IDENTICAL TRANSFORMABILITY OF BOTH STRANDS OF RECIPIENT DNA IN *Diplococcus pneumoniae*

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It has been recently demonstrated that, in *Diplococcus pneumoniae* as well as in *Bacillus subtilis* and *Hemophilus influenzae*, either strand of donor DNA can transform (Guild & Robinson, 1963 ; Roger, Beckman & Hotchkiss, 1966 ; Chilton, 1967 ; Rudner, Karkas & Chargaff, 1968 ; Notani &Goodgal, 1968 ; Gurney &Fox, 1968 ; Peterson& Guild, 1968). Thus, it was suggested that either strand of recipient DNA can be transformed. However, the possibility that the integration is performed preferentially into one of the two strands of recipient DNA has not yet been excluded.

We present here evidence that, in pneumococcus, the genetic information of transforming DNA can be transmitted with the same probability to either strand of the recipient genome.

MATERIALS AND METHODS

a) Principle :

The method consisted of an analysis of the frequencies of double transformants in a culture of *D. pneumoniae* treated by a DNA bearing two independent genetical markers.

The frequency of double transformants depends upon a variety of factors, one of them being the mode of integration of the markers involved : 1) if the transformation for a marker A modifies the two strands of the recipient genome, the transformation of this genome for a marker B will always lead to a double transformant, i. e. it will have a 100 % chance of giving a double transformant ; 2) if the two markers used modify only one strand of the genome, this chromosome will have only a 50 % chance of leading to a

double transformant, assuming that either strand of the recipient DNA can be equally transformed (see Figure 1).

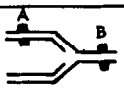
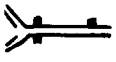
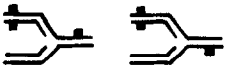
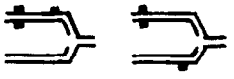
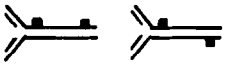
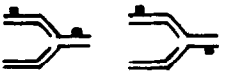
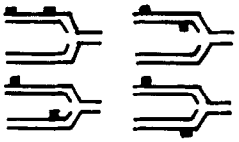
PAIRS OF MARKERS	MODIFICATION OF THE RECIPIENT CELL IF THE REPLICATION FORK IS:		
	BEFORE THE MARKERS	BETWEEN THE MARKERS	AFTER THE MARKERS
LE - LE	 $P_{AB} = 1.$	 $P_{AB} = 1.$	 $P_{AB} = 0,5$
LE - HE	 $P_{AB} = 1.$	 $P_{AB} = 0,5$	 $P_{AB} = 0,5$
HE - HE	 $P_{AB} = 0,5$	 $P_{AB} = 0,5$	 $P_{AB} = 0,25$

Figure 1 : Involvment of the replication on the probabilities of obtaining a double transformant.

In each case, the probability P_{AB} of obtaining a double transformant is indicated, assuming that a) either recipient strand can be transformed at random by HE markers, b) the LE marker is nearer the origin of replication than the HE marker for the pair LE-HE

In pneumococcus, the occurence of these two modes of integration has been demonstrated already (Ephrussi-Taylor, 1966 ; Louarn & Sicard, 1968) : the genetic information of low efficiency (LE) markers is transmitted into both strands of recipient DNA whereas that of high efficiency (HE) markers is mainly transmitted into a single strand. Thus, a comparison between the frequencies of double transformants for two HE and two LE markers would be useful in order to test the hypothesis of identical transformability for HE markers of both recipient chains. Such a comparison requires the following calculation :

Let N be the number of colony-forming units (c.f.u.) per ml., A the number of transformants per ml. for marker A, B for marker B, and D for markers A and B, the ratio $R = D.N/A.B$ is calculated. This ratio de-

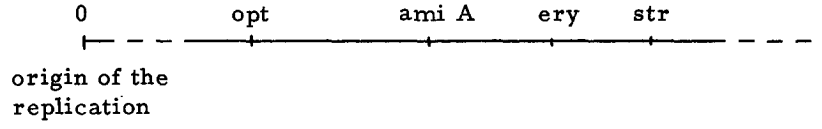
pends upon the percentage of competent cells (Goodgal and Herriott, 1961), the linkage between the markers if they are not genetically independent, and also the mode of integration of the markers involved. It can be seen in the appendix that, in a transformation involving pairs of HE and LE markers, the ratio $R_{HE\text{-}HE}/R_{LE\text{-}LE}$ is an estimate of the probability that a chromosome doubly transformed for two HE markers would lead to a stable double transformant. This probability would be 0,5 only if either recipient strands are equally transformable.

Thus, we have transformed wild-type bacteria with DNA bearing three to four independent markers belonging to the high and low efficiency classes and counted the number of single and double transformants for various combination of pairs of markers.

b) Markers used :

str-r 41 : resistant to 2 mg/ml streptomycin, the HE reference marker ; ami A-r 1 (HE) and ami A-r 9 (LE) : two closely linked markers belonging to the same ami A locus, resistant to 10^{-5} M. aminopterin ; ery-r 2 (HE) : resistant to 1 µg/ml erythromycin ; opt-r 2 (LE) : resistant to 5 µg/ml optochin.

