INTEGRATION AND EXPRESSION OF DIFFERENT LENGTHS OF DNA DURING THE TRANSFORMATION OF PNEUMOCOCCUS TO ERYTHROMYCIN RESISTANCE¹

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TRANSFORMATION in bacteria has provided us with a means by which mutations in genetically determinative molecules of deoxyribonucleic acid (DNA) can be ordered or mapped, and in recent years this has been attempted both in pneumococcus (LACKS and HOTCHKISS 1960; ROTHEIM and RAVIN 1961) and in *Bacillus subtilis* (EPHRATI-ELIZUR, SRINIVASAN and ZAMENHOF 1961; ANAGNOSTOPOULUS and CRAWFORD 1961). The mapping of DNA molecules is subject to certain uncertainties (LACKS and HOTCHKISS 1960; RAVIN and IYER 1961, 1962; EPHRATI-ELIZUR, SRINIVASAN and ZAMENHOF 1961), for it is based on the frequency with which recombinant classes of transformed bacteria are observed; the recovery of these recombinants depends not only upon the use of appropriate selective techniques, but also upon the successful completion of a series of stages in the host bacterium that has made an effective contact with a transforming DNA molecule. The outcome of mixing a transformable population of bacteria with transforming DNA depends on (a) the irreversible fixation of the DNA by the bacterium, (b) the integration of the specificity of *part* of the donor DNA molecule into the genome of the recipient or its progeny and (c) the expression of the phenotype determined by such an integration. Thus, the frequency of intramolecular recombination will depend on the susceptibility of each of these stages to the intracellular environment of the host.

In an earlier report on linked transformations of pneumococci to erythromycin resistance (RAVIN and IYER 1961), certain anomalies were reported. It was noted that in the transformation of sensitive pneumococci by transforming preparations bearing linked mutations, the frequency of occurrence of the multiply-marked transformants could be greater than one of the singly marked ones. Subsequent studies have confirmed these findings and have also revealed that the ratios of the transformant classes obtained in such experiments are considerably influenced by the state of the recipient cell population. Similar observations have been made in the transformation of pneumococci to sulfonamide resistance by

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HOTCHKISS and EVANS (1958) whose findings suggested that the size of the genetic region integrated in transformation was affected by metabolic factors and also that there was a direct relationship between the competence (ability to be transformed) of a culture and the size of the region integrated. The present study examines the influence of the intracellular environment of the host in determining the magnitude of the region to be integrated from the transforming molecule. More specifically we ask: are the intercellular conditions influencing the magnitude of an integrated region identical to those that determine the competence of a population? We are next concerned with the events that follow integration. Specifically: can intracellular conditions influence the postintegrative expression of a transformant? Is the rate of expression of the transformant influenced by the magnitude of the genetic region integrated?

MATERIALS AND METHODS

The capsule-deficient Rx strain of pneumococcus served as the recipient in all experiments described in this paper. The donor transforming DNA was prepared from each of the following erythromycin-resistant mutant strains derived from strain Rx or from strains bearing combinations of these mutations: *ery-r2*, *ery-r3*, *ery-r5*, *ery-r6*, and *ery-r7*. The origin of these strains and the methods used in preparing transforming DNA and culture media have been previously described in detail (RAVIN and IYER 1961).

The transformation procedure consisted first in securing a population of recipient cells which had developed the competence to transform. Rx cells from an exponentially growing culture were inoculated into Medium 3 of EPHRUSSTAYLOR (1951) supplemented with 0.2 percent beef serum albumin to yield approximately 10^5 colony-forming units per ml and incubated at 37°C. At 15 minute intervals during a phase when they were judged to be developing competence, aliquots of the culture were withdrawn and frozen immediately in a glycerol medium at -80°C (FOX and HOTCHKISS 1957; RAVIN and IYER 1961). Batches of competent cells frozen in this manner retained their competence for several months. To be transformed, a frozen batch of cells was thawed in the cold and diluted tenfold in Medium 3 plus 0.2 percent beef serum albumin to which was added a saturating concentration of the transforming DNA. The mixture was held for 15 minutes at 32°C and, as a rule, the reaction terminated by the addition of Mg^{++} activated pancreatic DNase. Following the addition of DNase, the transformed cells were allowed to express their phenotype in the liquid medium at 37°C before being challenged on antibiotic containing agar. The challenging concentration of antibiotic was always less than that to which the strain was maximally resistant. After acquisition of any one of the mutations *ery-r2*, *-r3*, *-r5*, or *-r6*, the period required for all transformants to develop resistance to 0.1 µg erythromycin per ml is usually one hour. The *ery-r7* transformants generally require three hours for expression under similar conditions. In a separate paper (IYER 1962), it is shown that cells newly acquiring the *ery-r7* marker grow

at the same rate as do untransformed cells in the medium in which expression occurs.

