Unstable Binary Capsulated Transformants in *Pneumococcus*

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Through transformation reactions, binary capsulated SI-III strains of pneumococci have been isolated that are unstable and lose the SI capsular genome at high frequency. The instability is caused by the specific mutation in the SIII capsular genome common to all of the unstable strains. In the family of strains studied, the SI capsular genome was integrated into the recipient chromosome in at least two loci: one apparently adjacent to the resident capsular genome and a second some distance from it. A hypothesis is presented to explain the instability of the strains on the basis of redundancy of genetic information.

Eleven binary capsulated SI-III strains of pneumococci which exhibit many characteristics in common were described in a previous publication (1). The strains, which produce simultaneously the capsular polysaccharides of type I and type III pneumococcus, were isolated from reactions in which the recipient cells carrying a normal or mutated type III capsular genome were transformed with deoxyribonucleic acid (DNA) from wild type I cells. The binary capsulated strains were all stable, and, in extracts of DNA prepared from them, no linkage could be demonstrated between the S-III capsular genome of the original recipient cell and the SI capsular genome introduced by the transformation reaction. In a series of sequential transformation reactions with recipient cells derived from diverse capsular types other than type I, the SI markers derived originally from SI-III cells continued to be integrated into the recipient chromosome at a locus other than that normally occupied by the capsular genome. All transformants were genetically binary whether they expressed phenotypically one capsular polysaccharide or two. Strains possessing SI markers that behaved in this manner were designated "atypical" or SI^A to distinguish them from wild-type or SI⁺ strains. Phenotypically, SI^A strains and SI⁺ strains were identical.

SI-III strains have been isolated that differ in several properties from the strains described previously. This report describes a family of strains that are unstable. Some of these strains evince linkage between the S-III and SI capsular genomes and give rise to wild type SI transformants; others, except for their instability, resemble strains described earlier.

MATERIALS AND METHODS

Nomenclature of pneumococcal strains. The system of nomenclature used in a previous publication (1) will be followed.

Briefly, the phenotype of fully capsulated strains will be designated by the letter S followed by a Roman numeral indicating capsular type; the phenotype of binary capsulated strains will be designated by the letter S followed by two Roman numerals separated by a hyphen, indicating the capsular polysaccharides they produce. The classification of Ephrussi-Taylor (4) will be used to describe the phenotype of mutant and transformant strains producing normal (SIII-N) or reduced (SIII-2) amounts of type III polysaccharide. No strains of the SIII-1 category of Ephrussi-Taylor will be encountered in this report.

The genotype of strains carrying a *single* capsular genome will be indicated as follows: capsulated strains, the letter S followed by a Roman numeral indicating capsular type and a superscript +; noncapsulated strains or strains producing less than the normal amounts of capsular polysaccharide, the designation S- followed by a subscript Roman numeral indicating the capsular type from which they were derived, and, when necessary, an Arabic subscript indicating strain number. Strains bearing the same Arabic number carry the same mutation in the capsular genome.

The genotype of strains carrying *two* capsular genomes is indicated by the genotypic designation of the parent strain (which is unchanged in binary strains) followed immediately by the genotypic designation of the second capsular genome. Since the second capsular genomes of the binary strains discussed in this report carry no apparent mutations, they will be represented by a Roman numeral indicating capsular type, followed by a superscript + or A to indicate whether DNA from the strain gives rise to wild-type or "atypical" transformants.

New strains. Strains 565, 791, 854, 1049, and 1073

are new strains for which the derivations, phenotypes, and genotypes are given in Table 1.

Preparation of DNA. In early experiments, DNA was prepared according to the method of MacLeod and Krauss (7). In later experiments, DNA was prepared from young unneutralized cultures (2). Treatment with ribonuclease was carried out according to the method of Marmur (8). The DNA concentration was determined by the method of Burton (3).

Ribonuclease. The ribonuclease used was a preparation (from Worthington Biochemical Corp., Freehold, N.J.) homogenous on polyacrylamide gel.

Recipient cells. Unless otherwise noted, frozen competent cells were grown as described previously (1).

