INTEGRATION EFFICIENCY IN DNA-INDUCED TRANSFORMATION OF PNEUMOCOCCUS. I. A METHOD OF TRANSFORMATION IN SOLID MEDIUM AND ITS USE FOR ISOLATION OF TRANSFORMATION-DEFICIENT AND RECOMBINATION-MODIFIED MUTANTS

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ABSTRACT

A method of transformation on solid medium especially adapted for pneumococcus has been developed. Under specific conditions, all colonies that are allowed to grow in the presence of transforming DNA for six hours give rise to transformed bacteria. Combined with replica plating this technique has been used to isolate mutants modified with regard to recombination. Most of the mutants found are transformation-defective and show a large diversity in their response to ultraviolet light. Some of these mutants have lost their ability to take up transforming DNA. One shows a reduced yield of transformants for a given quantity of DNA taken up. Mutants that manifest altered behavior with regard to marker efficiencies have also been isolated. One of these exhibits a decrease in the transformation efficiency of only the high efficiency markers and two mutants show a decrease in the transformation efficiency of the low efficiency markers.

BACTERIAL transformation requires several steps, starting with the binding of free DNA by the competent cells and ending with the alteration of the recipient genome. In terms of biochemical events, only some of the steps determining the fate of the DNA after uptake are characterized in *Diplococcus pneumoniae*. The nature of the physiological changes which occur during the development of competence is not completely understood. Also poorly understood in pneumococcal transformation is the nature of the process capable of discriminating between various markers. A striking feature emerging from the genetic studies in *Diplococcus pneumoniae* is the difference in the efficiencies with which markers representing point mutations are transferred by transformation. According to their individual integration efficiencies, auxotrophic markers or those conferring resistance to antibiotics can be divided into several classes. Two classes, one corresponding to high efficiency, the other to low efficiency, were found for mutations in the *ami-A* locus (EPHRUSSI-TAYLOR, SICARD and KAMEN 1965; GRAY and EPHRUSSI-TAYLOR 1967) and for mutants requiring uracil (MORSE and LERMAN 1969), whereas four classes were found for mutations in the amylomaltose locus (LACKS 1966) and for mutations in the structural gene of dihydrofolate reductase (SIROTNAK and HACHTEL 1969). Regardless of the exact number

of efficiency classes, two classes are considered especially, the first with high efficiency (HE), the second with low efficiency (LE). The difference in efficiency of these two classes is about a factor of 10. Results from LACKS (1965), EPHRUSSI-TAYLOR (1966, 1968), and LOUARN and SICARD (1968, 1969) suggest that the integration mechanism of LE markers is different from that of HE markers. In order to study the nature of the transient events occurring during pneumococcal transformation and the nature of the mechanism of discrimination between markers, attempts were made to isolate transformation-deficient mutants as well as mutants whose behavior in relation to the integration efficiency of markers is altered. With this in mind, a method of selection, based on the fact that pneumococcus can be transformed on solid medium, was developed. This method proved to be efficient for isolating both transformation-deficient mutants and recombination-modified mutants.

MATERIALS AND METHODS

Strains: The parent strain of *D. pneumoniae* referred to as "Clone 3" (Cl_3) was used as recipient in the transformation experiments. This strain originates from AVERY's strain R 36 A (AVERY, MACLEOD and MACCARTY 1944). Donor DNA was isolated from strain 69. This strain bears two HE markers, one conferring resistance to 2000 μg/ml streptomycin: *str-r 41* (HOTCH-KISS 1951), the other to 0.1 μg/ml erythromycin: *ery-r 6* (GREEN 1959), and also bears two LE mutations, the first conferring resistance to 2×10^{-5} M aminopterin: *ami A-r9* (SICARD 1964) and the second to 5 μg/ml optochin: *opt-r 2* (EPHRUSSI-TAYLOR 1958).

The thymidine marker (*Thy A_2*) from the thymidine-requiring strain (FRIEDMAN and RAVIN 1972), generously provided by A. RAVIN, was introduced into the wild-type strain, Cl_3 (BRUNEL, SICARD and SICARD 1971). From the strain Cl_3Td^- which requires 60 μg of thymidine per ml for growth, a mutant requiring only 5 μg/ml was isolated. The strain Cl_3Td^- requiring 5 μg/ml of thymidine and also bearing the *str-r 41* marker was used for the preparation of the DNA labeled with tritiated thymidine.

