THE INTEGRATION PROCESS IN THE BACILLUS SUBTILIS TRANSFORMATION SYSTEM

by

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Introduction

The transformation process of bacteria by DNA has two main phases: i) the uptake of donor DNA and ii) the integration of the introduced marker (s), followed by its expression. These two phases have been distinguished and studied by many authors. The characteristics of the first phase are rather clear, and we know some physiological (and genetical) factors such as competence, which control the uptake and penetration of donor DNA. The main interest from a genetical point of view, however, lies in the second phase. The term integration is used in a complex sense including all the features of transformation from the time of synapsis till the expression of the newly acquired character. Thus recombination is only one (important) feature of this process.

The fate of the introduced DNA has been followed in several experiments and it has been shown that its biological activity is lost immediately after the uptake and a short period is needed for the recovery of this activity. This "eclipse" period has been explained by L a c k s (1962). He has found that the DNA which penetrates the cells has a single stranded structure and has no transforming activity. The reappearance of this activity indicates that the donor DNA is in a native form once again. At the same time the ratio of the donor and recipient DNA's biological activity has been found (as studied in subsequent transformation tests) to reach rapidly a constant value, i.e. after that time the introduced and resident markers replicate at the same rate. This was regarded as a true sign of integration. Recombination as reflected by the linked transforming activity of resident and introduced markers follows some minutes after this time (F o x 1960).

Some other experimental results suggest, however, that integration (in a wider sense) is a more protracted process, because the transformed cells acquire an introduced character in two rounds of replication (E p h r u s s i — T a y l o r 1960).

The purpose of the experiments described in this paper was to elucidate these different features of integration.

The transformation rate in the indole auxotroph Bacillus subtilis was studied during the first few cell divisions after the end of exposure to trans-

forming indole prototrophic DNA, and the following questions were considered:

(1) What kind of relationship exists between the decrease of the transformation rate and the number of cell divisions completed after the DNA incubation?

(2) What is the origin of the observed difference in the decrease of transformation rate depending on the tryptophan (indole) content of the plating media?

Materials and Methods

(a) *Media*

Absolute minimal diluent (AMD) contained per 1000 ml of water: 14.0 g K_2HPO_4; 6.0 g KH_2PO_4; 1.0 g Sodium citrate·2 H_2O; 2.0 g $(NH_4)_2SO_4$; 0.22 g $MgSO_4 \cdot 7\ H_2O$. The pH was adjusted to 7.2 with NaOH.

Relative minimal diluent (RMD) contained all components of the AMD plus 0.01 g tryptophan, 0.1 g casamino acid, 5.0 g glucose and 0.0476 g $MgCl_2$.

Minimal medium (MM) contained all components of the AMD plus 0.050 g tryptophan, 0.2 g casamino acid and 5.0 g glucose.

Transformation medium (TM) was similar to RMD, but with a tryptophan content of 0.005 g.

Yeast-pepton medium (YPM) 200 g of baker's yeast plus 800 ml water was autoclaved at 120°C for 30 minutes, then the suspension was centrifuged — 200 ml of the supernatant was diluted with 800 ml water, 10.0 g pepton was added, the pH was adjusted with NaOH to 7.4 and autoclaved again.

Minimal plating medium (MPM) contained all components of the AMD plus 0.125 g casamino acid, 5.0 g glucose, 0.0476 g $MgCl_2$ and 20.0 g agar.

Complete plating medium (CPM) contained all components of the minimal plating medium plus 0.040 g tryptophan.

The casamino acid used for the preparation of diluents and media was an acid-hydrolitic product of casein and was perfectly free of tryptophan.

(b) *Culture and transformation techniques*

The bacterium used was the indole requiring mutant of *Bacillus subtilis* strain 168 which originated from the collection of the Microbiological Institute of the Med. Univ. of Szeged (Hungary).