Other experimental details are described where relevant in the following section.

RESULTS

Resistance of strains bearing ery-r mutations: Table 1 shows the level of resistance to erythromycin of the 'parent' Rx strain and strains bearing one or more of the *ery-r* mutations. The level of resistance is defined here as the maximum concentration of antibiotic which, in solid medium, permits the growth of as many colonies as a medium lacking antibiotic. This maximum concentration is readily determined by spreading from 10^2 to 10^3 cells on the surface of media containing increasing concentrations of erythromycin. The strains bearing two mutations were as a rule obtained by treating the less resistant strain with DNA prepared from the more resistant strain. Recognition of two or more specific mutations in a strain is based not only on the distinctive resistance level which possession of a combination of mutations may confer, but also on the ability of DNA prepared from the strain to transfer the mutations singly and in combination to the parental sensitive strain (RAVIN and IYER 1961).

Transformation of the sensitive strain by DNA bearing each of the ery-r mutations: The frequency of transformation of the sensitive strain to a level of resistance characteristic of the mutated donor strain was determined in the following type of experiment. Aliquots of sensitive cells drawn from a single competent batch were exposed under identical conditions to individual transforming DNAs each bearing a different *ery-r* mutation and, in addition, an unlinked streptomycin-resistance mutation, *str-r1*, which served as a reference marker. Following exposure, all potential transformants were allowed to express their resistance before being challenged with the antibiotics. A comparison of the ratio of erythromycin-resistant to streptomycin-resistant transformants obtained in each case

TABLE 1

Resistance to erythromycin of strains bearing different ery-r mutations or combinations of these mutations

Mutation(s)	Resistance ($\mu\text{g/ml}$)
nil (sensitive strain)	0.01 ± 0.005
<i>ery-r6</i>	0.1 ± 0.02
<i>ery-r2</i>	1.0 ± 0.2
<i>ery-r3</i>	1.0 ± 0.2
<i>ery-r5</i>	1.0 ± 0.2
<i>ery-r7</i>	10.0 ± 5.0
<i>ery-r2-r6</i>	4.0 ± 1.0
<i>ery-r3-r6</i>	15.0 ± 5.0
<i>ery-r2-r3</i>	40.0 ± 10.0

The estimate of variability indicated in the table expresses the highest and lowest levels of resistance that were observed in a series (three to six) of similar experiments.

provides a measure of the relative ability of each of the *ery-r* mutations to be integrated into the genome of the sensitive strain (Table 2).

The variability in the ratio of erythromycin-resistant to streptomycin-resistant transformants is quite low when a single batch of competent cells is used to test a given transforming preparation (Table 2). In Table 3 are recorded the results of another experiment in which a different batch of competent cells was used. In addition, several DNA preparations subjected to different conditions of storage and handling were tested with aliquots drawn from this new batch of cells. It may be seen from a comparison of Tables 2 and 3 that: (a) the use of different DNA preparations bearing the same *ery-r* and *str-r* markers, although stored for different periods of time and probably otherwise affected in their quality, does not cause significant differences in the ratio of erythromycin-resistant to streptomycin-resistant transformants so long as the same batch of competent cells is used; (b) although this ratio is altered when different batches of recipient cells

TABLE 2

Relative frequency of transformation to erythromycin resistance of the sensitive strain by DNA bearing each of the ery-r mutations

Mutation	Ratio of the frequency of <i>ery-r</i> transformants to <i>str-r1</i> transformants	Standard deviation
<i>ery-r2</i>	1.48	0.022
<i>ery-r3</i>	1.31	0.058
<i>ery-r6</i>	0.47	0.21
<i>ery-r7</i>	0.077	0.01
<i>ery-r5</i>	0.045	0.014

Aliquots of sensitive cells from a single competent batch were simultaneously exposed in quadruplicate to DNA preparations bearing each of the above *ery-r* mutations and a standard *str-r1* mutation.