Media: The media used for pneumococcal growth and transformation in liquid have been described in an earlier publication (SICARD 1964). The special solid transformation medium is prepared as follows: 7.5 g Difco Neopeptone, 7.5 g Difco Casamino acids and 13 g NaCl are dissolved in 1.5 liters distilled water. The pH is brought to 7.8 with 1N NaOH and the heavy flocculent precipitate is filtered off. The filtrate is distributed in fractions of 125 ml per flask to which is added 3 g of agar. The flasks are sterilized at 120° and stored at room temperature. Prior to use, this basal medium is melted, cooled to 55° and each is supplemented with 0.3 ml of 25% glucose and 50 ml of medium prepared as follows: 36 g of Difco Yeast Extract are dissolved in 330 ml distilled water and brought to pH 3 with 4N HCl. 15 g of charcoal (Billaut Charbon animal 15% cendres) are added with vigorous stirring. Absorption is allowed to take place for 2 hours at 4°. The charcoal is then filtered off on a large Buchner funnel, through Whatman #3 paper covered with a thin layer of filter-cell (Touzart et Matignon). The pH of the solution is then brought to 7.8 with 10 N NaOH and the heavy precipitate removed by filtration. Then 7.5 mg asparagine, 100 mg glutamine and 3 g fraction V bovine albumin are added. The volume is brought to 600 ml with distilled water and sterilized by passage through a membrane filter. This medium is kept frozen.

Preparation of ³H-labeled pneumococcal DNA: The low thymidine-requiring strain of pneumococcus carrying the streptomycin-resistant reference marker, *str-r 41*, was grown to a concentration of 2×10^8 colony-forming units/ml in 10 ml of complete neopeptone medium containing 500 μCi of (*Me-³H*) thymidine (1 Ci/mM, C.E.A. SACLAY). The cells were collected, washed and resuspended in 2 ml of 0.15M-NaCl 0.015M-EDTA (pH 8) with sodium deoxycholate to a concentration of 0.1%. After cell lysis, the nucleic acids were precipitated with one

volume of 95% ethanol. The fibers of nucleic acids were withdrawn with a Pasteur pipette and introduced into 4 ml 1/10 SSC (0.015M sodium chloride, 0.0015M sodium citrate). The tube was gently stirred at 37° until dissolution of the nucleic acid fibers.

Transformation procedure in liquid medium: The basic transformation techniques in liquid medium have been described previously (SICARD 1964). In some experiments competence was artificially induced by the pneumococcal-activator substance described by TOMASZ and HOTCHKISS (1964). Crude activator preparations were obtained by the method of TOMASZ and MOSSER (1966), as modified by VOVIS and BUTTIN (1970). Competent cells (1 ml) were treated with a solution of transforming DNA (0.1 μg/ml) for 15 min. at 37°. The bacteria were plated in non-selective medium for complete phenotypic expression. After 2½ hours' incubation at 37°, a second layer of nutrient agar containing the antibiotic was poured on the surface for selection, and the plates incubated overnight. The number of resistant colonies was then determined.

Uptake experiments: Two ml cultures of highly competent cells were prepared by the method using the pneumococcal activator substance. Tritium-labeled pneumococcal DNA was added to each culture at a final concentration of 0.25 μg/ml. After 20 minutes' incubation at 37°, bovine pancreas deoxyribonuclease (Worthington Biochemical Corp.) was added to a final concentration of 10 μg/ml and the incubation was continued for another 2 minutes. A sample was taken to determine the number of streptomycin-resistant transformants. Each radioactive DNA-cell complex was chilled in an ice bath for 2 minutes and washed 3 times with balanced salt medium (SICARD 1964). The last pellet was taken up in 1 ml 10% trichloroacetic acid and heated in boiling water for 30 minutes. After cooling, the proteins were removed by centrifugation, and a sample of the supernatant was added to 10 ml Bray's solution and counted in a liquid scintillator spectrometer (Nuclear Chicago).

