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A CONTRIBUTION TO THE KNOWLEDGE OF PNEUMOCOCCUS TRANSFORMATION DURING THE PERIOD BETWEEN THE INCORPORATION OF DEOXYRIBONUCLEIC ACID AND THE APPEARANCE OF STREPTOMYCIN RESISTANCE

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SUMMARY

1. In pneumococcus transformation, the appearance of SM-resistants from a population of sensitive cells which incorporated DNA at approximately the same time followed the normal frequency distribution during a period of 100 min after the exposure of cells to the DNA.

2. The phenomenon depended on temperature, the optimal temperature being 37° after the incorporation of DNA into the cells, and required an external supply of nutrients.

INTRODUCTION

In bacterial transformation, recipient cells express one or more of the genetic characteristics of the donor strain after the incorporation of DNA, where the DNA may at first be absorbed and incorporated into the growing cells of transformable phase and then fixed on the genetic apparatus of the cells to express its function[1]. Attention of several workers, in this connection, has been focussed on the competence of recipient cells[2,3], kinetic analysis of the reaction between the cells and DNA[3,4,5], or on the mechanism of incorporation of DNA by the cells[1]. No precise account has been reported of any attempt to elucidate the process during the period between the invasion of DNA and appearance of SM resistance. The present report deals with this process and shows that the time required for cells to develop SM resistance after the invasion

Abbreviations: DNA: deoxyribonucleate; RNA: ribonucleate; DNase: deoxyribonuclease; RNase: ribonuclease; SM: streptomycin.

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of DNA varies individually among the cells in a culture and that the appearance of resistant cells per unit time is an approximation to the normal frequency distribution by exposure of the cells to DNA.

MATERIALS AND METHODS

Organisms

SM-resistant strain *Diplococcus pneumoniae* RSt5b and SM-sensitive strain R19 were used as the donor and recipient of DNA, respectively. Both strains were provided by Dr. R. D. HOTCHKISS.

Chemicals

RNase, cryst. from ethyl alcohol, Worthington Chem. Corp. was used. Crude DNase, prepared from beef pancreas according to KUNITZ[6], was used. Crystallized DNase, Worthington Chem. Corp. was also used to confirm our crude preparation giving no different results. Meat infusion was prepared from fresh meat by the conventional method. Heart Infusion Broth, Difco, Yeast Extract, Difco, Neopepton, Difco and Bovine albumin, Armour Lab., were used for the medium. Decolorizing carbon, Norit S.X. 30, was used; this was heated just prior to use.

Media

The organisms for the extraction of DNA were grown in a meat infusion broth, pH 7.6–7.8, containing 1 % Neopepton, 0.25 % $Na_2HPO_4 \cdot 2H_2O$ and 0.1 % Yeast Extract. The basal medium for transformation reaction consisted of 1 % Heart Infusion, 1 % Neopepton and 0.25 % $Na_2HPO_4 \cdot 2H_2O$ to give the final pH of 7.7. Before the addition of phosphate the medium was treated with Norit at pH 5.5. For the determination of total cells and transformants count, solid medium of the same composition as above was used with the addition of 1 % agar and 10 % horse serum heated at 56° for 30 min.

Preparation of DNA (transforming principle)

Cultivation of cells and extraction of DNA were carried out according to MACLEOD AND KRAUSS[7] in order to obtain partially purified DNA preparation from which RNA was removed by the method of AVERY *et al.*[8]. Sometimes, RNA was removed by incubation with RNase followed by adsorption twice with Norit charcoal. After the removal of RNA, DNA was precipitated with twice as much alcohol and redissolved in saline. After repeated precipitation and dissolution, the final DNA preparation in sterilized saline containing about 200 μg DNA per ml was obtained and stored in refrigerator.

Transformation reaction

Inoculum cells were grown for 16 h at 37° in the basal medium with 0.05 % glucose. From this preculture, about 10^5 cells were transferred to 10 ml of the basal medium with 0.2 % glucose and 0.2 % albumin and allowed to grow for 4–5 h at 37°, to give what could be used as competent cells. Nine parts of the competent culture and 1 part of DNA solution (20–50 μg) were mixed at 37° and incubated for 5 min;

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this was followed by the addition of DNase dissolved in 0.03 M $MgSO_4$ and incubation was continued at 37°, unless otherwise indicated.

Assay of transformants

A suitable aliquot of the culture being transformed at each time interval was pipetted out and diluted to an appropriate proportion in the cold basal medium and distributed in the solid medium containing 150 μg per ml of SM-sulfate and horse serum. After the incubation for 24 h at 37°, the colonies of SM-resistants were enumerated.

