TRANSFORMATION REACTIONS WITH TWO NON-ALLELIC R MUTANTS OF THE SAME STRAIN OF PNEUMOCOCCUS TYPE VIII

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(Received for publication, February 1, 1956)

Most studies of pneumococcal transformation reactions have dealt with the transfer to recipient cells of characters phenotypically expressed in the donor strain. Ephrussi-Taylor's "allogenic" transformations (1) are a partial exception to this statement. She found that a transforming extract prepared from a mutant of pneumococcus type III, intermediate with respect to capsular size, when applied to a different intermediate mutant of the same type gave rise to transformed cells that produced larger amounts of SIII than either of the parents and resembled normal type III strains in this respect. The same transforming extract applied to non-encapsulated (R) pneumococci resulted in transformed cells whose production of SIII was similar to that of the intermediate donor cells.

While studying the "competence" of R mutants derived from various encapsulated (S) pneumococci to undergo transformations, an R mutant of a strain of type VIII was encountered which possessed properties not previously observed. Exposure of this strain, VIII-R1, to extracts prepared from type IIS pneumococci yielded small numbers of IIS cells and much larger numbers of type VIIIS cells. A second R mutant, VIII-R13, selected 3 years earlier from the same smooth strain of type VIII pneumococcus as VIII-R1 reacted similarly in the presence of type IIS transforming extract. Of equal interest is the observation that extracts prepared from strain VIII-R1 cause transformation of VIII-R13 to type VIIIS and *vice versa*. Neither R mutant is transformed to type VIIIS by autologous extracts. Desoxyribonucleic acid (DNA) extracts of pneumococcus type IIIS gave rise only to type IIIS cells when applied to either R mutant.

Materials and Methods

Preparation of Transforming Extracts.—The method employed for preparation of transforming extracts was that previously described (2).

Transformation Reactions.—0.1 ml. of a 10^{-8} dilution in broth of an 18 hour rabbit blood broth culture of R pneumococci was seeded into charcoal-absorbed beef heart infusion broth (3) containing 0.1 ml. of an appropriate dilution of transforming extract and 10 per cent human pleural fluid which had been heated at 60°C. for 30 minutes. The final volume was 2 ml.

The tubes were incubated at 37°C. for 16 to 24 hours and a loopful of supernatant culture was streaked on the surface of a blood agar plate. The loop transferred approximately 0.001 ml. of fluid. After incubation at 37°C., S colonies were picked for identification by quellung reaction. One S type only was ever detected by direct pickings from plates. To detect a second S type if present, 0.2 ml. of supernate was seeded into 2 ml. of broth containing rabbit anti-S serum in order to agglutinate organisms of the first, more frequently occurring type, and to permit the second type to grow out in the supernate. After incubation for 16 to 24 hours, the supernate was streaked on blood agar and the S colonies that appeared were identified by quellung reaction.

When there was a possibility that the number of S cells in the supernate of a transformation or a control tube might be so small that they would not be detected if a loopful only was plated, 0.2 ml. of culture was inoculated into 2 ml. of anti-R serum broth. After incubation overnight the supernate of the anti-R serum broth was examined for S organisms by plating on blood agar.

Strains of Pneumococcus.—VIII-Henrique, a fully encapsulated strain of pneumococcus type VIII.

VIII-R13, an unencapsulated mutant isolated in 1949 from strain VIII-Henrique by Dr. Robert Austrian in this laboratory. Strain VIII-Henrique was transferred twice in broth containing type VIII antiserum, subcultured on a blood agar plate, and a single R colony selected. The culture was lyophilized for storage. 3 years later the culture was placed in blood broth and after overnight incubation streaked on a blood agar plate. Three serial single colony isolates were made on blood agar followed by three serial transfers of a shaken loopful of culture in anti-R serum broth to determine whether any S organisms were present. None were found.

VIII-R1, an unencapsulated mutant isolated in 1952 from strain VIII-Henrique after two successive transfers in broth containing type VIII antiserum. VIII-R1 was transferred in blood broth at monthly intervals for a year. Following 3 successive single colony isolations from blood agar plates it was used in transformation reactions.