TABLE 3

Transformation of the sensitive strain by different DNA preparations all bearing an ery-r mutation and the str-r1 mutation but subjected to different periods of storage and handling

Mutations in transforming DNA	DNA preparation number	Period between preparation of DNA and test (months)	Average number of transformants in 0.1 ml sample		Ratio of <i>ery-r</i> to <i>str-r</i> transformants
			<i>ery-r</i>	<i>str-r</i>	
<i>ery-r2 str-r1</i>	1	2	138.5	138.0	1.0
	2	3	151.0	146.0	1.03
	3	7	84.5	91.0	0.92
	4	10	67.5	55.5	1.21
					Avg. 1.04
<i>ery-r5 str-r1</i>	1	2	51.0	1480	0.034
	2	6	55.5	1200	0.046
	3	12	30.5	980	0.031
					Avg. 0.037

Aliquots from a single batch of competent sensitive cells were simultaneously exposed, under identical conditions, to each of the DNA preparations. The batch of competent cells used in this experiment was different from that used in the experiments summarized in Table 2.

are used, the relative difference between two *ery-r* markers remains constant. Thus, by changing the batch of competent cells, the ratio of *ery-r* to *str-r1* transformants was changed to the same extent for the *ery-r2* and *ery-r5* markers.

It is observed from Table 2 that the five *ery-r* mutations studied may, under similar conditions, yield different numbers of transformants, showing that they are integrated with different degrees of efficiency. The relative ability to be integrated can thus be determined by the nature of the mutation being transferred.

Transformation of the sensitive strain by DNA bearing two linked mutations in the region ery-r2-r6-r3: Evidence for the close linkage of the mutations *ery-r2*, *ery-r6* and *ery-r3* has been presented earlier (RAVIN and IYER 1961). When the sensitive strain was treated with DNA bearing the mutations *ery-r2-r6* or *ery-r3-r6*, it was observed that the proportion of the doubly marked transformant class *ery-r2-r6* or *ery-r3-r6* was often greater than the proportion of a transformant class bearing a single mutation. This observation has been confirmed and also extended to transforming DNA bearing the mutations *ery-r2-r3*. Table 4 shows the results of these experiments in which three different batches of sensitive cells were used. Examination of the results indicate the following: (1) in the different experiments using the same batch of recipient cells, the relative proportions of the various classes of transformants are generally quite similar from one experiment to the next; (2) a significant alteration in these ratios often occurs with the use of a different batch of recipient cells.

It should be pointed out that the relative proportion of the doubly marked *ery-r* transformants recovered in a given experiment is not a reflection of different rates of expression for single and combined *ery-r* markers. As will be shown below (Tables 5-7), the rates of expression of these markers, individually or in com-

TABLE 4

Proportion of transformant classes arising from the transformation of sensitive cells by DNA bearing two ery-r mutations

Mutations in donor DNA	Experiment no.	Batch of competent sensitive recipient cells	Frequency of all erythromycin-resistant transformants	Proportion of each transformant class among all transformants obtained			
				No. tested	Class <i>ery-r6</i>	Class <i>ery-r2</i>	Class <i>ery-r2-r6</i>
<i>ery-r2-r6</i>	1	A	4.1×10^{-4}	50	5	36	9
	2	A	4.0×10^{-4}	50	1	28	21
	3	B	9.8×10^{-4}	59	0	42	17
	4	B	3.1×10^{-4}	57	0	43	14
	5	B	2.6×10^{-4}	57	0	50	7
<i>ery-r3-r6</i>	1	A	4.0×10^{-4}	No. tested 59	Class <i>ery-r6</i> 40	Class <i>ery-r3</i> 2	Class <i>ery-r3-r6</i> 17
	2	A	1.7×10^{-4}	76	55	3	18
	3	B	4.1×10^{-4}	59	0	6	53
<i>ery-r2-r3</i>	1	B	6.6×10^{-4}	No. tested 58	Class <i>ery-r2</i> or <i>ery-r3</i> 24	Class <i>ery-r2-r3</i> 34	
	2	C	3.9×10^{-4}	115	100	15	

The use of a similar letter in designating batches of competent recipient cells means that an identical batch provided aliquots for the indicated experiments, which were carried out at different times. Different batches were obtained from cultures grown on different days.