The transforming DNA was prepared from the prototrophic strain 168 (derived from the 168 ind^- strain by transformation) according to the method of Marmur (1961), and diethylpyrocarbonate was used during the preparation as a nuclease inhibitor (Fedorcsák & Turtóczky 1966, Fedorcsák & Ehrenberg 1966).

The procedure of the transformation was a slightly modified method of Anagnostopoulos & Spizizen (1961): *Bacillus subtilis* 168 ind^- was grown in test tubes on nutrient agar for 14 – 16 hours at 37°C. Then the cells were collected in 3 ml of minimal medium. The suspension was poured into an Erlenmayer flask and was violently shaken for 15 minutes. Then it was diluted with MM to get a suspension containing $5 \cdot 10^7 - 10^8$ cells/ml, and shaken for 285 minutes at 37°C.

One millilitre of this suspension was centrifuged and resuspended in 5 ml of TM and shaken again for 40 minutes at 37°C.

Then 0.5 ml of transforming DNA solution (containing 200 μg DNA per ml) was added and the shaking continued for a further 60 minutes at 37°C. The interaction of DNA with the cells was stopped by adding 0.5 ml of DN ase solution (1 mg DNase per ml). After two minutes the suspension was centrifuged and resuspended in 20 ml of TM plus 2 ml of YPM, while shaking was continued at 37°C for 210 minutes.

At various intervals during this incubation samples were taken:

a) One part of the sample was diluted with RMD and was plated on CPM for the determination of the number of cell divisions completed after the end of exposure to transforming DNA.

b) Another part of the sample was washed in AMD three times by means of centrifugation and

1. one part of the washed sample was resuspended and diluted in AMD and plated on MPM (=AM-plating, i.e. absolute minimal plating).
2. The other part of the washed sample was resuspended, diluted in RMD and plated on MPM (=RM plating, i.e. relative minimal plating, due to the small amount of tryptophan in RMD) as well as on CPM (=C plating i.e. complete plating).

This way we have counted the number of colonies on absolute minimal, relative minimal and complete media, and we have calculated the transformation rate under these conditions. To be able to compare the results from different experiments we have introduced the term "comparative transformation rate", that is, in each experiment the 0 time transformation rate (calculated from RM-C plating) was taken as 100 arbitrary units and the transformation rates found after different times of incubation were expressed as the percentages of this value.

Results and Discussion

Data from a representative experiment are given in Table 1. The results shown in Fig. 1 indicate that the transformation rate decreases as a function of the number of cell divisions.

Taking the transformation rate at 0 minutes as 100 (counted in RM-C), after one cell division the transformation rate (comparative transformation rate) was 50, after two cell divisions it decreased to 25, and after three cell divisions it was about 12.5.

On the other hand the comparative transformation rate in the AM plating was found to be 50 at 0 time and after the first cell division it was 25. Between the first and second generation the comparative transformation rate was maintained at the same level and after the second division it corresponded to the RM-C value. These results were highly reproducible.

As to the origin of this regular decrease of transformation rate first we had to test the possibility, whether the rate of multiplication in ind^- and ind^+ strains was different:

Table I.

Dependence of the rate of transformation on the plating media and the time elapsed since the termination of DNA incubation

(Data from one representative experiment)

RM, AM and C plating-see Materials and Methods. Transformation rate: percentage of transformation to prototrophy counted from the RM − C and from the AM − C values, respectively. The comparative transformation rate is expressed as the percentage of the transformation rate found in RM − C plating at 0 minutes. The cell division was counted separately without washing.

Time (minutes)		0			50			95			155			195	
Plating		RM	AM	C	RM	AM	C	RM	AM	C	RM	AM	C	RM	C
Number of colonies counted	10^2 times diluted sample	70	40		67	33		72	66		75	77		102	
36 hours after the plating of 0.05 ml	10^5 times diluted sample			20			39			69			163		283
Transformation rate		0.35	0.20		0.17	0.08		0.10	0.10		0.05	0.05		0.04	
Comparative transformation rate		100	57		49	22		30	27		13	13		10	
Number of cell divisions completed after the DNA incubation			0			1.0			1.7			3.0		3.8	

We mixed cells of ind$^-$ and ind$^+$ strains (the latter taken from a transformed 168 ind$^-$ strain preserved as a "homozygous" pure culture) in the proportion of 1000 : 3 which corresponds to the usual transformation rate.