Mutagenic treatment: One of the most active mutagenic agents for microorganisms was used: 1 methyl-3-nitro-1-nitrosoguanidine (NG). The following method of obtaining mutants was used: 1 ml of a culture of Cl_3 was taken 20 minutes after the competence peak and treated with 50 or 75 μg of NG for 30 minutes at 37°. After centrifugation and washing, the culture was diluted 500-fold into the growth medium, and 1 ml fractions were distributed into 10 small tubes After 8 hours of growth at 37°, glycerol was added to the cells at a final concentration of 12%. The number of *ami-r* and *opt-r* bacteria was then determined from only two tubes. Under these conditions the frequencies of mutants resistant to aminopterin (2 $\times$ 10^{-5} M/ml), optochin (5 μg/ml), erythromycin (0.1 μg/ml) or streptomycin (150 g/ml) were, respectively, 2 $\times$ 10^{-2}; 1 $\times$ 10^{-4}; 2.5 $\times$ 10^{-5} and 1 $\times$ 10^{-5}.

Ultraviolet irradiation of cells: Exponentially-growing cells, cultivated in the standard medium, were centrifuged, washed once and resuspended in balanced salt medium (SICARD 1964) at a concentration of about 1 $\times$ 10^{7} colony-forming units/ml. Two ml of cell suspension were poured into a petri dish of 5 cm diameter and immediately irradiated with stirring under a 15W General Electric germicidal lamp at a dose rate of 14 erg/mm^2 per sec. Samples were withdrawn at various times and plated in nutrient agar medium after appropriate dilutions. All these operations were made in the dark to avoid possible photoreactivation.

RESULTS

Procedure for obtaining transformation on solid medium: Bacteria from a Cl_3 culture diluted in order to obtain 100 to 150 colonies per plate are spread onto plates containing 10 ml of solid transformation medium. After 18 hours' incubation at 37°, a second thin layer of 3 ml transformation medium supplemented with 0.80% agar and containing saturating amounts of transforming DNA is slowly poured onto the surface of the plates. After 6 hours of incubation to allow transformation to take place, another layer of 10 ml rich medium containing 1.5% agar, 1% horse blood and the selective agent at the appropriate concentration, is cautiously poured onto the previous layers. After 48 hours of incubation

at 37° the colonies containing transformed bacteria resistant to the selective agent can easily be identified by their larger size and the color change in the blood.

This procedure for detecting transformed cells on the plate is preferable to a replica-plating method, since pneumococcus colonies are very small and transformants cannot be reliably picked up by the velvet.

Optimal transformation is obtained when the pH of the medium is between 7.8 and 8. Bovine albumin is an absolute requirement but horse blood should be added only after completion of transformation because it may contain DNA-inactivating nucleases.

In these conditions, 100% of the colonies treated with DNA bearing markers *str-r 41* (HE) and *opt-r 2* (LE) grow in the presence of streptomycin and 90% to 100% of the colonies grow in the presence of optochin. Without DNA no growth is observed in streptomycin plates, whereas about 2% of the colonies increase in size in optochin plates.

Procedure for the isolation of recombination-modified mutants: After a mutagenic treatment, a wild-type culture will contain cells modified in their transformability. The method designed to detect these mutants is based on changes in the frequencies of singly- and doubly-transformed bacteria after transformation of individual cells by DNA bearing unlinked markers. If, for instance, the frequency of transformed cells in a wild-type colony is 10^{-2} for high efficiency markers such as *str-r 41* and *ery-r6* and 10^{-3} for low efficiency markers like *opt-r2* and *ami A-r9*, then the frequency of doubly-transformed cells will be 10^{-4} for the *str-2 ery-r* couple and only 10^{-6} for the *opt-r ami A-r* markers couple. This latter frequency is so low that a colony will very seldom yield such doubly-transformed cells for low efficiency markers after transformation on plate. A mutant which integrates all markers with high efficiency will yield doubly-transformed cells even with the *opt-r ami A-r* couple.

The experimental procedure used is shown in Figure 1. Master plates are prepared from a standard wild-type culture treated with nitrosoguanidine as described in MATERIALS AND METHODS. Suspensions of mutagenized cells are diluted and plated. After 24 hours' incubation at 37° two replica-plating series are made from each of these master plates onto a total of six plates containing solid transformation medium. Then, before further incubation, the DNA from strain *69* bearing two high efficiency (*str-r 41* and *ery-r6*) and two low efficiency (*ami A-r9* and *opt-r2*) markers is added to the plates in a second layer of 3 ml transformation medium supplemented with 0.8% agar. Further steps of the procedure are the same as described above.