Type III depolymerase. Type III depolymerase was prepared by the method of Torriani and Pappenheimer (10).

Transformation. In early experiments, transformation was carried out in Neopeptone-infusion broth containing 5% heated normal human plasma. In later experiments, transformation was carried out in CH medium (6) containing 4 ml of 4% albumin per 100 ml of medium. Cells were incubated with DNA at 32 C for 20 min, and the reaction was terminated by the addition of deoxyribonuclease. Transformation was scored as described previously (1), and quantitative results are reported as the ratio of capsular transformants to streptomycin-resistant transformants.

RESULTS

In an effort to obtain cells of the genotype SIII-N-I^A, cells of the wild type III strain A66

and cells of strain 565 were grown overnight in the presence of DNA from an SI⁺ strain and the enzyme type III depolymerase. SI⁺ and SI-III transformants of both recipient strains were isolated. The SI-III strain recovered from strain A66 as the recipient conformed to the pattern of previously isolated SI-III strains and was described in an earlier report (1). The SI-III transformant recovered from strain 565 as the recipient, and designated strain 791, behaved anomalously.

Characteristics of strain 791. Phenotypically, strain 791 was similar to other SI-III strains. The colonies were large and mucoid, and resembled those of type III pneumococci. In quellung preparations, the cells exhibited a large refractile halo when exposed to type III antiserum. The size of the refractile zone with type I antiserum was intermediate between that seen in other SI-III strains and that seen with type I⁺ cells.

Strain 791 is unstable. Upon plating after several subcultures in liquid medium, about 30% of the colonies were very small and flat, although obviously still of the "smooth" category. Cells from these colonies no longer reacted with type I antiserum; with type III antiserum, they exhibited a refractile zone smaller than that seen with cells of strain 791. The cells grew diffusely in the presence of antibody to noncapsulated pneumococci, and from their properties could be assigned to the SIII-2 phenotype in the classification of Ephrussi-

TABLE 1. Derivation of new strains

- $$\text{SIII}^+ \xrightarrow{\text{mutation}} \text{S-III}^{\text{A}} \xrightarrow[\text{S-II } str-r]{\text{tr.}^b \text{ DNA}} \text{S-III}_{24} str-r \xrightarrow[\text{SIII}^+]{\text{tr. DNA}} \text{SIII}^+ str-r \xrightarrow{\text{mutation}} \text{S-III}_{24} str-r$$

(strain 565) (phenotype, SIII-2)^c
- $$\text{SIII}^+ str-r \xrightarrow[\text{SI}^+]{\text{tr. DNA}} \text{SI}^+ str-r \text{ and } \text{S-III}_{24}\text{I}^+ str-r \xrightarrow[\text{of SI-III}]{\text{segregation}} \text{S-III}_{24} str-r$$

(strain 565) (phenotype, SI-III) (strain 791) (phenotype, SIII-2)^c
- $$\text{S-II} \xrightarrow[\text{S-III}_{24}\text{I}^+ str-r]{\text{tr. DNA}} \text{SI}^+, \text{S-III}_{24}^c \text{ and } \text{S-III}_{24}\text{I}^+ \xrightarrow[\text{of SI-III}]{\text{segregation}} (\text{phenotype, SIII-2})^d$$

(phenotype, SIII-2) (strain 854) (phenotype, SI-III)
- $$\text{S-III}_{24} \xrightarrow[\text{SI}^+]{\text{tr. DNA}} \text{SI}^+ \text{ and } \text{S-III}_{24}\text{I}^+ \xrightarrow[\text{of SI-III}]{\text{segregation}} (\text{phenotype, SIII-2})^d$$

(phenotype, SIII-2) (strain 854) (phenotype, SI-III) (strain 1049)
- $$\text{S-III}_{24} \xrightarrow[\text{S-II}^{\text{A}}]{\text{tr. DNA}} \text{S-III}_{24}\text{I}^{\text{A}} \xrightarrow[\text{of SII-II}]{\text{segregation}} (\text{phenotype, SIII-2})^d$$

(phenotype, (phenotype, SI) (phenotype, SI-III) (strain 854) (strain 1073)

^a Strain deficient in uridine diphosphate glucose dehydrogenase.