These four loci have been located along the pneumococcal chromosome by clonal analysis (Louarn, unpublished) in the following order :



We found that the probability of observing a replication fork between the loci opt and str is about 0,6, and about 0,2 between the loci ami A and str. Even if the relationship between this probability and the distance along the chromosome is not yet established, it seems likely that these markers are separated by large amounts of the chromosome and are borne by different particles when the DNA is extracted. This is in good agreement with the results obtained by Martin and Ephrussi-Taylor (1963) using a different technique. Although the precise position of ery-r 2 is not well established, it seems closer to ami A than to str and therefore is probably on a different DNA particle than str-r 41. This could be in conflict with the results of Ravin (1965).

c) Transformation procedures :

The methods used in the transformation technique have been described previously (Sicard, 1964). Competent wild-type cells (Cl. 3) were treated with a saturating concentration of DNA for 15 mn. The bacteria were then plated and transformants selected by a second layer of agar containing the antibiotics after for incubation 2 1/2 hours at 37° for complete phenotypic expression. All double transformants were selected with the same concentration of antibiotics as single transformants, except the pair ery-r 2 ami A-r, which is less resistant to aminopterin in presence of erythromycin than the single transformants.

d) Statistical analysis :

The statistical analysis has been performed by the method of Kimball (1961). A final precision of 10 % on R values required the counting of about 1,000 colonies for each class.

RESULTS AND DISCUSSION

The results of four independent experiments are presented in the table, in which are shown the values of the ratio R = D.N./A.B. computed for different pairs of markers. For a given pair of markers, this ratio fluctuates from experiment to experiment, suggesting that the number of transformable chromosomes per colony-forming unit is somewhat variable.

Therefore, let us consider each experiment separatedly. The markers can be grouped into three different pairs : LE-LE, HE-HE and HE-LE.

In experiment n°1, the recipient culture was divided into two batches, one (A) transformed by a DNA bearing two LE markers (ami A-r 9) and opt-r2) and one HE marker (str-r 41), the other (B) transformed by a DNA bearing two HE markers (ami A-r1 and str-r41) and one LE marker (opt-r2). In experiment n°2, the DNA used contained two HE markers (ery-r2 and str-r41) and two LE markers (opt-r2 and ami A-r9). In experiments n°3 and 4, the behavior of several HE markers has been tested in combinations HE-HE and HE-LE.

The main result was that the ratio R_{HE-HE}/R_{LE-LE} is about 0,5 (exp. 1 and 2). This would be expected if both recipient strands can be transformed with the same efficiency by HE markers. Indeed, this result implies that at least one of the HE markers involved in experiments 1 and 2 modify either recipient strand at random (fig. 1). As, when three HE markers (ami-A-r1, str-r41 and ery-r2) were used (exp. 3 and 4), similar results were obtained, at least for two out of these three markers both recipient strands are identically transformable. This can be related to the results of Peterson and Guild (1968), who found that for str-r41 and ery-r2, each donor strand possess the same efficiency of transformation.

Another result is that the ratios R_{HE-HE}/R_{LE-HE} were significantly higher than 0,5. This could be due to an interaction between the replication and the transformation processes in the bacteria : if the LE marker is nearer the origin of replication than the HE marker (and this is indeed the case for the markers studied here), the occurence of a replication fork between the two loci at the time of the integration of the markers would lead to a probability of 0,5 instead of 1 of obtaining a stable double transformant (figure 1). The presence of a replication fork between two HE or two LE markers would not modify the probability of getting a double transformant. Thus, one can expect in these experiments values of R_{LE-HE} lower than values of R_{LE-LE}.

In fact, the involvment of the replication is not restricted to the R_{LE-HE} values. The probability of obtaining a double transformant would depend upon the number of copies of the two loci in the transformed cell (figure 1). Using two markers with the same mode of integration, the value of R would be lower if the two markers are located near the origin of the replication than if one marker is located near the terminus, as already pointed out by Porter & Guild (1969). In our experiments, this effect of the replication is probably neglectable, since the genes str, ami A and ery are located in the same region of the chromosome (unpublished results).

During this discussion, we have made the implicit assumption that all the cells of the culture are transformable by HE as well as by LE markers. As a matter of fact, it is not excluded that the culture is physiologically heterogeneous, containing, for instance, cells transformable by HE markers only, cells transformable by LE markers only and, of course

Pairs of markers	Efficiencies of markers	R values in transformations				
		1 A	1 B	2	3	4
opt - am9	LE-LE	0,23 ± 0,02	-	0,48 ± 0,05	-	-
aml - str	HE-HE	-	0,11 ± 0,01	-	0,28 ± 0,03	0,38 ± 0,03
aml - ery	"	-	-	-	0,34 ±0,03	0,36 ± 0,03
str - ery	"	-	-	0,20 ± 0,02	0,27 ± 0,03	0,38 ± 0,03
opt - str	LE-HE	0,16 ± 0,02	0,16 ± 0,02	0,33 ± 0,03	0,55 ± 0,06	0,55 ± 0,05
opt - ery	"	-	-	0,35 ± 0,03	0,57 ± 0,06	0,53 ± 0,05
opt - aml	"	-	0,14 ± 0,02	-	0,61 ± 0,06	0,48 ± 0,05
am9 - str	"	0,18 ± 0,02	-	0,33 ± 0,03	-	-