Colonies that appear to be modified in their transformability are picked from the plates without antibiotic and small cultures are prepared. Each culture is made competent by the pneumococcal activator substance and transformed by DNA from strain *69* to check the results obtained on plates.

Table 1 summarizes the expected behavior of transformation-deficient and some recombination-modified mutants.

appropriate dilutions

1 streptomycin
First replica plating

2 streptomycin
erythromycin

3 no antibiotic

over night
incubation

Second replica plating

Master plate
(100 to 150 colonies)

culture of wild-type
cells treated with nitrosoguanidine

4 optochin
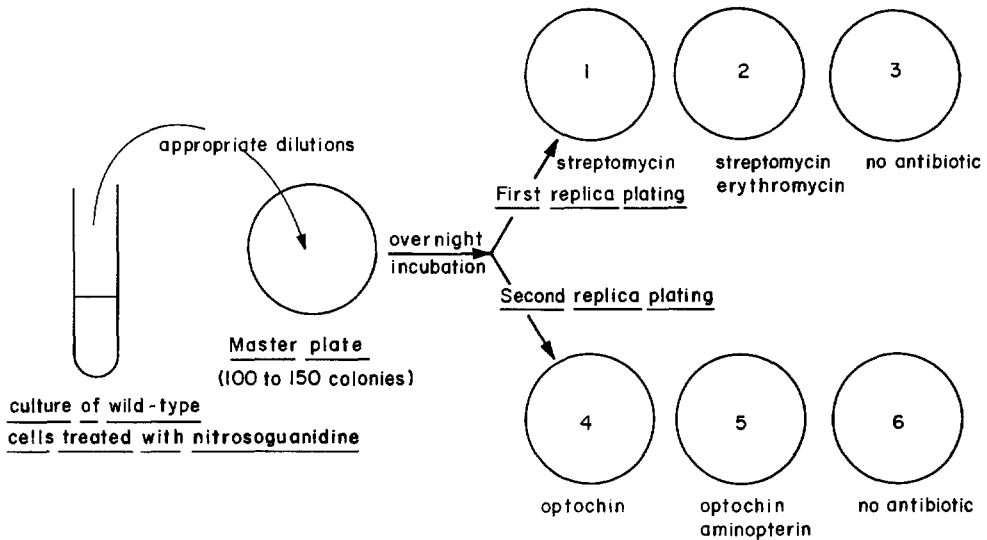
5 optochin
aminopterin

6 no antibiotic

FIGURE 1.—Experimental procedure for isolation of transformation-deficient and recombination-modified mutants of *Diplococcus pneumoniae*. Transforming DNA from strain *69* bearing *str-r41*, *ery-r6*, *ami A-r9*, *opt-r2* markers, is added in a second layer immediately after the replica plating has been made on all six plates. The combination of antibiotics shown in the figure is supplied, 6 hours later, in a third layer.

TABLE 1

*Expected behavior for transformation-deficient and recombination-modified mutants after transformation on plate**

Nature of the clone on the master plate	1	2	3	4	5	6	Classes of mutants
Wild-type cells	+	+	+	+	—	+	.
Non-transformable mutants	—	—	+	—	—	+	1
Poorly transformable mutants	+	—	+	—	—	+	2
Mutants integrating all the markers with a high efficiency	+	+	+	+	+	+	3
Mutants integrating all the markers with a low efficiency	+	—	+	+	—	+	4
Mutants no longer integrating high efficiency markers	—	—	+	+	—	+	5
Mutants no longer integrating low efficiency markers	+	+	+	—	—	+	6

* Plate numbers are as indicated in Figure 1. (+) indicates that the colony grows to a large size on the indicated plate (see text); (—) indicates that it does not.

Isolation of mutants: A variety of mutants were obtained using the procedure described above.

