-s* strain. Corresponding to this phenotypic difference is a genotypic difference between FR 1 and FR 2. The latter contains the *str-d1* mutation plus a suppressor different from either *su_a* or *su_b*, and hence termed *su_c*. FR 1 is genetically *str-s*, as no *str-d1* or *str-r* mutation can be detected in DNA extracted from this strain.

Although no attempt was made to obtain an accurate estimate of the frequency of reversion to the FR 1 type, it was observed that one third of the revertants obtained by selection method II exhibited the phenotype of FR 1.

DISCUSSION

The *str-d1* mutation, of spontaneous origin in a Group A streptococcal strain and transferred into pneumococcus, maps within the complex locus in which streptomycin resistance (*str-r*) mutations arise. Moreover, like all of the *str-r* mutations which confer high levels of resistance (i.e., 100% plating efficiency at a streptomycin concentration exceeding 500 $\mu\text{g/ml}$), *str-d1* behaves as a multi-site mutation. The data we have reported show that *str-d1* behaves as an allele of four *str-r* mutations: *str-r2*, *-r3*, *-r1* and *-r51*. The former two are capable of recombining with each other, while the latter two do not recombine with the others (ROTHEIM and RAVIN 1964). The *str-d1* marker has been shown in the present studies to be replaceable by the *str-r2*, *-r3*, *-r1* and *-r51* mutations, while recombinations leading to *str-s* genotypes occur extremely rarely, if at all (certainly with a frequency less than 10^{-2} *ery-r* transformations). The reciprocal process, namely, replacement by the *str-d1* marker, has been shown to occur in crosses in which *str-r3* and *str-r42* cells have served as recipients. In addition, streptomycin-sensitive transformants have been obtained in crosses involving the *str-d1* marker and either the *str-r42* and *str-r48* markers. Thus, the data are consistent with the genetic map shown in Figure 2: in this map, the *str-d1* mutation "covers" the *str-r2* and *-r3* mutations, is in turn "covered" by the *str-r1* and *str-r51* mutations, and fails to "cover" (or be "covered") by the *str-r42* and *str-r48* mutations.

The *str-d2* mutation, which arose spontaneously in the *str-r3* strain of pneumococcus, has not been as extensively mapped as the *str-d1* mutation. Because

FIGURE 2.—The location of the *str-d1* mutation in the streptomycin resistance locus of pneumococcus. Map based on previous findings (ROTHEIM and RAVIN 1964, 1965) in addition to results described in this report. The various mutations are drawn to indicate, by their relative lengths, the approximate levels of streptomycin resistance they confer. The distances between the sites of mutation do not accurately reflect the frequencies of recombination between them, except that the following rules are observed: (1) mutations occupying sites that do not overlap are capable of recombining; (2) mutations occupying overlapping sites fail to recombine. Differently numbered *str-r* mutations are of independent origin. The designation "*ex-r*#" means that the mutation arose as a secondary mutation in the mutant strain *str-r*#. Assignment of the position of the *str-d2* mutation is tentative.

streptomycin-sensitive transformants are recoverable in a cross between the two dependent strains, the *str-d1* and *-d2* mutations apparently do not overlap.

The *str-d1* and *-d2* mutations are transferred discretely into *str-s* recipients. The efficiency of recovering *str-d1* transformants from *str-s* recipients, relative to the integration efficiency of the *ery-r2* marker, has a value of about 0.31 which is slightly lower than that (1.0) observed for the most efficiently integrated *str-r* markers (ROTHEIM and RAVIN 1964; RAVIN 1965). It is possible that we may not be recovering all of the *str-d1* transformants because expression of the dependent phenotype must take place in a streptomycin-free medium, which is eventually lethal to dependent transformants. The absence of streptomycin may be more toxic to a newly arising transformant than to a streptomycin-dependent cell being transferred from an environment containing streptomycin to one without it, for in the latter case streptomycin previously bound by the dependent cells would insure continued growth for a brief period (Figure 1). This possibility would gain support if our preliminary findings are confirmed that the replacement of the *d1* mutation by its wild-type allele has a relative efficiency of 1.0. Other workers (EPHRUSSI-TAYLOR, SICARD and KAMEN 1965; LACKS 1965) have found that the integration efficiency at a given DNA site is usually the same in reciprocal crosses. It appears, therefore, that we may be losing at most 70% of the dependent transformants in the treatment of sensitive cells with *str-d1* DNA. The *str-d2* mutation is transferred into *str-s* recipients with about the same efficiency (Table 1).

