DETERMINATION OF TYPE IN CAPSULATED TRANSFORMANTS OF PNEUMOCOCCUS BY THE GENOME OF NON-CAPSULATED DONOR AND RECIPIENT STRAINS*

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Smooth transformants which produce type VIII capsular polysaccharide have been obtained by reciprocal transformations between strains VIII-R1 and VIII-R13 of pneumococcus (1). Both these strains are R (rough, non-capsulated) mutants of the S (smooth, capsulated) strain VIII-S Henrique.

When extracts of S strains of different capsular types were applied to VIII-R1 or VIII-R13, S transformants of type VIII in many cases were detected in addition to the expected S transformants of the same type as the donor strain. In the type VIII transformants capsular type was evidently determined by the genome of the recipient R strain.

The present work was undertaken to explore the possibility of producing S transformants by reactions between pairs of R mutants, firstly from different S strains of the same type and secondly from S strains of different types, and also to search for more examples of the determination of type in S transformants by the genome of R recipients.

Materials and Methods

Strains of Pneumococcus.—All the rough strains used had been isolated after cultivating S strains in broth containing antiserum of homologous type. II-Rat R, a mutant of the S strain II-Rat, was isolated in 1947. II-R36, II-D39R19, II-D39MVR30, and II-RB are all R mutants of II-D39S that were isolated at different times. This paper reports the first work carried out with II-RB, which was isolated in this laboratory in 1957 by Miss Jane Gutteron; the other mutants of II-D39S have been extensively studied in transformation experiments (2, 3). VIII-R1 and VIII-R13 are mutants of VIII-S Henrique, isolated in 1952 and 1949 respectively (1). Also used were transforming extracts made from the capsulated strains I-SV1, II-D39S, III-A66S, VIII-S Henrique, XIV-S Elders, and 1 strain each of type VIIS and type XVIIIS.

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Cultural Methods.—Beef heart infusion broth containing 1 per cent neopeptone, pH 7.4 to 7.5, was used as the basis of all liquid media. For stock cultures 0.3 ml. of defibrinated normal rabbit blood was added to a tube containing 7 ml. of broth. These cultures were stored at 5°C. for periods up to 1 month. Transformation trials and cultures grown for preparation of transforming extracts were inoculated from parent cultures grown in blood broth seeded from stock cultures.

Nutrient blood agar was prepared by adding 5 per cent of citrated normal human blood to brain heart infusion agar supplied by the Baltimore Biological Laboratory, Baltimore.

All cultures were incubated at 37°C.

Preparation of Transforming Extracts.—For each preparation of transforming extract, 100 ml. of broth were inoculated with 0.3 ml. of a 7 hour blood broth culture, and incubated for 16 to 18 hours. Glucose solution was then added to a concentration of 1 per cent, with 0.1 ml. of phenol red (0.5 per cent in 95 per cent ethanol) as indicator, and incubation was continued. The tomato-red color imparted by the indicator was maintained by the addition of small amounts of 10 per cent sodium hydroxide solution at suitable intervals. When about 3.5 ml. of sodium hydroxide solution had been added (usually after about 6 hours), samples of the culture were removed for testing as described below, and 3 gm. of sodium citrate dissolved in 10 ml. water were added. The resultant citrate concentration of 0.1 M is inhibitory to the destruction of transforming principle by desoxyribonuclease in the crude bacterial lysate (4). Sodium desoxycholate solution was then added to a concentration of 0.05 per cent; lysis of the cells rapidly ensued.

Within 30 minutes 1 volume of ethanol was added to the lysed culture and the mixture was manually shaken. The white, fibrous precipitate which appeared was sedimented by briefly centrifuging at 850 *g*, and the supernatant liquid was discarded. The precipitate was dissolved in 10 ml. of 0.1 M sodium chloride solution, and protein was removed by shaking for 15 minutes with 2 successive lots of 2 ml. of chloroform plus 0.1 ml. of octanol. The chloroform layer was discarded after being separated by centrifuging for 5 minutes at 1,100 *g*. The aqueous phase was transferred to a sterile tube and after reprecipitation with 1 volume of ethanol, the transforming extract was stored at 5°C. Before use the precipitate was sedimented by briefly centrifuging at 1,100 *g*, the supernate was discarded and the precipitate was redissolved in 10 ml. of sterile 0.1 M sodium chloride solution.