bination, are indistinguishable. The results in Table 4 demonstrate, therefore, that the integration of a pair of linked mutations from donor genetic material may, under certain conditions, be more efficient than the integration of a member of the pair. If one adopts the reasonable assumption that integration of two closely linked markers involves a longer segment of donor genetic material than does the integration of a single one of these markers (НОТЧКИСС 1958), we may conclude that integration of a long segment of genetic material may be more efficient than that of a shorter segment. This conclusion is amply supported by the results

TABLE 5

Expression of transformants integrating the mutations ery-r2, ery-r3 or ery-r2-r3 following the exposure of sensitive cells to DNA bearing the mutations ery-r2-r3 str-r1*

Period of expression (minutes)	Frequency of <i>str-r1</i> transformants	Frequency of all <i>ery-r</i> transformants	Proportion of <i>ery-r2-r3</i> transformants among all <i>ery-r</i> transformants	
			Number tested	Number in <i>ery-r2-r3</i> class
< 1	3.4×10^{-5}	2.3×10^{-5}	31	23
5	4.5×10^{-4}	4.3×10^{-4}	36	26
10	2.4×10^{-3}	1.6×10^{-3}	36	29
15	2.4×10^{-3}	1.5×10^{-3}	36	27

* Sensitive cells used in this experiment were from a population capable of expressing antibiotic-resistance rapidly.

TABLE 6

Expression of transformants integrating the mutations ery-r2, ery-r6 or ery-r2-r6 following the exposure of sensitive cells to DNA bearing the mutations ery-r2-r6 str-r1*

Period of expression (minutes)	Frequency of <i>str-r1</i> transformants	Frequency of all <i>ery-r</i> transformants	Proportion of the three transformant classes among all <i>ery-r</i> transformants			
			Number tested	Class <i>ery-r2</i>	Class <i>ery-r6</i>	Class <i>ery-r2-r6</i>
< 1	2.6×10^{-5}	5.3×10^{-6}	71	11	2	58
2	1.1×10^{-4}	7.0×10^{-5}	..	..	..	..
10	1.8×10^{-4}	1.2×10^{-4}	..	..	..	..
15	1.4×10^{-4}	2.3×10^{-4}	..	..	..	..
30	1.4×10^{-4}	2.4×10^{-4}	64	12	1	51

* Sensitive cells used in this experiment were from a population capable of expressing antibiotic-resistance rapidly.

TABLE 7

Expression of transformants integrating the mutations ery-r3, ery-r6 or ery-r3-r6 following the exposure of sensitive cells to DNA bearing the mutations ery-r3-r6 str-r1

Period of expression (minutes)	Frequency of <i>str-r1</i> transformants	Frequency of all <i>ery-r</i> transformants	Proportion of the three transformant classes among all <i>ery-r</i> transformants			
			Number tested	Class <i>ery-r3</i>	Class <i>ery-r6</i>	Class <i>ery-r3-r6</i>
10	1.1×10^{-4}	3.4×10^{-6}	11	5	2	4
15	1.1×10^{-4}	4.1×10^{-6}	..	..	..	..
30	3.4×10^{-4}	7.9×10^{-5}	47	22	10	15
45	3.0×10^{-4}	3.7×10^{-4}	..	..	..	..
60	3.4×10^{-4}	3.4×10^{-4}	46	20	8	17

of three-factor crosses (RAVIN and IYER 1961, 1962) in which double- and even triple-marker integration occurs more frequently than integration of the medially located marker.

The results show, moreover, that the integration of mutated regions from a donor molecule is not exclusively determined by the nature of those regions. The physiology of the recipient cell can play a decisive role in determining the length of the genetic region to be integrated in transformation.

Although it may be expected to have some influence on the relative frequency of transfer of linked markers, the quality of the DNA preparation appears to play a minor role, if any, under the conditions of our experiments. Different preparations of DNA bearing the same markers, but produced by similar methods, were used in some experiments involving the same batch of competent cells (for example, in Table 4, experiments 3, 4, and 5 involving the *ery-r2-r6* markers); no significant difference in the distribution of the recombinant classes was noted.