Average $R_{HE\text{-}HE} / R_{LE\text{-}LE}$ (exp. 1 and 2) = 0,45

Average $R_{HE\text{-}HE} / R_{LE\text{-}HE}$ (exp. 1 to 4) = 0,63

Table 1 : Influence of the efficiency of markers upon the frequency of doubly transformed bacteria

opt = opt-r2 ; str = str-r41 ; ery = ery-r2 ; aml = amA-r1 ; am9 = amA-r9

The frequency of transformation was above 5 % in each experiment for str-r41

(since HE-LE double transformants are found), cells transformable by HE and LE markers. Accordingly, R values would depend upon the size of each of these three fractions of the transformable population. Then, the double transformants HE-LE being restricted to one class whereas single trans-

formants are found into two classes, it can be calculated that R_{HE-LE} values would be always lower than R_{HE-HE} and R_{LE-LE} values if the mode of integration of the markers is the same, whatever efficiency they have (see appendix). This is not observed : when a HE-LE pair is involved, the R values remain always higher than the ones observed when both markers are HE.

Thus, is seems unlikely that any limitation of the integrations of LE markers to a part of the competent bacteria plays a role on the values of R. Indeed, these results do suggest not only that both recipient strands are identically transformable by HE markers, but also that the integrations of LE markers can be performed in all the transformable chromosomes.

Incidently, our work shows that it not safe to assume linkage between genes on the basis of observed small variations of the R ratio : the mode of integration of each marker must be established beforehand.

Moreover, the determination of R for LE-LE pairs, which is independent of the presence of replication fork (or R_{HE-HE} with a correction factor of 2), offers the possibility to estimate the number of transformable chromosome per colony-forming unit. This number ranges between 1,5 and 4. Such a figure agrees with the average number of cocci per pneumococcal chain in our culture media. Thus, it is likely that all chromosomes are transformable in these bacterial populations.

In summary, our results concerning double transformants for independent genes are consistent with the existence of two modes of integration for low and high efficiency markers. They show that genetic information of HE markers is transmitted into either recipient strand at random.

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APPENDIX Theoretical analysis of the frequencies of double transformants for two independent markers

This is a partial development of the analysis presented by Balassa and Prevost (1961). The effect of the replication is not taken in account.

Let us consider a competent culture treated by a DNA bearing markers A and B with respectively A and B single transformants and D double transformants per ml.

Among DNA particules, let a and b be the frequencies of those bearing respectively markers A and B, i the mean number of transforming particules penetrating into a coccus, and μ_A and μ_B the respective probabilities of integration of markers A and B ; then the proportion of transfor - mants for A will be :

$$t_A = i.\ a.\ \mu_A \qquad (1)$$

and in the culture, if N is the number of c.f.u./ml and n the number of trans formable chromosomes per c.f.u. :

$$A = n.N.\ t_A \qquad (2)$$

$$B = n.N.\ t_B \qquad (3)$$

When a marker is integrated, its genetic information is transmitted into one, or the other, or both strands of recipient DNA. Let α, β, γ, be the respective probabilities of these events, with :

$$\alpha + \beta + \gamma = 1 \qquad (4)$$

If there are integrations of markers A and B in the same chromosome, the probability to get a stable doubly transformed chromosome is :

$$P_{AB} = \alpha_A\ (\alpha_B + \gamma_B) + \beta_A\ (\beta_B + \gamma_B) + \gamma_A \qquad (5)$$

and the number of double transformants is :

$$D = n.N.t_A.t_B.P_{AB} \qquad (6)$$

By comparison between (2), (3) and (6) :

$$R = D.N./A.B. = P_{AB}/n$$

Thus, for a same competent culture treated by DNA bearing several markers, the determination of R leads to a comparison of the different P_{AB} values.

An alternative hypothesis is that the transformation culture is heterogeneous and that HE and LE markers modify the recipient genome in the same way. Among the n transformable chromosomes per c.f.u., there are x transformable only by HE markers, y only by LE markers and z by HE and

LE markers. It is easily shown that :

$$R_{HE-HE} = \frac{1}{x+z} P_{HE-HE} \; ; \quad R_{LE-LE} = \frac{1}{y+z} P_{LE-LE}$$

$$R_{LE-HE} = \frac{z}{x+z} P_{LE-HE}$$

Since $P_{HE-HE} = P_{LE-LE} = P_{LE-HE}$:

$$\frac{R_{LE-HE}}{R_{HE-HE}} = \frac{z}{y+z} < 1 \; ; \quad \frac{R_{LE-HE}}{R_{LE-LE}} = \frac{z}{x+z} < 1$$

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