It was important to prove that S cultures at the time of lysis were free from R mutants and, more important, that R cultures were free from S mutants. In the case of capsulated strains, homogeneity was tested by streaking a loopful of culture on blood agar, incubating overnight and examining the colonies formed. These were invariably found to be a homogeneous, smooth population and to give a *quellung* reaction with the appropriate type antiserum. R strains were similarly tested, and a homogeneous, rough population was invariably found. This test was not considered to be sufficiently rigorous and an additional test was run in parallel. A loopful of culture was inoculated into 2 ml. of 10 per cent human serum broth, which agglutinates R organisms and gives scope for the multiplication of S organisms in the supernate; after incubating overnight, a loopful of the supernate was streaked on blood agar and incubated for 18 hours; smooth colonies were never found.

Transformation Reactions.—Serum factors which consist presumably of albumin and anti-R agglutinins (2, 5) were supplied in the form of normal human serum prepared by heating citrated normal human plasma at 60° for 30 minutes, centrifuging for 1 hour at 850 *g*, and discarding the precipitate. The plasma was prepared from recently outdated blood from a blood bank.

In a typical experiment, 40 ml. of 10 per cent human serum broth was inoculated with 0.2 ml. of a 100-fold dilution of a blood broth culture of the recipient strain that had been stored at 5°C. for not more than 5 days, and after mixing 2 ml. aliquots were dispensed into 18 tubes

of 11 mm. internal diameter. Three control tubes received no extract. Five transforming extracts were tested, each in 3 tubes, by adding an aliquot of 0.1 ml. of extract solution. Experience with numerous pneumococcal extracts has shown that this amount is in excess of that required for the maximum rate of transformation. The tubes were incubated for 17 to 19 hours, after which most of the untransformed R organisms had been agglutinated and had sedimented to the bottom. A loopful of the supernate was streaked on blood agar. The rest of the supernate was carefully pipetted off for further testing; it was stored at 5°C. after adding a drop of defibrinated rabbit blood. After incubating for 24 hours the streak on blood agar was examined for the presence of smooth colonies. In many cases, even when the supernate of the transformation tube was not detectably turbid, as well as in all cases in which turbidity was present, many smooth colonies were observed. (It must be pointed out that the operation of selection pressures makes the number of S colonies an uncertain index of transformation frequency; the method as used was not intended for the precise measurement of transformation frequencies.) If no smooth colonies were found in the first streak on blood agar, 0.5 ml. of supernate from the transformation tubes was added to 2 ml. of 10 per cent human serum broth to give a still better opportunity for the growth of any S transformants. Control tubes to which extract had not been added were always examined in the same way. When S colonies were found in the streaks from the culture supernate type specificity of the pneumococci was determined by *quellung* reaction. When the reacting strains came from different types, S transformants were tested for *quellung* reactions with antisera of both types. Occasionally, colonies of both types were found in the same streak, but in no case did any individual colony react with antisera of both types. When only 1 of the possible transformant types was found, the other type was searched for by cultivating 0.2 ml. of the original supernate in 2 ml. of broth containing 10 per cent of antiserum of the type already found in order to select against it; the supernate of this culture was streaked on blood agar, and any smooth colonies found were tested for *quellung* reactions. This technique often revealed transformants of the second type.

EXPERIMENTAL

Controls in Transformation Experiments.—A total of 362 control tubes to which transforming extract had not been added were included in a total of 119 experiments. In only 3 tubes, each in a different experiment, were S organisms found; in 2 instances, the strain in question was II-RB; in the third, it was VIII-R1. S organisms found in tubes containing transforming extract in these experiments were not regarded as transformants.

In 6 tubes containing transforming extracts in 2 different experiments and also in 1 control tube in 1 of these experiments, organisms of strain II-D39R19 failed to agglutinate in 10 per cent human serum broth. The colonial morphology appeared to be normally rough, but there was evidently a trace of type II polysaccharide produced since an agglutinated disc was formed when a loopful of the turbid culture was inoculated into 2 ml. of 10 per cent type II serum broth and incubated overnight. The status of these organisms as transformants was considered doubtful, and they were not counted as such.

These results are considered to provide a sound guarantee for the transformation results reported below.

Transformations to Type IIS between Pairs of IIR Strains of Pneumococcus.—Each of 5 IIR strains was separately exposed to transforming extracts from

each of the other 4, and the cultures were examined for the presence of capsulated transformants.