Differentiation of the host conditions determining frequency of transformation from those determining the length of the genetic region integrated: The physiological state which determines the ability of a cell to be transformed is termed competence. For any given transforming DNA, differences in the frequency of transformation of a recipient population may therefore be attributed to differences in the competence of the population. The question to be resolved is whether the cellular factors that determine competence (i.e. the total ability to be transformed) also determine the length of the genetic region to be integrated from the donor molecule. The results presented in Table 4 suggest no such relationship; that is, the degree of competence of a population does not appear to be associated with an altered probability of integration of a larger region of the donor molecule. This conclusion has been verified as follows. Aliquots of a growing population of sensitive cells were frozen at different stages of growth corresponding to different degrees of competence. Each aliquot was thawed in the cold and exposed separately to a DNA preparation bearing the linked mutations *ery-r2-r3* and the unlinked *str-r1* mutation. Transformants were quantitatively detected on media containing concentrations of antibiotic that permitted the growth of *all* classes of transformants. The proportion of doubly-marked *ery-r2-r3* transformants among the total erythromycin-resistant transformants was next determined. This was done by transferring to antibiotic-free agar a large sample of the transformants originally selected, and then distinguishing the *ery-r2-r3* transformants by their characteristic higher resistance upon velvet replication (LEDERBERG and LEDERBERG 1952) to plates containing an increasing concentration of erythromycin.

Figure 1 illustrates the results of this experiment. Under the growth conditions obtaining, the population of sensitive cells had gone through a single peak of competence. The proportion of *ery-r2-r3* transformants among the total erythromycin-resistant transformants is high even at periods of low competence and does not undergo any significant change with a tenfold increase in competence, as the population passes through its competence peak. The host conditions that

FIGURE 1.—Transformation of populations of erythromycin-sensitive pneumococci by DNA *ery-r2-r3 str-r1*. Indicated in the boxes are the proportions of *ery-r2-r3* transformants among all *ery-r* transformants produced in populations at different stages of growth and competence.

determine competence are, therefore, not identical with those determining the magnitude of the genetic region integrated. In fact, the conditions that determine the length of the region to be integrated operate over an appreciably longer period than those conditions that determine competence.

The expression of resistance following the integration of different ery-r mutations: It was revealed in our earlier studies (RAVIN AND IYER 1961) that the period required for development of the ability of all transformants to resist 0.1 μ g erythromycin per ml was one hour for each of the mutations *ery-r2*, *ery-r5*, *ery-r3*, and *ery-r6*, whereas this period was three hours for the mutation *ery-r7*.

In subsequent experiments using different batches of sensitive recipient cells, it was observed that expression could occur much earlier than one hour (compare Tables 5 and 6 with Table 7). This implies that the expression of a transformant can be influenced by the state of the recipient cells. Furthermore, the factor or factors contributing to this state do not specifically influence the molecule bearing the *ery-r* mutations, as can be seen from the fact that the expression of the unlinked *str-r* marker is likewise influenced.

We observe that the ability of a cell population to express the transformed character relatively early or late is rather stable, for it persists through several serial transfers involving many cell generations. It is therefore likely that such states have some genetic basis. Notwithstanding these variations in the specific period required for expression, the distinctive nature of the mutation *ery-r7* is preserved, because when aliquots from any single batch of sensitive cells are simultaneously but separately transformed by DNAs bearing different *ery-r* mutations, the expression of *ery-r7* is consistently and significantly slower than that of all the other *ery-r* mutations. Thus, the postintegrative expression of a transformant is determined both by the recipient as well as by certain qualities of the region that is integrated.

The expression of resistance following the integration of regions of different length: When the sensitive strain of pneumococcus is transformed by DNA bearing any two linked mutations in the region *ery-r2-r6-r3*, one may recover transformants that bear either one of the single mutations or transformants that bear both linked mutations. A useful question that one might ask is whether the expression of transformants bearing both linked mutations, and therefore a longer integrated region of the molecule, is slower than the expression of transformants bearing a smaller component region. To determine this, a competent batch of sensitive cells was exposed to transforming DNA bearing the mutations *ery-r2-r3 str-r1*. At various periods after exposure, the population was challenged with a low concentration of erythromycin permitting the development of all transformant classes. As is to be expected, the total number of transformants that could be recovered on the erythromycin agar increased with increasing periods of expression until all transformants had expressed themselves. Transformant colonies obtained at various intervals during the period of incomplete expression and after complete expression had occurred, were next examined for the proportion of doubly marked (*ery-r2-r3*) transformants among them. This was accomplished by regrowing a sample of the colonies in the absence of the antibiotic and testing their resistance by velvet replication (LEDERBERG and LEDERBERG 1952). If the period required for the expression of doubly marked transformants is different from that of the singly marked transformants, one would expect this to be reflected in the proportion of doubly marked transformants among the total transformants examined at various stages of expression. The results presented in Table 5 show that throughout all stages of expression, from the earliest time when expression could be detected to a time when expression is complete, the proportion of doubly marked transformants does not undergo any appreciable change. Similar results are obtained when the sensitive strain is exposed to DNA bearing