BALASSA and GABOR (1964; GABOR 1965) have performed transformations with a streptomycin-dependence mutation in *Rhizobium*. This mutation is very poorly

transferred into streptomycin-sensitive recipients, and some of the dependent transformants are not phenotypically identical to the donor strain. These findings suggested that the mutation they were dealing with was multi-site in nature (GABOR 1965), but mapping studies were not done showing that the mutation fails to recombine with two or more recombinable sites. Indeed, because of the low frequency of transformation and the relatively long period of exposure of recipients to transforming DNA (24 hours or more), the differences between the dependent transformants and the dependent donor may be ascribable to modifier mutations in the sensitive recipient population.

In fact, like the modifier mutations which enhance or suppress the action of *str-r* mutations (BRYAN 1961; ROTHEIM and RAVIN 1961; RAVIN and DE SA 1964), modifiers arise spontaneously that affect the expression of streptomycin-dependence. The ones described in this report act like suppressors, for they either permit strains carrying the *str-d1* mutation to grow in the presence of lower concentrations of streptomycin than the *str-d1* mutation would allow by itself, or they allow growth in the absence of streptomycin. Indeed, in the case of the latter type of modifier (*su_c*), the rate of growth in the absence of streptomycin is indistinguishable from that of the wild-type, streptomycin-sensitive strain. The modifiers of the *str-d1* mutation may not be the same, however, as the modifiers of the *str-r2* and *-r3* mutations discovered in the *str-d1* recipient population during its transformation by DNA containing either the *str-r2* or *-r3* marker. This caution is suggested by the observation that a much larger fraction of the *str-r2* or *-r3* transformants are found to possess the modifier than can be accounted for by the frequency of "modified dependents" in the *str-d1* population.

One of the more interesting findings, however, is that the *str-d1* mutation is capable of reverting to a state indistinguishable from the *str-s* allele. That is to say, some revertants (like FR 1) in the *str-d1* strain are not only indistinguishable by a number of phenotypic criteria from the *str-s* wild-type strain, but they show no evidence, by test of genetic recombination, of possessing the *str-d1* mutation. The sensitivity-conferring locus in such revertants may not, of course, be identical in nucleotide sequence to the wild-type locus. If, however, a suppressor were present in such revertants, it would be one so tightly linked to the *str-d1* mutation that their separation by recombination could not be detected by the fine resolving power of transformation, and in this regard it would be more tightly linked than any of the three known suppressors. It is more reasonable to assume that back mutations at the site of the *str-d1* mutation are the cause of the FR 1 type of revertant. The occurrence of back mutations would be difficult to reconcile with the notion that *str-d1*, like all other multi-site mutations, is a deletion of a segment within the *str* locus of the pneumococcal genome. However, it may be necessary to recognize a class of multi-site mutations that are in fact capable of back mutation (SUYAMA, MUNKRES and WOODWARD 1959; ISHIKAWA 1962; ROTHEIM and RAVIN 1964).

The phenotypic expression of streptomycin-dependence in sensitive recipients, and of sensitivity in dependent recipients, merit discussion. Dependence is expressed with the same delay characteristic of a number of streptomycin-resistance

markers (FOX 1959; GREEN 1959; EPHRUSSI-TAYLOR 1962). It is likely, therefore, that dependence is expressed in sensitive recipients by the same two-stage process observed in the case of streptomycin-resistance (EPHRUSSI-TAYLOR 1962) and erythromycin-resistance (IYER 1962). When a sensitive recipient acquires a donor *str-r* (or *ery-r*) marker, the first phenotypic change, which is expressed early, is an ability to survive the selective streptomycin challenge. Such transformants cannot yet multiply in the presence of the selective streptomycin concentration; it is the development of this ability which requires one to two hours of growth in the absence of streptomycin. On the basis of the SPOTTS and STANIER (1961) hypothesis that streptomycin-resistance mutations make streptomycin-resistant ribosomes, EPHRUSSI-TAYLOR (1962) proposed that the two stages in the development of streptomycin-resistance (ability to survive followed by ability to multiply in the presence of streptomycin) depend on the relative proportions of streptomycin-sensitive and -resistant ribosomes in the recipient which has just acquired the *str-r* mutation. When the majority of its ribosomes are of the sensitive type and only a few are of the resistant type, the bacterium can survive but not multiply in the presence of streptomycin. Presumably the few resistant ribosomes cannot compete efficiently with the sensitive ribosomes, in a streptomycin medium, to permit the continued synthesis of resistant ribosomes. However, in the absence of streptomycin, streptomycin-resistant ribosomes can be made, and when the proportion of resistant ribosomes becomes large enough, the power to multiply in a streptomycin medium becomes manifest. This hypothesis of the expression of streptomycin-resistance in transformed cells seems highly plausible in the light of recent biochemical studies on the streptomycin-resistance of ribosomes from streptomycin-resistant strains (FLAKS *et al.* 1962; DAVIES 1964). In fact, *in vitro* protein synthesis by ribosomes from streptomycin-sensitive pneumococci has been shown to be more sensitive to streptomycin than similar synthesis performed by ribosomes from several resistant mutants discussed in this report (*str-r42*, *-r48*, *-r2*, and *-r51*; STUART, unpublished).