II-R36NC, a transformable colonial variant of the R mutant R36 originally selected from the smooth strain II-D39S (3).

Transforming extracts were prepared also from the following S strains of pneumococcus: I-SVI, II-D39S, III-A66, VII, XIV, and XVIII.

EXPERIMENTAL

Transformation to Type IIS and Type VIIIS When Strains VIII-R1 and VIII-R13 Were Exposed to DNA Extracts of Pneumococcus Type II.—Strain VIII-R1 was exposed to the transforming extract of pneumococcus type II, strain II-D39S. After 18 hours' incubation at 37°C., a loopful of culture supernate was streaked on blood agar. A mixture of S and R colonies developed on the plate. Quellung reactions carried out on S colonies unexpectedly showed them to consist only of type VIIIS organisms instead of type IIS from which the extract was prepared. Type IIS pneumococci were never detected by direct examination of colonies on blood agar plates that had been seeded by loop directly from the supernate of transformation tubes. However, when 0.2 ml. of supernate was inoculated into 2 ml. of broth containing type VIII antiserum and incubated overnight, type II pneumococci were generally found in the supernate.

Exposure of strain VIII-R13 to a type II extract yielded results similar to those found with strain VIII-R1 (Table I).

TABLE I

Transformation Reactions with Two R Mutants of Pneumococcus Type VIIIS and DNA Extracts Prepared from Various Pneumococci

Donor strain for DNA extract	Receptor strain	Transformations observed	Comment
IIS (D39S)	VIII-R1	VIIIS; IIS	IIS cells recovered only after culture supernate was subcultured in presence of type VIIIS antiserum.
	VIII-R13	VIIIS; IIS	
	II-R36NC	IIS	
IIIS (A66)	VIII-R1	IIIS	No type VIIIS cells recovered on any occasion even when supernate cultivated in type IIIS antiserum.
	VIII-R13	IIIS	
	II-R36NC	IIIS	
VIII-R1	VIII-R1	None	
	VIII-R13	VIIIS	
	II-R36NC	None	
VIII-R13	VIII-R1	VIIIS	
	VIII-R13	None	
	II-R36NC	"	
II-R36NC	VIII-R1	None	
	VIII-R13	"	
	II-R36NC	"	
IS (SV-1)	VIII-R1	VIIIS; IS	In some tubes type VIIIS was predominant, in others type IS.
	VIII-R13	R	
	II-R36NC*	IS	
VIIS	VIII-R1	VIIIS; VIIS	VIIS found only after transfer in type VIIIS antiserum.
	VIII-R13	R	
	II-R36NC	VIIS	
VIIIS	VIII-R1	VIIIS	
	VIII-R13	VIIIS	
	II-R36NC	VIIIS	
XIVS	VIII-R1	VIIIS; XIVS	In some tubes VIIIS was predominant, in others XIVS.
	VIII-R13	XIVS	
	II-R36NC	XIVS	
XVIIIS	VIII-R1	VIIIS; XVIIIS	XVIIIS recovered only after transfer in type VIII antiserum.
	VIII-R13	XVIIIS	Supernate not transferred in type XVIII antiserum.
	II-R36NC	XVIIIS	
—	VIII-R1	None	
	VIII-R13	"	
	II-R36NC	"	

* When receptor strain II-R36NC was reacted with DNA extracts of types IS, VIIS, VIIIS, and XIVS, respectively, transformed cells of the type of the extract only were recovered. IIS cells were not found after transfer of the supernate in S antiserum corresponding to the S type of the DNA extract.

It should be emphasized that in experiments of this kind repeated numerous times, on no occasion was an S colony found to contain pneumococci of more than one S type as indicated by direct quellung reactions.

The addition of crystalline bovine pancreatic desoxyribonuclease to the transformation tubes just before incubation prevented the appearance of S organisms of either type VIII or type II, both when the supernate was streaked directly on blood agar or subcultured into anti-R serum broth. Moreover, tubes from which transforming extract was omitted did not show S organisms.

These experiments indicate, therefore, that the S organisms present, whether type VIII or type II, appeared because of the act of transformation and not through spontaneous mutation.