the mutations *ery-r2-r6 str-r1* or *ery-r3-r6 str-r1*. The proportion of all three transformant classes remains unaltered during different stages of expression (Tables 6 and 7). It may be concluded from these findings that the expression of resistance following the integration of one or more mutated sites in the region *ery-r2-r6-r3* is independent of the magnitude of the integrated region. This further suggests that the delayed expression observed in the case of *ery-r7*, a mutation linked to *ery-r2-r6-r3* and presumably located in the same molecule, may be due to some quality of this mutated region other than size.

DISCUSSION

It is now known that in any population of transformable pneumococci, every coccus possesses the potential ability to be transformed under suitable conditions (RAVIN 1954; SCHAEFFER 1957). At any one time, however, it is only a fraction of the population that is susceptible to transformation and the term *competence* has been historically used in a relative sense to connote the ability of a population to undergo transformation to a particular genetic characteristic under suitable conditions. HOTCHKISS (1954) and THOMAS (1955) have shown that competence is a physiological property of the cell that arises during a particular period of growth and division, persisting for only a fraction of the generation time of the cell. Subsequently, FOX (1957) and LERMAN and TOLMACH (1957) found a simple proportionality, independent of several experimental variables, between transformation frequency and irreversible P^{32} uptake. The latter also noted that in the presence of a constant amount of labeled pneumococcal DNA and varying amounts of unlabeled calf thymus or *E. coli* DNA, while pneumococcal transformation is reduced, the ratio of P^{32} uptake to transformation frequency is preserved. On the basis of these observations, it was suggested (LERMAN and TOLMACH 1957) that the term competence might appropriately be applied to the capacity of a cell to bind DNA and it has been used on occasion to denote this specific ability (EPHRUSSI-TAYLOR 1958).

However, in more recent studies, both on pneumococcus (LERMAN and TOLMACH 1959) and on *Bacillus subtilis* (YOUNG and SPIZIZEN 1961) significant deviations from proportionality between P^{32} uptake and transformation frequency have been observed, suggesting that the uptake of DNA is not the sole process determining transformation frequency. These observations are not altogether unexpected. It would indeed have been surprising if a strict invariant proportionality between DNA uptake and transformation frequency to any observed character had been observed under all conditions. After all, during transformation the uptake of DNA is followed by processes (integration and expression), each of which is susceptible to variation and capable, therefore, of influencing a population's competence, as broadly defined.

The observations of HOTCHKISS (1954) and FOX and HOTCHKISS (1957) have shown that physiological ability of a pneumococcus to bind and take up DNA persists for only a few minutes. On the other hand, the present studies have indicated the existence of a distinct host condition that influences the magnitude

of the genetic region which the recipient genome is able to integrate from the donor molecule. Unlike the ability to take up DNA, this condition persists for several generations. This condition, moreover, appears to be physiological in nature, for it varies considerably, but in a nonprogressive way with time, in batches of recipient cells prepared on different occasions from the same strain. These different occasions may differ with respect to small, uncontrolled changes in medium composition, rate of freezing the cells, etc. At the present time, we have no tested explanation for these physiological variations affecting the relative length of the donor DNA molecule that can be integrated.