We suggest that dependence also arises by the same two-stage process as for resistance, but that the second stage is manifested in a requirement for streptomycin to maintain viability. This would, of course, explain why dependent cells eventually die in the absence of streptomycin (Figure 1) and why the number of dependent transformants arising in a sensitive population at first increases and then decreases after the second stage of the phenotypic change is fully expressed (Table 1).

The ribosomal hypothesis does not make any necessary prediction about the expression of the ability to multiply in a streptomycin-free medium, arising by transformation in a dependent strain. One might assume that this ability would also be delayed in its expression because of the need to replace a predominantly dependent population of ribosomes by a predominantly sensitive (or resistant) population. Our results are not inconsistent with this assumption. In the transformation of dependent cells by sensitive DNA, the recovery of transformants capable of growing in streptomycin-free agar is maximal by selection immediately after the brief (15 minute) exposure to transforming DNA (Table 2). This

experiment was complicated by the loss of sensitive transformants upon continued incubation in the medium in which exposure to DNA occurred; this medium contained a small amount of streptomycin which had been carried over with the inoculum of competent dependent cells into the transforming mixture. (We were not successful in diluting the competent culture of its streptomycin without causing loss of competence.) The argument that the maximum yield of sensitive transformants had been recovered by selecting immediately after exposure to DNA was based on an estimate calculated from the number of erythromycin-resistant transformants obtained in the same experiment. Fortunately, there is no such complication when dependent cells are treated with resistant DNA (Table 3). In this case, there is no loss of resistant transformants upon continued exposure to streptomycin in the transforming mixture. The number of resistant transformants, selected in streptomycin-free agar, increases at the same rate as the population as a whole, starting immediately after exposure to DNA. This rapid expression of the ability to multiply at a normal rate indefinitely in the absence of streptomycin may be explained as follows. Preformed dependent ribosomes continue to make protein for an hour or so in an environment free of streptomycin as a result of the binding of streptomycin acquired by previous growth in the presence of the antibiotic. During this period of time, nondependent ribosomes are manufactured and attain a number capable of continuing protein synthesis without diminution of the growth rate.

The authors acknowledge with pleasure the expert technical assistance of Miss RUTH MARY HOHMAN and the help of Mrs. LINDA FRIEDMAN, who performed the experiments with the *str-r48* mutation.

SUMMARY

A mutation conferring dependence on streptomycin (*str-d1*), which arose originally in a streptococcal strain and was transferred into pneumococcus, maps as a multi-site mutation within the locus of the pneumococcal genome in which streptomycin-resistance (*str-r*) mutations occur. Another dependence-conferring mutation (*str-d2*), which arose in a pneumococcus already bearing an *str-r* mutation, is closely-linked to but at a separate site from that of *str-d1*.—The mutations are transferred discretely into streptomycin-sensitive recipients. The reverse transformation, dependence to sensitivity, is also possible, and the efficiency of replacement of the dependent mutation by the sensitivity-conferring allele is quite high.—When dependent cells are transformed by DNA from either sensitive or resistant cells, the ability to multiply in the absence of streptomycin is expressed very rapidly, and is in sharp contrast to the rate of development of the ability to multiply in the presence of streptomycin when resistant or dependent transformants are being produced in a sensitive strain. This finding is explained by the probable continued binding of streptomycin to dependent ribosomes, after transfer to a streptomycin-free environment.—The dependent *str-d1* strain may undergo spontaneous mutation at a number of different sites, resulting in an altered phenotype. Two different suppressor mutations have been distinguished which result in altered patterns of dependence. Another suppressor mutation has

been discovered which confers an ability to grow in the absence of streptomycin along with a slight resistance to the antibiotic. In addition, the dependent strain is capable of reverting to a phenotypic state indistinguishable from that of the wild-type (*str-s*) population. The DNA of this reverted strain shows no evidence of containing the original *str-d1* mutation, and behaves like that of the *str-s* strain.