The mixture was incubated and the percentage of ind$^+$ cells present in the mixture was tested in the course of 4 generations. The number of the ind$^-$ cells expressed as the percentage of total cell number remained constant. Consequently the decrease of the transformation rate cannot be explained by the unequal division rate of the ind$^+$ and ind$^-$ cells.

It can be seen from the data presented in Fig. 1 that the absolute number of the transformed cells was approximately constant while the total viable count indicated three cell generations. This can be explained in two different ways:

(i) the transformed cells — due to the transformation — were in a physiological state i.e. in a lag phase, which delayed the onset of their division;

(ii) the transformed cells similarly due to the transformation were in a genetical (heterozygotic like) state, which caused a lag in the increase of their number, because the introduced marker segregated in the progeny.

The main difference between the two explanations is, that according to the first one the transformed cells do not, while according to the second one they do divide.

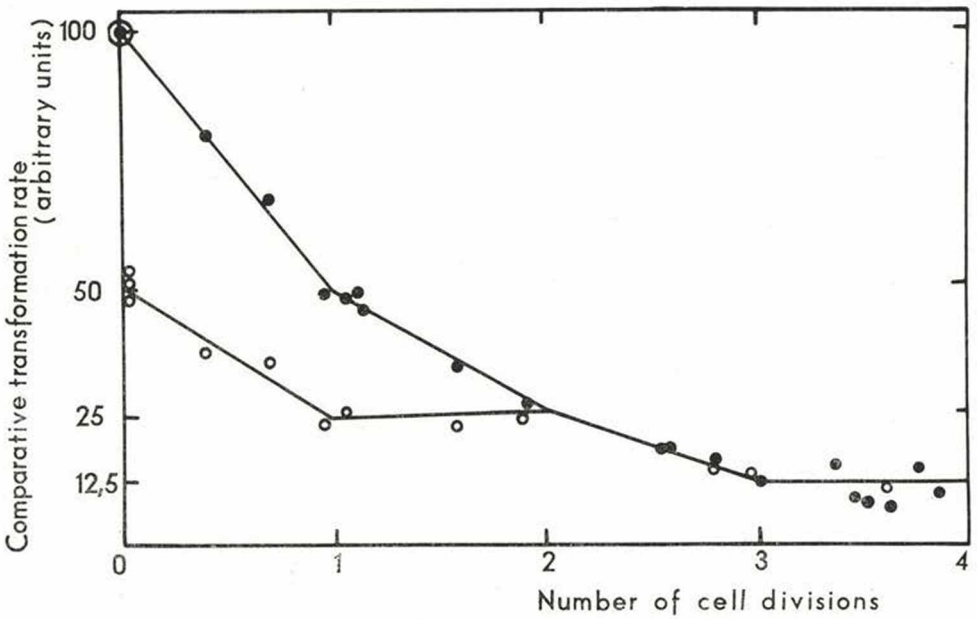

Fig. 1. The comparative rate of transformation plotted as a function of the number of cell divisions. The plotted points represent an average value of three different experiments. The range of variation in the comparative transformation rate was consistently below 8%
–●–●–, values counted from the RM – C plating; – ○ – ○ –, values counted from the AM – C plating

Recently K a m m e n et al. (1966) have studied the stabilization of "potential transformants" in *Bacillus subtilis*. The authors published in Fig. 4 the results which are in accordance with our experiments. If we translate the data of Fig. 4 of K a m m e n et al. (1966) into our terms we may see that the absolute number of transformed cells did not change for three cell generations, i.e. the comparative transformation rate decreased in accordance with our data. N e s t e r & S t o c k e r (1963) explain their result by supposing a biosynthetic latency (a not dividing period) in the transformed cells.