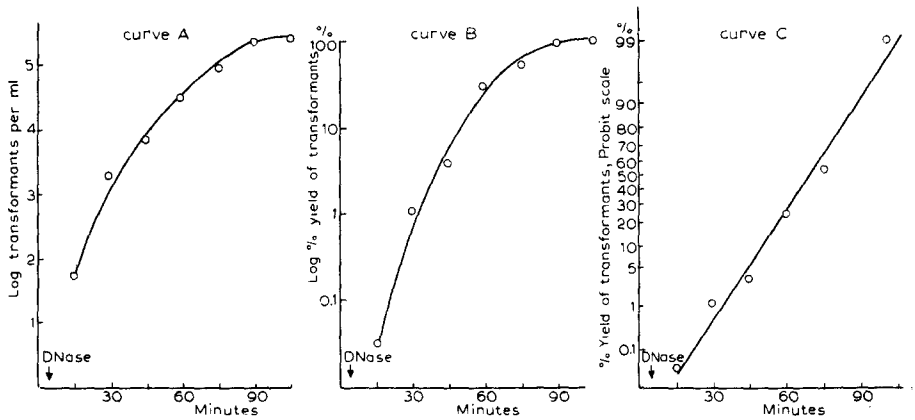

Fig. 1. Appearance of SM-resistants after incorporation of DNA into cells. DNase was added at 5 min after the addition of DNA. The number of transformants is plotted on a logarithmic scale in curve A. The percentage yield of transformants is plotted on a logarithmic scale in curve B and on a probit scale in curve C.

RESULTS

Appearance of transformants after the exposure of cells to DNA

In order to make clear the figures for the appearance of SM-resistants after the limited time exposure of cells to DNA, the following typical transformation experiments were carried out. Contact of cells with DNA for 5 min at 37° was terminated by the addition of DNase and the appropriate aliquots were taken up at 15 min intervals to assay the SM-resistants. The typical result obtained is given in Fig. 1. In curve A, the increments of SM-resistants were plotted on a logarithmic scale as a function of sampling time after the addition of DNA. As can be seen, SM-resistants increase even during the first 15 min and rise until they reach to the maximal yield at 90–100 min, showing that the curve has no lag period. In curve B, the yield, expressed in percentage of the total transformants was plotted on a logarithmic scale as a function of sampling time. If the percentage yield is plotted on a probit scale as a function of sampling time, the curve C is given. It is apparent that the probit values follow a linear function of sampling time. In other words, the appearance of transformants per unit time is normally distributed during the time course of 100 min following the incorporation of DNA. The same experiments carried out more than 10 times showed identical results without exception.

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The effect of environment on the appearance of SM-resistants

In order to discover the factors determining the above-mentioned distribution of duration of the expression of SM resistance among the cells, the following experiments were carried out, in which the effect of the environment of the cells on the distribution curve were examined.

(a) *The effect of temperature.* After the exposure of 8 tubes of cells to DNA for 5 min at 37°, the tubes were divided into 4 groups (duplicate tubes for a given temperature) and immersed in water-baths kept at 37°, 25°, 15°, and 0°, respectively. At each time-interval, an aliquot was taken from tubes of the 4 groups, diluted and mixed with SM agar to assay SM-resistants. Being treated as in the case of Fig. 1, the percentage yield of SM-resistants was plotted on a probit scale as a function of sampling time. The results are shown in Fig. 2. The slope of the curve is maximum at 37° and declines as the temperature is lowered. At temperatures higher than 37°,

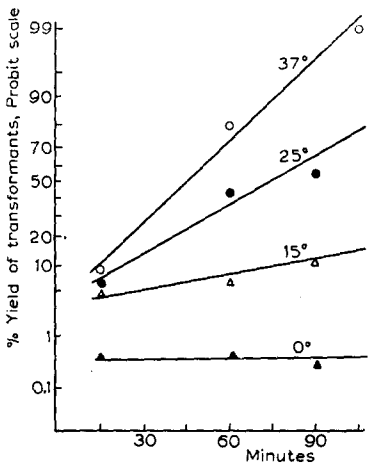

Fig. 2. Temperature dependence of the appearance of SM resistance after the incorporation of DNA into cells.

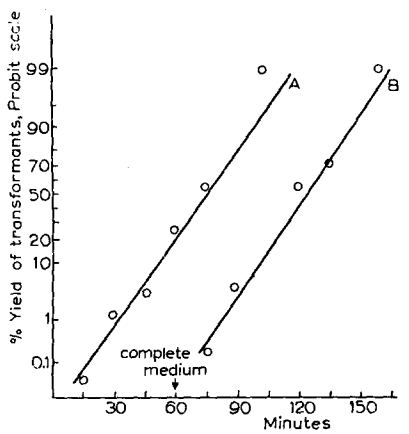

Fig. 3. Effect of external nutrition. A: Control, B: DNA-incorporated cells were suspended in saline for 60 min, centrifuged and resuspended in complete medium.

for instance 42°, the linear relationship does not hold. It seemed that the optimal temperature for growth is required for the maximal expression of SM resistance.