Type IIS transformants were found in 9 of the 20 trials (Table I), the 5 negative results in trials of a strain tested against its own extract being discounted. Of the 11 negative results, 8 involved the strain II-R36, which was entirely inactive both as a recipient and as a donor. All the other strains were active in at least 1 instance both as donors and recipients. Transformants were obtained from all pairings except the pairing between II-D39R19 and II-D39MVR30. Reciprocal transformations occurred in all the other pairings except II-Rat R with II-RB; II-Rat R transformed II-RB, but was not transformed by II-RB extract.

TABLE I

IIS Transformants Found after Exposing Cells of IIR Strains of Pneumococcus to Extracts of Other IIR Strains

Recipient strain	Donor strain from which transforming extract was prepared				
	II-Rat R	II-RB	II-D39R19	II-D39MVR30	II-R36
II-Rat R	0	0	+ (2/6)	+ (3/6)	0
II-RB	+ (6/6)	0	+ (6/6)	+ (6/9)	0
II-D39R19	+ (4/6)	+ (2/9)	0	0	0
II-D39MVR30	+ (2/9)	+ (1/12)	0	0	0
II-R36	0	0	0	0	0

The symbol (+) signifies that S transformants were found. The denominator of the fraction in brackets is the total number of tubes in which tests were made; the numerator is the number of these tubes in which S transformants were found.

All S transformants were proved by *quellung* reactions to be of type II.

The symbol 0 signifies that no transformants were found; for each trial, not less than 3 tubes were tested in each of 3 experiments.

It is notable that transformants of II-D39MVR30 were found in 3 tubes only. This is in line with the poor receptivity of this strain with extracts of capsulated strains (Table IV). More surprising was the low activity of II-RB extract with IIR recipients especially since this extract readily transformed VIII-R1 to VIIIS (Table II). The absence of II-R36 transformants is presumably not due to poor receptivity, since the strain is readily transformable by extracts of capsulated strains (Table IV).

Transformations between IIR and VIIIR Strains of Pneumococcus.—The strains VIII-R1 and VIII-R13 were tested as donors and recipients in transformation reactions with the 5 IIR strains (Tables II and III).

As in trials among the IIR strains, no transformations were found with II-R36 either as donor or as recipient. Type VIIIS transformants were found when each of the other IIR extracts was applied to VIII-R1 cells (Table II) or, with the

TABLE II

Transformants Found in Reactions between IIR Strains and Strain VIII-R1 of Pneumococcus

IIR strain	VIII-R1 as donor strain		VIII-R1 as recipient strain	
	VIIIS	IIS	VIIIS	IIS
II-Rat R	+ (2/9)	0	+ (15/15)	0
II-RB	+ (7/12)	+ (7/11)	+ (12/12)	0
II-D39R19	+ (1/9)	0	+ (15/15)	0
II-D39MVR30	0	0	+ (15/15)	0
II-R36	0	0	0	0

For each trial, not less than 3 tubes were tested in each of 3 experiments.

The symbol (+) signifies that S transformants of the specified type were found. The denominator of the fraction in brackets is the total number of tubes in which tests were made; the numerator is the number of these tubes in which S transformants of the specified type were found.

The symbol 0 signifies that no transformants were found.

TABLE III

Transformants Found in Reactions between IIR Strains and Strain VIII-R13 of Pneumococcus

IIR strain	VIII-R13 as donor strain		VIII-R13 as recipient strain	
	VIIIS	IIS	VIIIS	IIS
II-Rat R	0	0	+ (3/9)	0
II-RB	+ (5/9)	+ (8/9)	0	0
II-D39R19	0	0	+ (4/12)	0
II-D39MVR30	0	0	+ (2/12)	0
II-R36	0	0	0	0

For each trial not less than 3 tubes were tested in each of 3 experiments.

The symbol (+) signifies that S transformants of the specified type were found. The denominator of the fraction in brackets is the total number of tubes in which tests were made; the numerator is the number of these tubes in which S transformants of the specified type were found.

The symbol 0 signifies that no transformants were found.

exception of II-RB extract, when applied to VIII-R13 (Table III). Type VIIIS transformants were also found when VIII-R1 extract was tested with II-Rat R and II-D39R19 as recipients.

The most striking feature of these observations is that type IIS transformants, in addition to those of type VIIIS, were found when extracts of VIII-R1 and VIII-R13 were applied to II-RB; progeny of 2 different capsular types arose by reaction between 2 non-capsulated strains.