Since many authors refer to these basic results of N e s t e r & S t o c k e r it seems necessary to discuss their results in greater detail.

N e s t e r & S t o c k e r used penicillin to discriminate between growing and resting cells. It has been shown by the above authors that the competent cells of *Bacillus subtilis* are resistant to the action of penicillin. This means that they are in a resting (not dividing) state. The important question is now whether or not this latency is continued after the DNA uptake.

We have to refer to Fig. 4 of N e s t e r & S t o c k e r's paper which proves without doubt that the transformed and untrasformed cells are equally sensitive to penicillin treatment: they are dividing at the same rate at 37°C. The rate of transformation remained constant throughout the experiment under these conditions. So the answer can be given to the above question that the

transformed population at 37°C does not inherit the latency and penicillin resistance of the competent cells. Still in the control experiment – without penicillin – the number of the transformed cells started to rise only after a three cell division lag. A similar experiment carried out at 40°C gave the same results: although the "lag" in the multiplication of transformants was shorter in time but this time corresponded also to three cell divisions.

Though the time of the "lag" is shorter at higher temperatures the duration of the lag appears to be dependent on the growth rate of the recipient population and not on the temperature per se. This non-dependence of temperature is somewhat against a biosynthetic lag-explanation.

The same kind of experiments performed at 32°C provided data somewhat at variance with those described above. At 32°C in the absence of penicillin the number of transformants remained constant for eight cell generations. This longer lag can be explained by the experiments demonstrated in Fig. 3 of Nester & Stocker's paper: as reflected by their penicillin resistance the transformed cells started to divide after 5 cell generations; consequently only the last 3rd cell division lag, characterized by penicillin sensitivity is identical with the lag observed at 37°C or 40°C. From these results it can be concluded that at 32°C the resting state of competent cells is continued after the DNA uptake for 5 cell generations (presumably because of the lower temperature; its effect is also shown by the slower growth of the population), but the 3 cell generation long "virtual lag" was clearly distinguishable at 32°C, too. The difference between these two kinds of lag is also emphasized by the fact that the expression of the introduced try^+ marker (measured by tryptophan synthetase activity) starts at about the same time as the cells become sensitive to penicillin.

All these results indicate that the transformed cells have to pass through a "virtual lag" period, which lasts – irrespective of the growth conditions – for three cell generations. During this time the transformed cells are dividing, and yet they do not increase in number.

Voll & Goodgal (1961) following the biological activity of the introduced DNA have shown that after a one generation lag, the donor transforming factor (new marker) and the recipient transforming factor (resident marker) increased at the same rate. A similar but even shorter period was recorded by Fox (1960) for the same phenomenon.

These results indicate that the intracellular replication of the donor marker starts after about one cell division in spite of the well-known fact (demonstrated by Voll & Goodgal, too), that the absolute number of the transformed cells increases after a three cell division lag only. Similar results were obtained by Bodmer (1965) who by using a ^2H and ^{15}N labeling and picnographic distinction method has demonstrated that the replication of the donor DNA activity as shown by its shift to heavier (hybrid and heavy) regions, occurs during the second and third DNA cycle, respectively. The absolute number of the transformants, however, starts to increase only after the third cell division.

All these experiments suggest a genetical rather than physiological background of the three cell generation long "virtual lag" found by several authors, and which was demonstrated by us as the decrease of the comparative transformation rate.

As a further test of the possibility of a physiological lag we have performed an experiment in which after the DNase termination of DNA treatment the cells were frozen in 10% glycerine medium and stored at −20°C. After 24 hours the experiment was continued. The cells were transferred into TM-YPM medium and were shaken at 37°C for 210 minutes. At various intervals samples were taken and the transformation rate was determined as in the earlier experiments. The results were identical with those demonstrated in Fig. 1. This means that such a drastic treatment as the interruption of the physiological functions by freezing (in the whole population!) did not influence the three cell generation "lag".