^b tr. = transformation reaction.

^c The site of deficiency in this strain has not been established biochemically.

^d The genotype of this strain has not been determined.

Taylor (4). An established strain has been designated S-_{III24} in this laboratory.

Genetic behavior of strain 791. When S-_{II} cells were exposed to saturating concentrations of DNA from strain 791, three types of capsulated transformants, SI, SIII-2, and SI-III, were recovered in high yield, as shown in experiments 1 and 2 of Table 2.

The high yield of SI-III transformants indicated linkage between the SIII and SI capsular genomes and further distinguished strain 791 from SI-III strains described earlier. To preclude any possibility that the SI-III transformants arose from two transformational events, the experiments were repeated with nonsaturating levels of DNA, and the DNA was further purified by treatment with ribonuclease (experiments 3 and 4, Table 1).

It was thought possible that the excess recovery of SIII-2 transformants over SI and SI-III phenotypes exemplified in experiments 1 and 2 was caused by the instability of strain 791 and the loss of the SI capsular genome from cells during the growth of cultures from which DNA was to be extracted. For preparation of the DNA used in experiments 3 and 4, a tube of blood broth was inoculated in the morning with bacteria from 20 verified SI-III colonies; in the evening, a sample of the culture was used to inoculate 100 ml of broth. The next morning, 1 liter of broth was inoculated with the 100-ml culture and was allowed to grow for 3.5 hr. Both the 100-ml and the 1-liter cultures were incubated in an atmosphere of 5% CO₂, and the enzyme type III depolymerase was added to reduce the amount of type III polysaccharide in the lysate. The extracted DNA was maintained in 70% alcohol until the day before it was to be used, for it was noted that, in previous preparations, the frequency of recovery of SI-III transformants fell as the DNA solution aged.

It can be seen in experiments 3 and 4 that, with the precautions noted above in the preparation of DNA, there was no longer an excess

yield of SIII-2 transformants; instead, SI-III cells were recovered at a higher frequency.

The SI transformants recovered in these experiments were of the wild type, and their DNA transformed all S-_{III} strains tested to capsular types SI and SI-III. DNA was prepared from 11 clones of SI transformants, each arising from an independent transformational event; all behaved in an identical manner. Results from DNA of eight clones isolated in experiment 1 are presented in Table 3.

The SIII-2 transformants recovered after transformation of strain S-_{II} with DNA from strain 791 resembled in all respects the SIII-2 segregant clones found in cultures of strain 791. The occurrence of SIII-2 transformants and SIII-2 segregants suggested that, in the original transformation reaction from which strain 791 was isolated, the recipient cell was not an SIII-N cell of strain 565, but was a hitherto unsuspected mutant SIII-2 cell. Subsequently, such an SIII-2 clone was isolated from strain 565. The DNA of all of the SIII-2 strains behaved in an identical manner in transformation reactions, and, so far as could be determined, the segregant clones and SIII-2 transformants were carrying no part of the SI capsular genome. The DNA of each strain transformed S-_I strains only to SIII-2. SI segregants have never been recovered from strain 791.

In addition to S-_{II} cells, S-_I, and S-_{VII} strains have been transformed to SI-III with DNA prepared from strain 791. All SI-III transformants exhibited the instability of strain 791 and DNA prepared from them yielded SI-III transformants at a high frequency.

The genetic behavior of DNA from strain 791 indicates that the genotype of the strain is S-_{III24}I⁺; the phenotype, SI-III.