Comment has already been made on the poor receptivity of strain II-D39-MVR30. Strain VIII-R13 showed equally poor recipient activity.

Transformations of IIR Strains of Pneumococcus by Extracts of S Strains of Various Capsular Types.—The 5 IIR strains were exposed to transforming extracts of S strains of types I, II, III, VII, VIII, XIV, and XVIII. Transformants of donor type were found in most trials, including those with II-R36 as recipient for all donor types except VIIS (Table IV). This contrasts with the

TABLE IV

S Transformants Found after Exposing IIR Strains of Pneumococcus to Extracts of S Strains of Different Pneumococcal Types

Recipient strain	Capsular type of donor strain from which transforming extract was prepared						
	IS	IIS	IIIS	VIIS	VIIIS	XIVS	XVIIIS
II-Rat R	D (4/10) d (8/10)	D = II (6/6)	D (9/9)	D (8/9) II (5/9)	D (11/15)	D (9/10)	D (10/10) II (1/10)
II-RB	D (2/12) d (6/12) II (1/12)	D = II (6/6)	D (21/21)	D (2/9) II (9/9)	D (13/15) II (15/15)	D (14/16)	D (7/9) II (8/9)
II-D39R19	d (1/9)	D = II (6/6)	D (9/9)	II (1/9)	D (9/9) II (5/9)	II (2/9)	D (6/12) II (2/12)
II-D39MVR30	0 (0/8)	D = II (4/6)	D (3/9)	0 (0/9)	D (6/12) II (4/12)	0 (0/8)	0 (0/9)
II-R36	D (4/9) d (5/9)	D = II (3/6)	D (9/9)	0 (0/9)	D (8/9)	D (5/9)	D (7/9)

The symbol D signifies that fully encapsulated transformants of the same type as the donor strain were found; the symbol d signifies that transformants producing only a small amount of donor-type polysaccharide were found.

The symbol II signifies that transformants of type II were found.

The denominator of the fraction in brackets is the total number of tubes in which tests were made; the numerator is the number of these tubes in which the specified transformants were found. The figures for type II transformants found after applying type XVIII extract are minimal; no type XVIII serum was available for selecting against the type XVIII transformants which tended to predominate.

The symbol 0 signifies that no transformants were found.

inactivity of II-R36 with other IIR strains and with VIIIR strains. IIS transformants of II-R36 were never found except with IIS extract. On the other hand each of the other IIR strains gave IIS transformants with at least 1 extract of heterologous S type.

Each of the S extracts, with the important exception of IIIS, transformed at least 1 of the IIR strains to IIS.

The poor receptivity of II-D39MVR30 may be held responsible for its failure to transform with some extracts, and this is probably also the case with II-D39R19.

In addition to fully capsulated transformants of type I, transformants pro-

ducing a smaller amount of type I polysaccharide were often found after exposing IIR cells to IS extract. Although the morphology of these partially capsulated transformants was similar to that of the IIR recipients, they were not agglutinated by anti-R serum. The presence of type I polysaccharide could not be demonstrated by *quellung* reaction, but was made evident by the formation of an agglutinated disc after a single colony from a blood agar plate was inoculated into 10 per cent type I serum broth and incubated overnight.

DISCUSSION

The reciprocal transformations to IIS between strains II-RB and II-D39R19 and between II-RB and II-D39MVR30, all of which are R mutants of the same S strain II-D39S, constitute a parallel case to the reciprocal transformations to VIIIS between VIII-R1 and VIII-R13 (1). Further, the reciprocal transformations to IIS between II-Rat R and II-D39R19 or II-D39MVR30 and the transformation of II-RB to IIS by II-Rat R provide examples of capsular transformation between R mutants of *different* S strains of the same type. Evidently II-Rat R, II-RB, and II-D39R19 are non-identical mutants of IIS. It seems possible that II-D39R19 and II-D39MVR30 are identical mutants as far as capsulation is concerned.

The experiments reported do not permit a decision as to whether the non-identical mutations are concerned with different functions involved in capsular synthesis or whether they are mutations at different sites in a single gene locus affecting only 1 function (6). In this connection, Smith and Mills (7) have examined strains VIII-R1 and VIII-R13 for possible biochemical blocks along the path of synthesis of uridine diphosphate glucuronic acid (UDPGA),[1] which is presumed to be an essential intermediate in the synthesis of capsular polysaccharides which contain glucuronic acid residues (8). Both strains yielded enzyme extracts which catalyse the reactions:

uridine triphosphate + glucose-1-phosphate →
uridine diphosphate glucose →
uridine diphosphate glucuronic acid

Large amounts of UDPGA are synthesised by cells of both strains, and their failure to form capsules of type VIII polysaccharide is probably due to deficiency at a later stage of its synthesis. In this case also, whether the non-identical mutations are at different sites of the same locus or in different loci remains unknown.