If the explanation were right, that the biosynthetic latency of the competent cells is preserved after the DNA treatment and it causes a decrease of the transformation rate, then bringing the majority of the population on the same level of activity – either by higher degree of competence or e.g. freezing – would abolish this decrease.

On the basis of a genetical explanation and using all the known facts an integration model, illustrated in Fig. 2 can be offered. According to our model the transformation and integration occurs in single stranded DNA. The existence of such a structure i.e. a double helix, composed of a donor and a recipient strand, has been shown by many authors (Fox 1966, Notani & Goodgal 1966, Bodmer 1966). The integration (and recombination) is complete at the first cell division and the replication of the donor marker starts only during the second DNA cycle. Since the DNA of a transformed nuclear body is heterozygous after the integration with respect to the introduced marker, a strand segregation of this marker will be expected in the second division cycle. However, *Bacillus subtilis* has two nuclear bodies (Vinter 1966) therefore a further segregation (nuclear segregation) is also to be expected during the third division.

The segregation model illustrated in Fig. 2 (part A) explains the decrease of the transformation rate which is shown in Fig. 1 (RM-C plating). The model agrees with the results of Ephrussi-Taylor (1960), Voll & Goodgal (1961), Lacks (1962), Nester & Stocker (1963) and Bodmer (1965) concerning the replication of the introduced marker(s).

As to the results of Nester & Stocker we can conclude that the segregation is responsible for penicillin sensitivity without any detectable increase in transformants. But if segregation causes the decrease of the transformation rate it might be expected that the ratio of transformed and untransformed cells would decrease even in the presence of penicillin. However this ratio is unchanged. The evident explanation is that since penicillin acts on dividing cells it does not allow the expression of any segregation.

The integration model (Fig. 2 part B) also explains the fact that the number of transformants in our experiments was lower in absolutely tryptophan-free milieu during the first two divisions (Fig. 1 AM-C plating). As can be seen the difference in the two kinds of platings disappears at the second cell division.

As the transformation occurs in single stranded DNA and it is not predetermined as to which of the strands will be incorporated and integrated

(cf. Fox 1966) two kinds of transformants should occur with equal probaility: transformed in the codon and in the anticodon.

Results by Bautz & Hall (1962) and Champe & Benzer (1962) showed that only one of the strands of DNA is copied by messenger RNA.

Evidently in the double stranded DNA molecule where a certain part of "A" strand codes for a "c" trait, the corresponding segment of the complementary "B" could not code for this "c" trait. Neither is it possible that this segment of "B" could code for another "d" trait because in that case an alteration of this segment (mutation) would alter both "c" and "d" and only double transformants or mutants would occur. Accordingly we can designate the two chains for a definite marker as the anticode and code strand. If the code strand is the chain which serves as a template for the messenger RNA, then in the case of anticodon transformation the "dominant phenotype" is expressed only if the new code strand is allowed to turn up (by the first replication of the segment). This makes the start of messenger RNA and protein synthesis, responsible for the indole production possible. Evidently until this time the cell transformed in this anticodon is in need of some exogeneous tryptophan.

In our experiment the basis of distinction between absolute minimal and relative minimal plating was that in the case of relative minimal plating we diluted and plated the washed cell suspension in the presence of a small amount of tryptophan, where both types of transformants could grow. On the other hand in the case of absolute minimal plating the same cell suspension was diluted and plated in an absolute tryptophan-free milieu where the cells transformed in the anticodon were lost.

Segregation of cells transformed in codon and in anticodon, respectively, is shown in Fig. 2 part A. B. According to this model until the second cell division twice as much colonies will be received in RM plating than in AM (tryptophan-free) plating. In the AM plating only cells, transformed in the codon i.e. transformed in the geno- and phenotype can multiply and the cells transformed in the anticodon (i.e. transformed in genotype only) are eliminated. This difference between RM and AM platings disappears after the second division.