Transformation reactions of strain S-_{III24}. Many unsuccessful attempts were made to transform SIII-2 segregant clones and the SIII-2 variant isolated from strain 565. The strains were incompetent. One competent SIII-2 trans-

TABLE 2. Transformation of strain S-_{II} with DNA from strain 791^a

Expt no.	SI/ <i>str-r</i>	SI-III/ <i>str-r</i>	SIII-2/ <i>str-r</i>	Frequency of <i>str-r</i> transformants
1	3.3×10^{-3}	9.8×10^{-3}	2.1×10^{-2}	4.6×10^{-2}
2	9.7×10^{-4}	1.1×10^{-3}	3.3×10^{-3}	1.3×10^{-1}
3	9.0×10^{-4}	3.86×10^{-3}	9.0×10^{-4}	4.1×10^{-2}
4	8.8×10^{-4}	2.5×10^{-3}	8.8×10^{-4}	5.1×10^{-2}

^a Recipient: frozen competent cells of strain R₆. Experiment 1: transformation carried out in Neopeptone infusion broth + 5% heated normal plasma. Experiments 2, 3, and 4: transformation carried out in CH medium. DNA concentration was saturating in experiments 1 and 2, and 0.05 µg/ml in experiments 3 and 4. DNA in experiment 4 was treated with ribonuclease.

TABLE 3. Testing of strains of *S*-II *str-r* transformed to capsular type I with DNA from strain 791^a (recipient strain, *S*-III₈)

DNA from strain	SI/no. of samples	SI-III/no. samples
976	7/10	2/10
977	6/10	4/10
978	4/10	3/10
979	5/10	6/10
984	4/10	5/10
985	4/10	7/10
986	6/10	5/10
987	4/10	3/10
972 ^b	0/10	10/10
973 ^b	0/10	10/10

^a The dilution tested was 1:100.

^b *S*-II^I_A *str-r* strains tested in the same experiment.

formant of strain *S*-II, strain 854 (see Table 1), has been found, and this strain has been used as the recipient in several experiments.

For transformation, cells were grown in CH medium with and without type III depolymerase. No depolymerase was added to the transformation tubes, but the enzyme was added to all tubes containing antibody to noncapsulated pneumococci in which the transformed cells were grown to allow expression of the capsule. Cells grown to competence in the absence of depolymerase manifested transformation, but the apparent rate of transformation was approximately twice as high with cells grown in the presence of the enzyme. Table 4 sets forth the results of an experiment in which frozen competent cells of strain 854 were transformed with DNA from an *SI*⁺ *str-r* strain. Transformation to *SI* and *SI*-III was comparable to that found with other *S*-III strains as recipients.

The *SI*-III transformants were highly unstable, more so than was strain 791. From one experiment, six single clones of *SI*-III transformants of strain 854, each arising from an independent transformational event, were transferred daily on blood-agar plates. After 15 transfers of single, verified *SI*-III colonies, *SI*-2 segregants were still found in all lines. It has been possible to carry as a stock strain one clone of an *SI*-III transformant which exhibits the same degree of stability as does strain 791.

Strain 854 (*S*-III₂₄) was transformed also with DNA prepared from strain 908, *S*-II^I_A *str-r*. Strain 854 behaved in this reaction like all *S*-III strains except strain *S*-III₁₅ (2) and yielded only *SI*-III transformants. These transformants were unstable also, and *SI*-2 segregants were found regularly in all cultures. It has been possible to

carry, as stock cultures, several *SI*-III clones which arose in separate transformation events.

Comparisons of strains of *S*-III₂₄^I⁺ and *S*-III₂₄^I^A. DNA was prepared from each of two strains of 854 transformed to phenotype *SI*-III: strain 1049, in which the *SI* capsular genome was donated by an *SI*⁺ strain, and strain 1073, in which the source of the *SI* capsular genome was an *S*-II^I_A strain. Both strains were unstable, and the precautions noted in the preparation of DNA from strain 791 were observed. Table 5 presents the results of an experiment in which *S*-II cells were exposed to nonsaturating levels of the two DNA preparations.

It can readily be seen that DNA of strain 1049 resembled that of strain 791 in manifesting a high degree of linkage between the *SI*⁺ and *S*-III₂₄ capsular genomes. Strain 1073, on the other hand, showed no linkage, under the experimental conditions employed, between the two capsular genomes, and behaved genetically like binary strains described previously (1). When the same experiment was carried out with 0.5 μg of DNA from strain 1073 per ml, a saturating concentration, some *SI*-III transformants of strain *S*-II were recovered. Further experiments must be performed to determine whether there is a low degree of linkage between the *S*-III₂₄ and the *SI* capsular genomes of strain 1073.