The capsular transformations between IIR and VIIIR strains (Tables II and III) are relevant to the question of genetic determination of capsular type. It has been postulated that the synthesis of pneumococcal capsular polysac-

[1] UDPGA, uridine diphosphate glucuronic acid.

charides of different types is dependent on structural determination by templates under the control of single allelomorphic genes (see review by Wilkinson (9)). The transformation of II-RB to IIS by VIII-R1 (Table II) and VIII-R13 (Table III) seems difficult to account for on this hypothesis, since it appears that II-RB fails to synthesise type II polysaccharide because of mutational deficiency which can be made good by VIII-R1 and VIII-R13. An alternative hypothesis is that the structure of capsular polysaccharide is determined by the action of a number of enzymes, each controlled by a different gene. On this basis it would be more reasonable that VIII-R1 and VIII-R13 should possess intact a gene which has mutated in II-RB. It seems likely that some of the same enzymes are involved in the synthesis of different type-specific polysaccharides, even at stages subsequent to the synthesis of UDPGA, but the possession of the same gene by different types need not require that the gene be biochemically active in all types.

It is clear that R strains in general are not blank sheets on which capsule production is imposed solely by the genome of S donor strains. The determination of capsular type by an R donor in the transformations to VIIIS of II-Rat R, II-RB and II-D39R19 by extracts of VIII-R1, and of II-RB by extracts of VIII-R13, are very striking illustrations of this fact. Table IV contains numerous examples of the determination of capsular type by R recipients. Mention has already been made of the similar results reported for VIIIR recipients (1). Strain II-R36 is unusual in that it never gave rise to IIS transformants either as a donor or as a recipient except when IIS extract was applied to it. It appears that II-R36 has multiple genetic deficiencies affecting the synthesis of type II polysaccharide, possibly in the form of a deletion.

Transformants of type IIS were never found when extract of III-A66S was applied to IIR strains, which suggests that III-A66S may be deficient in a way comparable with II-R36 in the genetic requirements for synthesis of the heterologous type II polysaccharide.

MacLeod and Krauss (1) found VIIIS transformants of VIII-R1 with extracts of S strains of capsular types I, II, VII, VIII, XIV, and XVIII, but not with extract of IIIS. The unique distinction of giving neither IIS transformants with IIR recipients, nor of VIIIS when applied to VIIIR belongs to IIIS. On the hypothesis that a number of enzymes, each under separate genetic control, is involved in the synthesis of each type-specific polysaccharide, it appears that all the S types tested except IIIS possess a gene which is concerned with synthesis of VIIIS polysaccharide, and a gene which is concerned with synthesis of IIS polysaccharide. Genetic evidence of this nature should be valuable in pointing the way for biochemical investigation of the pathways leading to the synthesis of capsular polysaccharides.

SUMMARY

Capsulated transformants producing type II polysaccharide have been obtained by reactions between a number of R mutants of type II pneumococcus.

All the IIR strains except II-R36 yielded extracts which transformed 2 VIIIR strains to VIIIS. Two of the IIR strains were transformed to VIIIS by an extract of 1 VIIIR strain.

The most striking result was the production of both IIS and VIIIS transformants by the action of extracts of both VIIIR strains applied to cells of II-RB. This suggests that the determination of capsular type in pneumococcus does not depend on single allelomorphic genes; it seems more likely that a number of genes may be required to determine type specificity, and that some genes may be common to different types, possibly in connection with common biochemical pathways for some stages of polysaccharide synthesis.

This is further indicated by the finding that extracts of capsulated strains of each of the types I, II, III, VII, VIII, XIV, and XVIII are capable of giving IIS transformants with 1 or more of the IIR strains in addition to the expected donor-type transformants.

Strain II-R36 was transformed by extracts of all S types except type VII, but the only transformants found were of the donor type. It appears that II-R36 may have multiple genetic deficiencies for type II capsulation, possibly in the form of a deletion. This may be the case also with IIIS strains since only IIIS transformants were found when IIIS extract was applied to any of the IIR strains.

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