Ratio of Type VIIIS to Type IIS Cells in Cultures of Strain VIII-R1 Exposed to Transforming Extracts of Type IIS.—In 43 different instances over a period of 12 months in which type IIS transforming extract was applied to strain VIII-R1, a total of 77 smooth colonies was picked from the surface of blood agar plates and tested for capsular swelling with type IIS and with type VIIIS antiserum. All these colonies showed type VIIIS pneumococci only. However, in 35 of the 43 experiments, type IIS pneumococci were found when a portion of the supernate of the transformation tube that had been incubated for 18 hours was subcultured in broth containing type VIIIS antiserum.

An approximation of the ratio of type VIIIS to type IIS cells was obtained in one experiment by enumeration after the transformation tube had been incubated for 20 hours.

0.1 ml. of various dilutions of culture supernate from the transformation tube and 0.1 ml. horse blood were mixed thoroughly with 2 ml. of soft meat infusion agar (0.85 per cent agar) previously melted and held at 45°C. The mixture was poured over the surface of a solidified layer of 1.7 per cent meat infusion agar in a Petri dish and incubated at 37°C. for 18 hours. Colonies of S organisms in the soft agar layer can be distinguished readily from R colonies because S colonies are larger and have a blurred, hazy outline in contrast to the sharp outline of R colonies.

In this experiment it was found that exposure of strain VIII-R1 to type IIS transforming extract over a period of 20 hours yielded 4×10^6 type VIIIS organisms (colony-producing units) per ml. of culture supernate.

The number of type II cells per ml. of culture supernate was approximated by subculturing 0.1 ml. of various dilutions of supernate in broth containing 10 per cent type VIIIS antiserum. Type IIS pneumococci were recovered from 6 of 10 tubes containing in all 1 ml. of 10^{-3} dilution of culture, and from 2 of 10 tubes containing in all 1 ml. of 10^{-4} dilution of culture. One ml. of 10^{-5} dilution yielded no type II organisms. It can be estimated, therefore, that there were about 10^4 type II cells per ml., and since approximately 10^6 type

VIII cells per ml. were found, the type VIII to type II ratio in this experiment was approximately 100 to 1.

Transformation Reactions with Extracts of Pneumococcus Type IIIS Applied to Strains VIII-R1 and VIII-R13.—Transforming extracts of pneumococcus type III, strain A66, were applied to the two R mutants of pneumococcus type VIII: VIII-R1 and VIII-R13. The results as shown in Table I were different from those found in experiments with extracts of any other S types that were tested. Examination of S colonies developing on blood agar plates streaked with a loopful from the supernate of transformation tubes, in all instances showed type IIIS cells only. Colonies of type VIIIS organisms were never observed, nor was it possible to isolate type VIIIS from broth containing type III antiserum that had been seeded with 0.2 ml. of supernate from transformation tubes. Further studies to prove the absence of type VIIIS cells were carried out in transformation reactions with strain VIII-R1.

Because of the immunological relationship between the capsular polysaccharides of types III and VIII it was considered that type IIIS antiserum might not be an effective selective agent. Accordingly, the type III antiserum was absorbed with a heavy suspension of heat-killed type VIIIS pneumococci to remove antibody capable of reacting with type VIIIS even though in this specimen of type IIIS rabbit antiserum type VIIIS antibody was not demonstrable by agglutination reactions. The use of absorbed antiserum as a selective agent, however, did not reveal the presence of type VIII cells in the culture supernate of transformation tubes that had been incubated for 18 to 20 hours before samples were removed for subculture.

In an additional attempt to demonstrate the absence of type VIIIS cells from transformation reactions employing strain VIII-R1 and an extract of pneumococcus type IIIS, 5 per cent type III antiserum that had been absorbed with heat-killed type VIII cells, was added to the transformation tubes at the beginning of the experiment. The presence of type IIIS antibody completely inhibited the appearance of type IIIS cells and should have facilitated greatly the recognition of type VIII cells if any were present. None were found, however.