The *SI* transformants isolated after transformation of strain *S*-II with DNA of strain 1073

TABLE 4. Transformation of strain 854 with DNA of *SI*⁺ *str-r*^a

<i>SI</i> / <i>str-r</i>	<i>SI</i> -III/ <i>str-r</i>	<i>SI</i> / <i>SI</i> -III	Frequency of <i>str-r</i> transformants
6.4×10^{-3}	3.6×10^{-4}	17.5	2×10^{-1}

^a Recipient: frozen competent cells grown with type III depolymerase. Results are the average of 50 samples from each of two transformation tubes.

TABLE 5. Transformation of strain *S*-II with DNA of strains 1049 and 1073^a

DNA	<i>SI</i> transformants/ml	<i>SI</i> -2 transformants/ml	<i>SI</i> -III transformants/ml
1049	0 ^b	0 ^b	1,500
1073	1,650	1,350	0

^a DNA concentration: 0.05 μg/ml. Donor strains did not carry *str-r* markers.

^b No *SI* or *SI*-2 transformants were found at the dilutions sampled; *SI*-2 transformants were recovered when lower dilutions were tested.

were of the "atypical" kind and transformed all S-III strains tested only to SI-III. The genotype of strain 1073 can be written as S-III₂₄I^A.

DISCUSSION

Strain 791 differs in several important respects from binary strains studied earlier. First, strain 791 is unstable, and SIII-2 segregants, such as S-III₂₄, appear with high frequency. Second, SI transformants recovered after exposure of S-III strains to DNA from strain 791 behave like wild type SI strains and transform other S-III strains to SI and SI-III. Third, the S-III₂₄ and SI⁺ capsular genomes are linked. Previously (1), binary strains were described that were stable, gave rise only to "atypical" SI transformants, and exhibited no linkage between the S-III and SI capsular genomes. These differences in behavior suggest strongly that the association of the SI capsular genome with the recipient chromosome is different in strain 791 from that in other SI-III strains. Whereas it was concluded that, in other strains, the SI capsular genome was integrated into the recipient chromosome at some distance from the resident capsular genome, the evidence presented in this report suggests that, in strain 791 and in related strains except for strain 1073, the SI capsular genome is located adjacent to or very near the S-III capsular genome. In strain 1073, as in binary strains described previously, it is not known exactly where the SI capsular genome resides, but it is apparently located between outside markers different from those in wild-type strains, for strain 1073 gives rise to "atypical" SI transformants.

Iyer has reported unstable genetic transformation in *Bacillus subtilis* to both prototrophy and erythromycin resistance (5). In these transformed cells of *B. subtilis*, clones became stabilized within a few generations after transformation. The conclusion was reached that the unstable transformants were heterogenotes in which the donor DNA could be transmitted linearly for several cell divisions before recombination with the recipient chromosome occurred.

The results in the present report of unstable genetic transformation in pneumococcus lead to different conclusions. Because of the high degree of linkage exhibited between the two capsular genomes in DNA preparations of strain 791, it must be assumed that the SI capsular genome is integrated covalently into the chromosome. Furthermore, the pneumococcal strains have remained unstable over a period of several years, and, indeed, a stable SI-III clone of strain 791 (or of other strains related to it) has never been isolated. The behavior of strain 791 re-

sembles that of gram-negative bacilli carrying an integrated episome more closely than it resembles any transformant strain previously described in pneumococcus or in other transformable species.

Strain 1073 fails to satisfy either criterion that has been used to argue for integration into the recipient chromosome of the second capsular genome in binary capsulated strains. DNA extracted from the strain does not show close linkage of the two capsular genomes, nor is the strain stable. Whether the SI-III transformants found when saturating concentrations of DNA were used to transform strain S-III were evidence for a low degree of linkage, or whether the transformants arose as a result of two transformational events, remains to be determined. Other binary strains that exhibit the same questionable linkage have been found and will be described in a separate communication. Despite the fact that strain 1073 is unstable, because it arose following transformation with DNA from an "atypical" SI strain and because it gives rise to "atypical" SI strains, it is believed that it is analogous to SI-III strains that have been studied previously (1, 2) and that the SI capsular genome is incorporated linearly into the recipient chromosome.