A. *Mutants modified in their transforming ability*

Typically, 1–2% of the colonies obtained from a culture treated with NG fail to give optochin and streptomycin transformants. Approximately thirty mutants
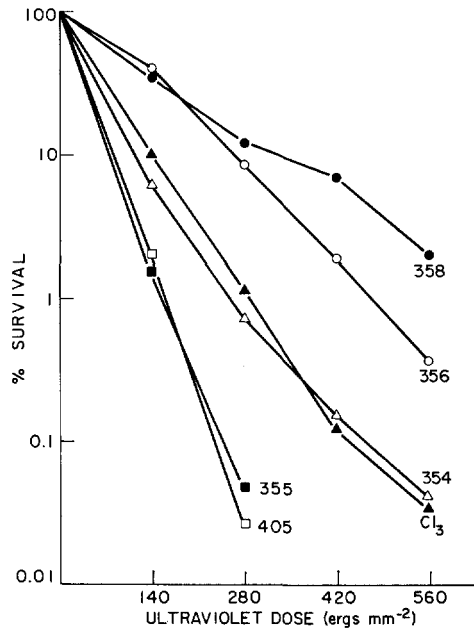
FIGURE 2.—Ultraviolet sensitivity of transformation-deficient mutants of *Diplococcus pneumoniae*. These mutants were isolated by the method of transformation in solid medium. The irradiation was carried out as described in MATERIALS AND METHODS.

that do not transform (class 1) or that transform poorly for both HE and LE markers (class 2) were isolated from five different samples of the same mutagenized culture. In a preliminary attempt to classify these mutants we determined their sensitivity to ultraviolet light. Figure 2 shows the rate of loss of colony-forming ability for some of the most representative ones. According to their response to UV, three groups can be distinguished: strain *358* and strain *356* are, respectively, 2.5 and 2 times more resistant than the standard wild type; strain *354* has the same sensitivity as the wild type; strains *355* and *405* are more sensitive than Cl_3. In order to determine the nature of the deficient steps, the transformation-deficient mutants, whose sensitivities are shown in Figure 2, were allowed to react with DNA from a streptomycin-resistant strain labelled with tritiated thymidine. The results of these experiments are presented in Table 2. It can be seen that the DNA fails to penetrate into strains *354*, *355* and *358* under conditions of high transformability of the wild-type strain. Strain *405* yields transformed cells, but fewer than the wild type. The relative efficiencies of integration of various markers are the same in strains *405* and Cl_3 (TIRABY 1971). The decrease in the number of transformed bacteria in strain *405* is partially due to a decrease in the integration of the transforming DNA into the genome of the cells, since the number of transformants per count of radioactivity incorporated is five times smaller for *405* than it is for Cl_3.

TABLE 2

*DNA uptake of transformation-deficient mutants**

Strain	Streptomycin transformants per ml (str-r)	Counts/min/ml (cpm)	Str-r/cpm
354	<20	80	—
355	<20	124	—
358	<20	112	—
405	2.1 × 10^5	4950	42
Cl_3	2.43 × 10^6	10,370	230
Non-competent Cl_3	<20	90	—

* An activated culture containing 7 × 10^7 colony-forming units/ml is treated by tritiated DNA bearing *str-r* marker. Measurements are made as described in MATERIALS AND METHODS. Control non-competent Cl_3 was made by lowering the pH of the transforming medium.

B. Mutants modified in their recombination ability

Mutants of great interest for studying recombination in pneumococcus are those that would modify the integration efficiency of HE and-or LE markers compared to the wild-type strain. A few such recombination-modified mutants were isolated by the method of transformation on plates.

In strain *500* the variation of the integration efficiency for various markers is less pronounced than in Cl_3 (Table 3). From the measure of the DNA uptake it appears that the efficiencies of mainly the HE markers are modified in this strain (Table 4). The efficiencies of LE markers are almost the same as in the wild type, but the efficiency of the streptomycin-resistant marker is lowered. The data in Table 3 imply that another HE marker tested, *ami A-r1*, behaves like *str-r 41*, since the efficiencies of both markers are the same in strain *500*. Based only on its behavior in transformation on plates, strain *500* falls into group 4 (Table 1).