(b) *The effect of external nutrition.* An experiment was undertaken to discover how the incorporated DNA and cells behave when extracellular nutrients are not available and whether or not the curve of appearance of SM-resistants changes under such conditions. Cells which have been exposed to DNA in the same way as described in (a) were cooled, centrifuged at 10,000 rev/min for 5 min, and resuspended in the original volume of saline. Control cells were resuspended in fresh complete medium. These two cell suspensions were incubated for 100 min at 37° and assayed for SM-resistants. The suspension in saline showed negligible amounts of SM-resistants which seemed to have appeared during the process of making the suspension, although the control suspension showed the existence of a sufficient amount of SM-resistants. If the cell suspension in saline was recentrifuged at 60 min, resuspended in complete medium and incubated at 37°, SM-resistants appeared. The percentage yield of the

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SM-resistants plotted as a function of time is presented in Fig. 3. The curve after the resuspension in complete medium is essentially identical with the typical transformation reaction, thus indicating that the expression of SM resistance in the re-incubated cells in complete medium follows the same processes as the normal one. It seemed therefore that the DNA incorporated into cells is not able to express SM resistance without an external supply of nutrition and that, within the cells suspended in saline, the incorporated DNA does not behave in such a manner as to modify the distribution curve.

DISCUSSION

It was observed that, in *D. pneumoniae*, the maximum number of SM-resistant transformants was reached at about 1.5–2 h after the exposure to DNA. It seemed natural to assume a lag phase as is the case in the one-step growth experiment of phage

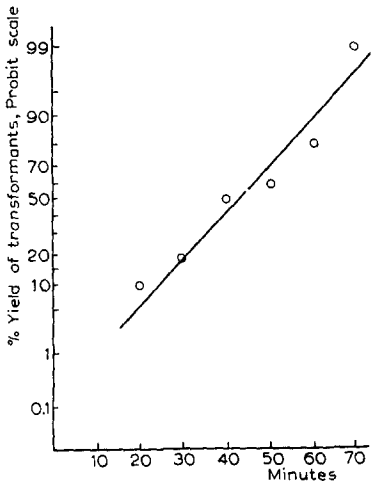

Fig. 4. Percentage yield of transformants on a probit scale. (Data derived from THOMAS[3] and re-calculated by us.)

infection. In fact, HOTCHKISS[9,10] described in his articles a sudden appearance of transformants after a lag of about 30 min during which drug resistance was not detected. The results presented here, by contrast, showed the existence of SM-resistants among cells even immediately after the exposure to DNA and of an increase in the number of such SM-resistants during subsequent incubation up to a certain maximal yield. A plot of percentage yield of SM-resistants on probit scale against sampling time gave a linear relationship, showing the normal distribution of SM-resistants per unit time throughout the period of the appearance of transformed cells. This fact would indicate that the time required for cells to become SM-resistant varied among individual cells in a culture. It may also be permissible to suppose that some factors participating in the reactions between the incorporated DNA and the cells or in the cellular responses to these interactions might be influenced by the law of frequency. Although THOMAS[3] recognized the observation of HOTCHKISS, the data in his figure are in accordance with our result (Fig. 4).

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It was also found in the present experiment that the duration of the process was dependent on temperature, and 37° was optimal. In addition, cells which incorporated DNA and were suspended in saline were not able to express drug resistance, but followed exactly the same process as normal ones when saline was replaced by complete medium. In other words, if external nutrients are not given to the DNA-incorporated cells, the cells would remain inert or exert an effect of undetectable level, if any, on the distribution curve. It may be evident that the phenomenon observed in this experiment is dependent on the growth of cells.

It has been believed that cell competence for the transformation is closely associated with the growth stage of cultures or division cycle of cells. Since the exposure of cells to DNA was limited to a short period in our present experiment, it might be considered that the cells used were uniformly competent in the sense of so-called transformability. In other words, the invasion of DNA might occur fairly synchronously. The fact that the appearance of SM-resistants, in spite of synchronous incorporation of DNA, is normally distributed at any given time during the period tested may suggest an interaction of some physiological factor(s) which can be distinguished from the "cell competence" for the transformation. Further work to analyse the process of these phenomena is now under way.

NOTE ADDED IN PROOF

After this manuscript was submitted, Dr. HOTCHKISS informed us by personal communication that FOX AND HOTCHKISS proposed briefly "the expression of SM-resistance distributed normally in time", at the International Biochemical Congress in Brussels in 1955. It coincides with our view. Since the work had not yet been published and was not known to us when the manuscript was written, we could not cite it.

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