By employing synthetic mixtures of the R mutant VIII-R1 and strain VIII-R1 transformed to type IIIS and type VIIIS (strains IIIS-VIII-R1 and VIIIS-VIII-R1 respectively) we have been able to show that very small numbers of type VIIIS cells can be selected in the presence of much larger numbers of a mixture of type IIIS cells and R cells. The culture medium consisted of charcoal-absorbed broth containing 10 per cent pleural fluid. At zero time each of 3 tubes containing 2 ml. of medium was seeded with 0.1 ml. of 10^{-3} dilution of an 18 hour culture of strain VIII-R1 (approximately 30,000 cells) and incubated at 37°C. After 4 hours, to tube 1 was added 0.1 ml. of 10^{-7} dilution of culture of strain VIIIS-VIII-R1 (approximately 3 cells) and

0.1 ml. of a 10^{-5} dilution of culture of strain IIIS-VIII-R1 (approximately 300 cells). After 5 and 6 hours incubation, respectively, a mixture containing the same relative numbers of type IIIS and type VIIIS cells as in the case of tube 1 was added to tubes 2 and 3.

Incubation of all tubes was continued for a total of 20 hours and a loopful of culture supernate from each tube was then streaked on the surface of a blood agar plate. On the plates streaked from tubes 1 to 3, type IIIS colonies were present in large numbers. 0.2 ml. of supernate of each tube was transferred to broth containing 20 per cent type IIIS antiserum. After 18 hours' incubation the supernate was streaked on blood agar and in all 3 instances showed the presence of type IIIS and type VIIIS colonies recognizable readily by their distinctive colonial morphology and confirmed by quellung reaction.

It can be concluded from these experiments that by using selective techniques small numbers of type VIIIS organisms can be identified in the presence of a mixture of large numbers of type IIIS and R cells. We believe that our failure to detect type VIIIS cells in the transformation reactions using extracts of type IIIS indicates that type VIIIS cells were not present.

Transformation of Strain VIII-R1 to Type VIIIS by Extract of VIII-R13 and vice versa.—As shown in Table I a transforming extract prepared from strain VIII-R1 caused transformation of strain VIII-R13 to type VIIIS. Similarly an extract of strain VIII-R13 transformed cells of strain VIII-R1 to type VIIIS. Neither of these transforming extracts, however, caused transformation of strain II-R36NC to type VIIIS, nor did extracts of strain II-R36NC transform either strain of VIII-R to type VIIIS (Table I).

One may conclude on the basis of these observations that the mutations in strains VIII-R1 and VIII-R13 which result in inability to synthesize type VIII polysaccharide are non-allelic so that an extract of either strain applied to the other is able to restore the capacity to produce type VIII polysaccharide. As might be expected autologous extracts did not cause transformation of either VIII-R strain to type VIIIS (Table I). Failure of extracts of strain II-R36NC to transform either of the VIII-R strains to type VIIIS is not explicable on the basis of the present experiments.

Transformation Reactions with Extracts of Pneumococcus Types IS, VIIS, XIVS and XVIIIS Applied to Strains VIII-R1 and VIII-R13.—The results of transformation reactions with extracts of types IS, VIIS, XIVS, and XVIIIS and strains VIII-R1 and VIII-R13 are shown in Table I.

An extract of type I applied to strain VIII-R1 yielded a predominance of type VIIIS cells in some transformation reactions, in others type IS predominated. This is in contrast to the results found with type IIS extracts and both VIII-R strains, since type VIIIS cells invariably predominated and the type IIS organisms could be detected only upon the use of a specific selective procedure—cultivation in type VIIIS antiserum. S cells, whether of type I or

type VIII were not recovered from transformation reactions employing strain VIII-R13 and type IS transforming extract.

It has been a consistent experience with all the transforming extracts used that mutant VIII-R13 is less "transformable" than mutant VIII-R1. That is to say, the same quantity of extract applied to strain VIII-R1 yields larger numbers of S colonies, whether type VIIIS or the type homologous to the donor strain used for preparing the DNA extract. Furthermore, transformation may fail entirely to occur with strain VIII-R13 although successful with strain VIII-R1.