LITERATURE CITED

- BALASSA, R., and M. GABOR, 1964 Transformation of streptomycin markers in rough strains of *Rhizobium lupini*. *Acta Microbiol. Hung.* **11**: 329-339.
- BRYAN, B. E., 1961 Genetic modifiers of streptomycin resistance in pneumococcus. *J. Bacteriol.* **82**: 461-470.
- DAVIES, J. E., 1964 Studies on the ribosomes of streptomycin-sensitive and resistant strains of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.* **51**: 659-664.
- EPHRUSSI-TAYLOR, H. E., 1951 Transformations allogenes du pneumocoque. *Exptl. Cell Research* **2**: 589-607. — 1962 Appearance of streptomycin resistance following the uptake of transforming deoxyribonucleic acid. *Nature* **196**: 748-752.
- EPHRUSSI-TAYLOR, H. E., A. M. SICARD, and R. KAMEN, 1965 Genetic recombination in DNA-induced transformation of pneumococcus. I. The problem of relative efficiency of transforming factors. *Genetics* **51**: 455-475.
- FLAKS, J. G., E. C. COX, M. L. WITTING, and J. R. WHITE, 1962 Polypeptide synthesis with ribosomes from streptomycin resistant and dependent *E. coli*. *Biochem. Biophys. Res. Comm.* **7**: 390-393.
- FOX, M. S., 1959 Phenotypic expression of a genetic property introduced by deoxyribonucleate. **42**: 737-748.
- FOX, M. S., and R. D. HOTCHKISS, 1957 Initiation of bacterial transformation. *Nature* **179**: 1322-1325.
- GABOR, M., 1965 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. *Genetics* **52**: 905-913.
- GREEN, D. McD., 1959 A host specific variation affecting relative frequency of transformation of two markers in pneumococcus. *Exptl. Cell Research* **18**: 466-480.
- ISHIKAWA, T., 1962 Genetic studies of *ad-8* mutants in *Neurospora crassa*. I. Genetic fine structure of the *ad-8* locus. *Genetics* **47**: 1147-1161.
- IYER, V. N., 1962 Application of the membrane filter for the quantitative study of transformations with special reference to phenotypic expression of an erythromycin resistance mutation. *J. Bacteriol.* **84**: 326-330.
- LACKS, S., 1965 Genetic recombination in pneumococcus. *Proc. Symposium on Mutational Process, Czechoslovak Academy of Sciences, Prague* (In press).
- LEDERBERG, J., and E. LEDERBERG, 1952 Replica plating and indirect selection of bacterial mutants. *J. Bacteriol.* **63**: 399-406.
- MISHRA, A. K., and A. W. RAVIN, 1966 Occurrence of streptomycin-dependent mutations in a streptomycin-resistant culture of pneumococcus. *J. Bacteriol.* **91**: 1389-1390.
- RAVIN, A. W., 1965 Intergenic and intragenic recombination during DNA-induced transformation. *Proc. Symposium on Mutational Process, Czechoslovak Academy of Sciences, Prague* (In press).
- RAVIN, A. W., and J. D. H. DE SA, 1964 Genetic linkage of mutational sites affecting similar characters in pneumococcus and streptococcus. *J. Bacteriol.* **87**: 86-96.

- RAVIN, A. W., and V. N. IYER, 1961 The genetic relationship and phenotypic expression of mutations endowing pneumococcus with resistance to erythromycin. *J. Gen. Microbiol.* **26**: 277-302.
- RAVIN, A. W., and A. K. MISHRA, 1965 Relative frequencies of different kinds of spontaneous and induced mutants of pneumococci and streptococci capable of growth in the presence of streptomycin. *J. Bacteriol.* **90**: 1161-1173.
- ROTHEIM, M. B., and A. W. RAVIN, 1961 The mapping of loci affecting streptomycin resistance in pneumococcus. *Genetics* **46**: 1619-1634. — 1964 Sites of breakage in the DNA molecule as determined by recombination analysis of streptomycin resistance mutations in pneumococcus. *Proc. Natl. Acad. Sci. U.S.* **41**: 344-354. — 1965 Low frequency of co-integration of two linked streptomycin resistance markers in pneumococcus. *Proc. Natl. Acad. Sci. U.S.* **54**: 1327-1332.
- SPOTTS, C. R., and R. Y. STANIER, 1961 The mechanism of streptomycin action on bacteria: a unitary hypothesis. *Nature* **192**: 633.
- SUYAMA, Y., K. MUNKRES, and V. W. WOODWARD, 1959 Genetic analysis of the *pyr-3* locus of *Neurospora crassa*: The bearing of recombination and gene conversion upon intra-allelic linearity. *Genetica* **30**: 293-311.