Mutants *T6* and *T16* represent another class of recombination-modified mutants. In these strains the differences in efficiencies between HE and LE

TABLE 3

*Relative efficiencies of integration of various markers in recombination-modified mutants and in the wild-type strain**

Markers	Cl_3	500	Strains T_6	401
ami A-r1	1.2 ± 0.1	1.09 ± 0.1	1.2	0.82 ± 0.03
ami A-r9	0.15 ± 0.05	0.046 ± 0.05	0.05	0.82 ± 0.03
opt-r2	0.18 ± 0.05	0.39 ± 0.06	0.05	0.48 ± 0.04
str-r41	1.00	1.00	1.00	1.00

* The integration of a given marker is determined by the ratio of the number of transformants for this marker to the number of transformants for the reference marker conferring resistance to streptomycin: *str-r41*. The different strains are transformed by DNA from strain *69* and by DNA bearing both markers *str-r41* and *ami A-r1*. The competent cells are allowed to react with DNA for 20 minutes, and then the cells are plated in a non-selective medium. After 150 minutes incubation at 37° for strains Cl_3, *500*, *401*, and *300* minutes incubation for strain T_6, the transformants are scored by pouring a second layer of medium containing the antibiotic over the first layer.

TABLE 4

*DNA uptake and transformation efficiency in strains Cl_3 (wild type)
and 500 (recombination-modified)* *

Strains	DNA concentration μg/ml	Counts/min/ml of culture (cpm)	Number of transformants per ml of culture		Str-r/cpm	Opt-r/cpm
			Streptomycin × 10⁴	Optochin × 10³		
Wild type	0.25	14930	313	563		
(Cl_3)	0.25	10370	243	437	1.00	1.00
	0.05	2510	57	103		
500	0.25	17500	87.5	341		
	0.25	9850	74.5	291	0.31	0.67
	0.05	2820	23	90		

* The measurements were made as described in MATERIALS AND METHODS. The values of
Str-r/cpm and opt-r/cpm are the averages of the values obtained with the three different experi-
ments reported in this table.

markers are amplified, compared to the wild-type strain (Table 3). In addition,
the delay in the complete phenotypic expression after transformation of the LE
markers tested *opt-r2* and *ami A-r9* is greatly increased in *T6* and *T16*, whereas
this delay is normal for HE markers *str-r41* and *ami-A r1*. Probably because LE
markers are not fully expressed at the time of the selection in transformation on
plates, strains *T6* and *T16* behave, by this method, like mutants from Class 6
(Table 1).

Mutants from class 3 (Table 1) in which LE marker efficiencies increase to
almost the level of HE marker efficiencies are known. Such mutants were iso-
lated fortuitously many years ago; they include strain *Rx* (RAVIN 1959), strain
84 (LITMAN 1961) and, more recently, the *Hex⁻* strains (LACKS 1970). LACKS
established that one specific mutation in the recipient strain was responsible for
the increase of the integration efficiencies of all LE markers. We have also isolated
such a mutant, strain *401*, which shows a reduced difference in efficiencies be-
tween markers (LOUARN, TIRABY and SICARD 1970). The properties of this
mutant are described in the accompanying paper. By measuring the uptake of
labelled DNA it will be shown that, in contrast to strain *500*, *401* increased the
efficiency principally of the LE markers. In addition, *401* and also *Rx* (Fox,
personal communication) and *Hex-1* (LACKS 1970) have the same level of resist-
ance to ultraviolet light as the wild-type strain, whereas the mutation which
modifies the efficiencies of markers in strain *500* confers sensitivity to radiation
(TIRABY 1971).

The method of transformation on plates was used to introduce the gene
accounting for the *401* phenotype into the wild-type strain. The experimental
procedure is as follows: Competent cells of Cl_3 were allowed to react with DNA
from strain *401*, and after a tenfold dilution, the cells were incubated for three
hours at 37° to allow segregation of the mutant phenotype. These cells were
tested for transformability on plates as described above and selected for double

LE-LE transformants resistant to aminopterin and optochin. Only six colonies developed in the presence of these two antimetabolites. Further tests showed that two of these colonies displayed the phenotype of the donor *401* strain.

DISCUSSION AND CONCLUSION

The method of transformation on petri dishes reported here and especially adapted for *Diplococcus pneumoniae* provides a means of isolating both transformation-deficient mutants and recombination-modified mutants. Recombination-modified mutants are those in which the pattern of integration of only one class of markers is altered compared to the wild type.