Our results can be interpreted as follows:

Messenger RNA is copied from only one of the two strands (the code strand, Champe & Benzer 1962).

Transformation is carried out by a single strand of DNA double helix (Lacks 1962) and there are two kinds of transformants: transformed in the codon and in the anticodon. These two types occur with the same probability (cf. Fox 1966).

The observation that the difference between AM and RM platings is reduced in the course of the second division can be interpreted by the fact that the first replication of the introduced segment occured only at the second cell generation. This is in contradiction with the insertion model of transformation. If the insertion model had been in action, then the segregation of donor and recipient strands should have been completed by the first cell division. Here it seems plausible to mention some other results which are somewhat against a breakage and reunion model. The finding of Fox (1966), that the inactiva-

tion rate of ^{32}P labeled DNA is the same before and after integration, suggests that no fragmentation occurs. Another interesting fact was shown by E p h r u s s i - T a y l o r (1966). They have found, that caffein, known to inhibit repair of ultraviolet damage (through the inhibition of exicision enzymes) does not influence the integration.

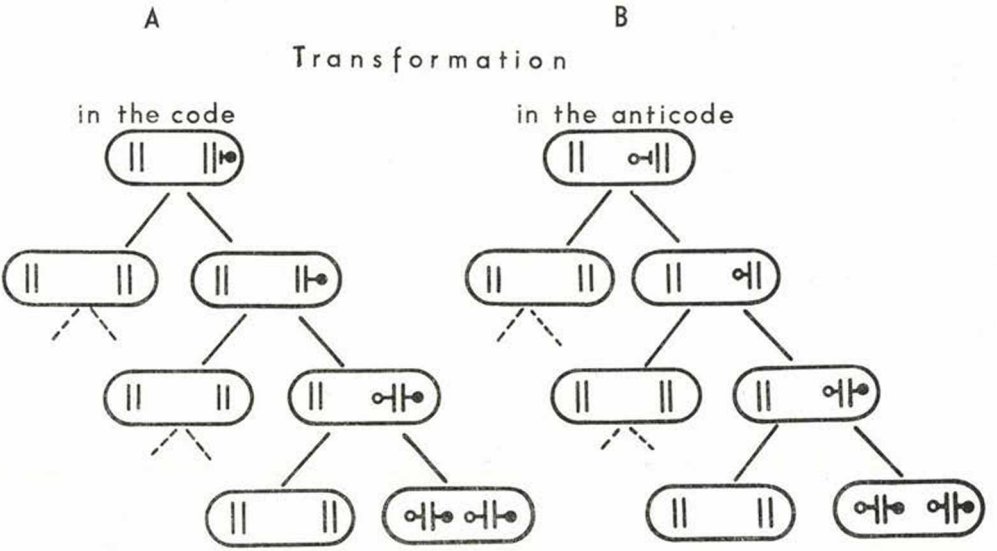

Fig. 2. Transformation in the code and in the anticode

| = auxotrophic DNA's strand; |–● = prototrophic DNA's code strand; ○| = prototrophic DNA's anticode strand

At the first DNA cycle the introduced segment appears as the preformed fragment of the replica to be newly synthesized and the other parts of the replica are templated from the recipient DNA and linkage appears between introduced and resident markers. Perhaps this requirement of synthesis explains why some minutes have to elapse after the reappearance of biological activity, before the linkage of introduced and resident markers becomes detectable (F o x 1960). On the basis of an insertion model it is difficult to explain this difference between integration and recombination.

Following integration the two strands are partly incomplementary and this incomplementarity is abolished at the second cell division.

The strength of this model is that it is in accordance with most of the known experimental facts of transformation.