The results observed with transforming extracts prepared from type VIIS pneumococcus applied to strain VIII-R1 were similar to those that occurred with type II extracts (Table I). Type VIII pneumococci predominated and could be identified directly on streaking the supernate of the culture from the transformation tube on blood agar. Type VIIS organisms could be found only after subculture in the presence of type VIIIS antiserum. Neither type VIIIS nor type VIIS organisms were found when type VIIS transforming extract was applied to strain VIII-R13.

A transforming extract prepared from type XIVS pneumococci and applied to strain VIII-R1 yielded a predominance of type VIIIS cells in some tubes, in others type XIVS cells predominated. Type XIVS cells only were found when the type XIV extract was applied to strain VIII-R13 (Table I).

When a type XVIIIS extract was applied to strain VIII-R1, type VIIIS cells predominated. Type XVIIIS was found only after subculture in the presence of type VIIIS antiserum. In tests with strain VIII-R13, type XVIIIS only was recovered on blood agar plates streaked from the transformation tubes. Subculture was not carried out in the presence of type XVIII antiserum.

Experiments to Determine Whether Cultures of Strains VIII-R1 and VIII-R13 Contained Type VIIIS Pneumococci.—Failure of the extracts of strain VIII-R1 or VIII-R13 to cause transformation of the autologous strain to type VIII or to transform strain II-R36NC to type VIII indicated that the cultures of the two VIII-R strains did not contain a significant number of type VIIIS organisms, and that significant back mutation to type VIIIS had not occurred during routine transfers in broth or on mass cultivation for the preparation of DNA extracts. Additional experiments were carried out, however, to investigate these possibilities.

Immediately before the addition of sodium desoxycholate to lyse the mass cultures used for preparing the transforming extracts, aliquots were removed. The viable count for both strain VIII-R1 and VIII-R13 was approximately 10^9 pneumococci per ml. Cultures were inoculated intraperitoneally in mice and in addition were inoculated into anti-R serum broth. Five mice were injected with 0.5 ml. of undiluted culture of each R strain (5×10^8 bacteria) and 5 mice with 0.1 ml. (10^8 bacteria). None of the 10 mice injected with strain VIII-R13 showed the

presence of type VIIIS pneumococci. Similarly none of 5 animals injected with 0.5 ml. of culture of VIII-R1 showed type VIIIS pneumococci, but one of the 5 injected with 0.1 ml. showed type VIIIS organisms in its heart blood at autopsy.

0.1 ml. of undiluted culture and 0.1 ml. of each of 7 successive 10-fold dilutions of culture of each strain were inoculated into 2 ml. of anti-R serum broth. The cultures were incubated at 37°C. overnight and the supernates then streaked on blood agar plates. No S colonies were observed.

These observations make it clear that the reciprocal transformations of strains VIII-R1 and VIII-R13 to type VIIIS were not caused by the presence of type VIII pneumococci in significant numbers in the cultures used for preparation of transforming extracts. The recovery of type VIIIS pneumococci in cultures of the blood of one mouse injected with 0.1 ml. of strain VIII-R1, but absence of smooth organisms in the other mice injected or their failure to appear in the highly selective medium containing anti-R serum, indicate that the culture of VIII-R1 probably contained not more than one type VIIIS pneumococcus per 10^8 to 10^9 R organisms or one type VIIIS pneumococcus per ml. This is not a significant number.

In addition to these tests for the presence of encapsulated type VIIIS pneumococci in the mass cultures of strains VIII-R1 and VIII-R13 used for preparation of the transforming extracts, a search for type VIIIS pneumococci was made in *stock cultures*. Serial transfer of both VIII-R strains in anti-R serum broth on no occasion showed the presence of S pneumococci. This method is capable of detecting one to five S pneumococci in the presence of 10^9 R organisms in a single culture passage (2). In addition, precipitin reactions on culture supernates of both R strains were negative for type VIII capsular polysaccharide. The type VIIIS antiserum used was capable of detecting type VIII polysaccharide in a dilution of 1:5,000,000.

Injection of 18 mice with 1 ml. of undiluted stock culture of strain VIII-R13 (10^8 organisms) or injection of 10 mice with one-tenth this number of bacteria did not reveal the presence of type VIIIS pneumococci. However, 2 of 10 mice injected with 1 ml. of a stock culture of strain VIII-R1 (10^8 pneumococci) showed type VIIIS pneumococci in their heart blood. None of 5 mice injected with 0.5 ml. of stock culture of VIII-R1 nor of 5 mice injected with 0.1 ml. culture showed the presence of type VIIIS pneumococcus.