Integration of the specificity of part of the donor DNA molecule appears to be dependent also upon the nature of the region to be integrated. This conclusion is supported by the quite different efficiencies with which the different *ery-r* markers are integrated into recipient cells of an identical state of competence. A thirtyfold difference in the ability to be integrated exists between the *ery-r2* marker, at one extreme, and the *ery-r5* marker, at the other (Table 2). It is possible that these different efficiencies of integration reflect the size of the mutation, for it is believed that in transformation, as a rule, long lengths of genetic material are less easily integrated than shorter lengths (HOTCHKISS 1958). The experimental evidence in support of this belief is reasonable and is drawn both from studies on the relative integration of multisite mutations and smaller mutations with which they are allelic (LACKS and HOTCHKISS 1960) and from numerous observations that the frequency of transformants acquiring two linked mutations is usually less than the frequency of transformants acquiring either one of the linked mutations (HOTCHKISS and EVANS 1958; BRYAN 1961; GOODGAL 1961; NESTER and LEDERBERG 1961; EPHRATI-ELIZUR, SRINIVASAN and ZAMENHOF 1961). The work of LACKS and HOTCHKISS (1960) also has drawn on physical evidence in support of this contention. The possibility should not be overlooked however, that the particular nucleotide composition of a mutation, rather than its length alone, may determine the ease of its integration in transformation. Size is obviously not the sole factor determining the relative frequency of integration of a pair of closely linked mutations. When two *ery-r* markers are present on the same donor DNA molecule, the integration of the pair of them often occurs more frequently than one of the members of the pair (example: *ery-r2-r6* in Table 4). This may be due to the fact that, when the distance between two linked markers is shorter than the length of one of them, the integration of the pair may be relatively very frequent (RAVIN and IYER 1962). In any case, integration efficiency should not be used, by itself, as an index of the size of a given mutation.

In another paper (IYER 1962) it is shown that the expression of erythromycin-resistant transformants, following integration, occurs in two stages: first, the development of the ability to withstand, but not to multiply in the presence of the antibiotic; second, the development of the ability to reproduce in the presence of the antibiotic. This result is similar to that observed by EPHRUSSI-TAYLOR (1958) with streptomycin resistance in pneumococci.

In any case, the period in which colony-forming ability in the presence of a specific concentration of erythromycin is expressed has been shown to depend

on at least two factors. One of these is apparently the genetic constitution of the recipient population, for certain populations transmit in a stable fashion through clonal growth the ability to express resistance very rapidly. In such populations all of the transformants express the ability to multiply in the presence of erythromycin within ten to 15 minutes after contact with the transforming DNA (Tables 5 and 6) although in other populations such expression requires from 45 to 60 minutes (Table 7, and RAVIN and IYER 1961). We believe that a gene (or genes) controlling the rate of expression differentiates such populations, but it has been difficult to verify this view. It is likely, however, that whatever condition is responsible for rapid expression, it is not specific since it affects expression of streptomycin resistance as well.

The other factor which affects expression is the nature of the mutation itself. The mutation *ery-r7* requires a considerably longer period of time to express resistance than do any of the other *ery-r* markers. This obtains whether the host condition for rapid expression is present or not. The evidence suggests that it is not the size of the *ery-7* mutation that is responsible for the slowness of its expression. In the first place, the rate of expression appears to be largely independent of length in the *ery-r2-r6-r3* mutated region. Secondly, studies on the genetic linkage of the five *ery-r* mutations reveal that *ery-r7* is only loosely linked to the other four mutations which form a more tightly linked cluster (RAVIN and IYER 1962). Thus, it may be that *ery-r7* belongs to a different cistron having a slightly different function in the production of the erythromycin-resistant phenotype. This different function may have an intrinsic rate of expression, slower than that of the adjacent cistron.

The totality of the results described in this paper stresses the importance of using controlled competent batches of recipient cells in the mapping of DNA molecules. Unless intramolecular recombination between mutations is followed within the same intracellular environment, interpretations drawn from the observed recombination frequencies are apt to be spurious.

SUMMARY

In the region of the DNA molecule controlling resistance to erythromycin, the efficiency of integration into the recipient genome depends on the nature of the mutated region being integrated. This efficiency is not clearly related to the length of the mutated regions, as long segments of the molecule are often more readily integrated than shorter, component segments. Physiological variations in the recipient cell do, however, have a significant influence on the length of the region to be integrated. These physiological variations are different in duration from those that control the ability of the pneumococcus to bind and take up transforming DNA.

Expression of erythromycin resistance, following integration, depends in part on the state of the host cell, for strains permitting very rapid expression have been isolated. Another important factor affecting expression, however, is the nature of the mutation that has been integrated. The rate of expression is largely independ-

ent of length in one closely linked cluster of mutations. In an adjacent region, a mutation has a considerably slower rate of expression.

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