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Summary

An indole requiring mutant of Bacillus subtilis strain 168 was transformed to prototrophy, and the expression of the introduced marker was studied for several cell generations. The transformation rate was found to decrease for three cell divisions. At the 0, 1st, 2nd and 3rd cell division (completed in a rich medium after the incubation with transforming DNA has been terminated by DNase) the rate of transformation was 1/1, 1/2, 1/4 and 1/8, respectively, when plated on relative minimal medium. However, when the cells were washed before plating on absolute tryptophan-free media, the respective transformation rates were found to be 1/2, 1/4, 1/4 and 1/8. Since freezing and storage of the DNA treated population did not alter these features, a genetical rather than a physiological explanation is offered. According to the suggested model, the transformation is carried out by single stranded DNA and the decrease of the transformation rate is due to strand segregation of the hybrid DNA. It is not predetermined which strand of the transforming DNA will be integrated, consequently there exist two kinds of transformants: cells transformed by the codon and cells transformed by the anticodon DNA strand, the first being transformed both in the genotype and phenotype, the second in the genotype only. Cells transformed only in the genotype require two divisions for the phenotypic expression of the introduced marker.

REFERENCES

Anagnostopoulos, C. Spizizen, J. 1961. Requirements for transformation in Bacillus subtilis. J. Bacteriol. **81**: 741–746.

Bautz, E. K. F. & Hall, B. D. 1962. The isolation of T4 specific RNA on a DNA cellulose column. Proc. Natl. Acad. Sci. U. S. **48**: 400–408.

Bodmer, W. F. 1965. Recombination and Integration in *Bacillus subtilis* transformation: Involvement of DNA synthesis. J. Mol. Biol. **14**: 534–557.

Bodmer, W. F. 1966. Integration of deoxyribonucleasetreated DNA in *Bacillus subtilis* transformation. J. Gen. Physiol. **49**: 233–258.

Champe, S. P. & Benzer, S. 1962. Reversal of mutant phenotypes by 5-fluorouracil: an approach to nucleotid sequences in messenger-RNA. Proc. Natl. Acad. Sci. U.S. **48**: 532–546.

Ephrussi-Taylor, H. E. 1960. On the biological function of deoxyribonucleic acid. Symp. Soc. Gen. Microbiol. **10**: 132–154.

Ephrussi Taylor, H. E. 1966. Genetic studies of recombining DNA in pneumococcal transformation. J. Gen. Physiol. **49**: 211–231.

Fedorcsák, I. & Turtóczky, I. 1966. Effects of the Diethyl Ester of Pyrocarbonic Acid on bacteriophage and transforming DNA. Nature **209**: 830–831.

Fedorcsák, I. & Ehrenberg, L. 1966. Effect of Diethylpyrocarbonate and methylmethanesulfonate on nucleic acids and nucleases. Acta Chem. Scand. **20**: 107–112.

Fox, M. S. 1960. Fate of transforming deoxyribonucleate following fixation by transformable bacteria. Nature **187**: 1002–1006.

Fox, M. S. 1966. On the mechanism of integration of transforming deoxyribonucleate. J. Gen. Physiol. **49**: 183–196.

Kammen, H. O., Beloff, R. H. & Canellakis, E. S. 1966. Transformation in Bacillus subtilis. Biochim. Biophys. Acta **123**: 39–55.

Lacks, S. 1962. Molecular fate of DNA in genetic transformation of Pneumococcus. J. Mol. Biol. **5:** 119 – 131.

Marmur, J. 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms J. Mol. Biol. **3:** 208 – 218.

Nester, E. W. & Stocker, B. A. D. 1963. Biosynthetic latency in early stage of deoxyribonucleic acid transformation in Bacillus subtilis. J. Bacteriol. **86:** 785 – 796.

Notani, N. & Goodgal, S. H. 1966. On the nature of recombinants formed during transformation in Haemophilus influenzae. J. Gen. Physiol. **49:** 197 – 209.

Vinter, V. 1966. Developmental cycles of sporeformers: a cellular type of differentiation in bacteria. Fol. Microbiol. **12:** 89 – 100.

Voll, M. J. & Goodgal, S. H. 1961. Recombination during transformation in Haemophilus influenzae. Proc. Natl. Acad. Sci. Wash. **47:** 505 – 512.