It is apparent from these tests that the presence of type VIIIS pneumococci in the cultures of the 2 strains of VIII-R pneumococci cannot account for the findings described in this paper. On no occasion were encapsulated organisms detected in cultures of strain VIII-R13, and the number present in cultures of VIII-R1 (a maximum of 1 S organism per 10^8 to 10^9 R total viable count) is so small that in our opinion it did not have any influence on the results.

DISCUSSION

Two R mutants of the same strain of type VIIIS pneumococcus are described in which the mutations leading to inability to synthesize type VIII polysaccharide appear to be at different gene loci since transforming extracts prepared from each and applied to the other strain cause transformation to

fully encapsulated type VIIIS pneumococcus. Autologous extracts do not cause transformation to type VIIIS. Two genes, both of which are concerned with production of type VIII polysaccharide can thus be recognized.

This is the first instance of which we are aware wherein the phenotypic character appearing in the cells transformed by DNA extracts is entirely absent from the donor strain. In the "allogenic" transformation to normal encapsulation described by Ephrussi-Taylor (1) the capacity to produce type III polysaccharide was present in both donor and recipient cells but in reduced amount. The present observations, however, are entirely similar to those of Stocker, Zinder, and Lederberg (4) in which flagellated derivatives of *Salmonella* O strains were produced by transduction with phage-containing lysates of other O strains.

Treatment of the two VIII-R strains with extracts prepared from various smooth pneumococci led to different results depending on the type of the donor strain. DNA extracts from type IIS applied to both R strains yielded mainly type VIIIS pneumococci in the transformed population. The proportion of type IIS cells found was only about 1 per cent of the type VIII population. It is apparent, therefore, that both in strain VIII-R1 and VIII-R13 the mutations are in genes not concerned with the immunological (and chemical) specificity of the type VIII polysaccharide since the ability to produce type VIII polysaccharide can be restored in both strains by an extract of type II pneumococcus in which it is highly unlikely that genes specific for type VIII polysaccharide would be present. Transformations using extracts of types IS, VIIS, XIVS, and XVIIIS, especially when applied to strain VIII-R1, gave generally similar results, although the proportion of type VIIIS cells relative to that of the type of the donor in the transformed population was variable.

In reactions employing DNA extracts from pneumococcus type IIIS, different results were obtained. On no occasion were type VIIIS pneumococci recovered despite careful search employing highly selective conditions. The transformed population consisted entirely of type IIIS cells in repeated experiments with both VIII-R mutants. From these observations it appears that under the conditions described the gene(s) responsible for type III specificity may supersede the expression of those bearing type VIII specificity. Alternatively it is possible that the type III DNA extracts do not contain the gene(s) necessary for restoration of type VIII polysaccharide synthesis in these 2 different VIII-R mutants, even though present in a variety of other type-specific strains.

SUMMARY

Two distinct R mutants of pneumococcus type VIIIS have been described in which the mutations leading to loss of type VIII polysaccharide production are non-allelic. A DNA extract prepared from either R strain restores the

capacity to produce type VIII polysaccharide in the other strain. Autologous extracts are inert in this respect.

DNA extracts from pneumococcus type IIS when applied to the two R mutants consistently yielded a high proportion of cells transformed to type VIIIS and relatively small numbers of type IIS cells.

When extracts prepared from other encapsulated pneumococci, types IS, VIIS, XIVS, and XVIIIS, were applied to R mutant VIII-R1, a mixture of type VIIIS cells and cells corresponding to the type of the donor appeared, although the proportion of type VIIIS cells was not uniformly in excess. The same extracts applied to the second R mutant, VIII-R13, yielded transformed cells of the type of the donor only, or else transformation did not occur.

Transforming extracts of a strain of pneumococcus type IIIS gave rise only to type IIIS pneumococci when applied to either strain. Type VIIIS pneumococci were never recovered from such interactions.

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