The first category of mutants isolated includes those which are not transformable or poorly transformable as assayed by two different transformation procedures: the one used for many years in our laboratory (SICARD 1964), or the more recent one in which the pneumococcal-activator substance is involved. CASTER, POSTEL and GOODGAL (1970) have isolated transformation-deficient mutants of *Haemophilus influenzae* by a similar method of transformation on plates. These mutants have been divided into four classes according to their abilities to bind and to be transformed by native and denatured DNA. Because pneumococcus is poorly transformable by denatured DNA, the transformation-deficient mutants of pneumococcus are not readily divided according to the same criterion used by CASTER, POSTEL and GOODGAL (1970). A preliminary study of these latter mutants revealed a great variety within this category. According to their UV sensitivity, several classes can be distinguished. Therefore genetic modifications of transformability do not necessarily involve a change in UV sensitivity.

The UV resistance of non-transformable bacteria that fail to take up DNA is unexpected. It is possible that these bacteria are mutated for one gene conferring resistance to UV and another gene controlling transformability. However, we isolated two other spontaneous mutants for their UV resistance (TIRABY 1971); they are also non-transformable. It might be, therefore, that these two properties depend upon one single gene.

Some non-transformable mutants that show the same UV sensitivity as the wild type were probably defective in the first steps of DNA uptake. These steps need several gene products since a number of non-detectable proteins (including the pneumococcal activator) in non-competent cells were found in the competent cells (TOMASZ 1970; TOMASZ and ZANATI 1971).

Studies on the physiological state of competence in streptococcus, an organism related to pneumococcus, are facilitated by the natural occurrence of a variety of strains of group H streptococcus, deficient in their ability to undergo transformation. PAKULA, SPENCER and GOLDSTEIN (1972) have shown that either the competence factor or a factor inactivating the donor DNA or both factors were missing in these transformation deficient mutants. Similarly, non-transformable or poorly-transformable mutants of pneumococcus could be of great help in solving the problem of competence for DNA uptake.

One of the UV-sensitive mutants (strain *405*) was found to be recombination-deficient. The properties of this stain (TIRABY and SICARD, in preparation) are similar to those of the recombination-deficient pneumococcal mutant *A5-5-9A* isolated by VOVIS and BUTTIN (1970). As in many other organisms, recombination-deficient mutants of pneumococcus could be of great interest for the genetical and biochemical studies of recombination mechanisms.

The second category of mutants includes strains modified in the integration efficiency of markers. The study of these mutants should provide some new insight into the mechanisms responsible for the specific efficiencies of markers. This study is being carried out in our laboratory and some results are presented in the accompanying paper.

LITERATURE CITED

AVERY, O. T., C. M. MACLEOD and M. MACCARTY, 1944   Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. J. Exptl. Med. **89**: 137–158.

BRUNEL, F., A. M. SICARD and N. SICARD, 1971   Transforming ability of bacterial DNA in relation to the marker efficiencies in *Diplococcus pneumoniae* during thymidine starvation. J. Bacteriol. **106**: 904–907.

CASTER, J. H., E. H. POSTEL and S. H. GOODGAL, 1970   Competence mutants: isolation of transformation deficient strains of *Haemophilus influenzae*. Nature (London) **227**: 515–517.

EPHRUSSI-TAYLOR, H., 1958   The mechanism of desoxyribonucleic-acid-induced transformations, In: *Recent Progress in Microbiology*. Edited by G. TUNEVALL. Almqvist & Wiksell, Stockholm. ——, 1966   Genetic recombination in DNA-induced transformation of pneumococcus. IV. The pattern of transmission and phenotypic expression of high and low efficiency donor sites in the *ami-A* locus. Genetics **54**: 211–222. ——, 1968   The mechanism of desoxyribonucleic-acid-induced transformation. Symposium of the VIIth Int. Cong. of Microbiology, Stockholm.

EPHRUSSI-TAYLOR, H., A. M. SICARD and R. KAMEN, 1965   Genetic recombination in DNA-induced transformation of pneumococcus. I. The Problem of relative efficiency of transforming factors. Genetics **51**: 455–475.