The results of experiments presented in this report suggest that the instability of the binary strains examined is caused by the specific mutation in the S-III₂₄ capsular genome or some other factor so closely linked that it is always cotransferred, in or out, together with the capsular genome. The particular S-III mutant capsular genome is common to all of the unstable strains, whatever their derivation. Furthermore, if strain 854 (S-III₂₄) is transformed to SI, SII, or SVI, thereby losing the S-III₂₄ capsular genome, the transformants are stable. One possible explanation for the instability of the SI-III strains carrying the S-III₂₄ mutation was suggested by Thomas (9). From experimental evidence and theoretical considerations, he suggested that, in DNA, the minimal number of consecutive complementary bases necessary for synapse is 12 (the actual number may be more than 12, but it is small). If, therefore, a double-stranded DNA molecule contains two identical stretches of at least 12 consecutive bases on each strand and single strand breaks occur, such as at time of replication, there may be synapsing between distant parts of the chromosome at the complementary regions. This occurrence would lead to a loss of the genetic material between the two repeating portions of the molecules. Any strain carrying such repeating units would be expected to be unstable.

FIG. 1. Postulated alignment of genes of capsular genomes of strain 791. *Epim_I*: uridine diphosphate glucuronic acid epimerase. *P_I*: polymerase(s) for the synthesis of type I polysaccharide. *P_{III}*: polymerase(s) for the synthesis of type III polysaccharide.

The only enzyme known to occur in common in the biosynthetic pathways of type I and type III pneumococcal capsular polysaccharides is uridine diphosphate glucose (UDPG) dehydrogenase. In none of many hundreds of transformation reactions, carried out over a 15-year period, has there been any evidence for intracistronic recombination in the UDPG dehydrogenase locus when one parent in the reaction was an SIII or S-III strain, and the other parent, an SI or S-I strain. No recombination in the locus could be demonstrated even when SI⁺ DNA was sheared so that less than 8% of intact SI⁺ markers survived. The simplest explanation for these findings is that the UDPG dehydrogenase loci of the type I and the type III capsular genomes are not homologous. In the terms discussed above, this argument would mean that nowhere in the genes are there 12 consecutive bases of identical sequence. It is possible, however, that a change in one base pair, as may occur in a point mutation, could convert a previously nonidentical stretch to an identical one. It may be that the mutation in strain S-III₂₄ is an example of such a mutation and that, in strain 791 and related binary strains, synapse can now occur between the UDPG dehydrogenase loci of the type III and type I capsular genomes, resulting in circularization and loss of genetic material. The fact that SI segregants have never been isolated from this family of strains would impose limitations on the possible alignment of genes in the two capsular genomes. Figure 1 presents an alignment which would allow for the experimental findings.

It might be expected that, if there is homology in the UDPG dehydrogenase loci of the S-III₂₄ and the SI capsular genomes, intracistronic crossing to give rise to SIII-N transformants should occur and binary SI-III strains should not be found. It seems likely, however, that the extent

of homology in the UDPG dehydrogenase loci is small in comparison to that of markers outside the capsular loci, which are believed to determine pairing for transformation to heterologous capsular type. Once the binary strain is established, however, the homology in the UDPG dehydrogenase loci may be sufficient to cause instability. Attempts have been made to look for rare SIII-N transformants from a reaction between strain 854 as recipient and DNA from an SI⁺ strain as donor, but technical difficulties have interfered. Reconstruction experiments were set up in which a few S-III₂₄ cells known to have been newly transformed to SIII-N were added to a large background of untransformed S-III₂₄ cells. In the presence of type III depolymerase and anti-R serum, the transformed cells were not recovered. In the absence of depolymerase, revertants of S-III₂₄ to SIII-N appeared so frequently as to make meaningless the search for rare SIII-N transformants. One SIII-N clone was recovered in a transformation reaction in which strain 854 was exposed to DNA from a wild type I strain, but it is impossible to know whether this clone arose from reversion or transformation.

Experiments are in progress to test the validity of the hypothesis for instability outlined above.

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