FRIEDMAN, L. R. and A. W. RAVIN, 1972   Genetic and biochemical properties of thymidine-dependent mutants of pneumococcus. J. Bacteriol. **109**: 459–475.

GRAY, T. C. and H. EPHRUSSI-TAYLOR   Genetic recombination in DNA-induced transformation of pneumococcus. V. The absence of interference and evidence for the selective elimination of certain donor sites from the final recombinants. Genetics **57**: 125–153.

GREEN, D. M., 1959   A host specific variation affecting frequency of transformation of two markers in pneumococcus. Exptl. Cell Res. **18**: 466–480.

HOTCHKISS, R. D., 1951   Transfer of penicillin resistance in pneumococcus by the deoxyribonucleate derived from resistant cultures. Cold Spring Harbor Symp. Quant. Biol. **16**: 457–460.

LACKS, S., 1965   Genetic recombination in pneumococcus. Pp. 159–164. In: *The physiology of gene and mutation expression*. Edited by M. KOHOUTOVA and J. HUBACEK. Academia, Prague.

——, 1966 Integration efficiency and genetic recombination in pneumococcal transformation. Genetics **53**: 207–235. ——, 1970 Mutants of *Diplococcus pneumoniae* that lack desoxyribonucleases and activities possibly pertinent to genetic transformation. J. Bacteriol. **101**: 373–383.

LITMAN, R. M., 1961 Genetic and chemical alteration in the transforming DNA of pneumococcus caused by ultraviolet light and by nitrous acid. J. Chimie Phys. **58**: 997–1004.

LOUARN, J. M. and A. M. SICARD, 1969 Identical transformability of both strands of recipient DNA in *Diplococcus pneumoniae*. Biochem. Biophys. Res. Commun. **30**: 101–109.

LOUARN, J. M., G. TIRABY and A. M. SICARD, 1970 Intégration des marqueurs à forte et faible efficacité chez le pneumocoque. Société Francaise de Génétique 36–48. Centre d'Hemotypologie du CNRS, CHU Purpan, 31-Toulouse (Editors).

MORSE, H. G. and L. S. LERMAN, 1969 A genetic analysis by transformation of a group of uracil-requiring mutants of *Diplococcus pneumoniae*. Genetics **61**: 41–60.

PAKULA, R., L. R. SPENCER and P. A. GOLDSTEIN, 1972 Deoxyribonuclease and competence factor activities of transformable and nontransformable group H streptococci. Can. J. Microbiol. **18**: 111–119.

RAVIN, A., 1959 Reciprocal capsular transformation of pneumococci. J. Bacteriol. **77**: 296–309.

SICARD, A. M., 1964 A new synthetic medium for *Diplococcus pneumoniae*, and its use for the study of reciprocal transformation at the *amiA* locus. Genetics **50**: 31–44.

SIROTNAK, F. M. and S. L. HACHTEL, 1969 Increased dihydrofolate reductase synthesis in *Diplococcus pneumoniae* following alteration of the structural gene. I. Genotype derivation and recombination analyses. Genetics **61**: 293–312.

TIRABY, G., 1971 Etude des mécanismes responsables des efficacités de transformation chez *Diplococcus pneumoniae*. Thesis. University of Toulouse, France.

TOMASZ, A., 1970 Cellular metabolism in genetic transformation of pneumococci requirement for protein synthesis during induction of competence. J. Bacteriol. **101**: 860–871.

TOMASZ, A. and R. D. HOTCHKISS, 1964 Regulation of the transformability of pneumococcal cultures by macromolecular cell products. Proc. Nat. Acad. Sci. U.S. **51**: 480–487.

TOMASZ, A. and J. L. MOSSER, 1966 On the nature of pneumococcal activator substance. Proc. Nat. Acad. Sci. U.S. **55**: 58–66.

TOMASZ, A. and E. ZANATI, 1971 Appearance of a protein "agglutinin" on the spheroplast membrane of pneumococci during induction of competence. J. Bacteriol. **105**: 1213–1215.

VOVIS, G. and G. BUTTIN, 1970 An ATP dependent desoxyribonuclease from *Diplococcus pneumoniae*. II. Evidence for its involvement in bacterial recombination. Biochim. Biophys. Acta **224**: 42–54.

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