A STUDY OF PNEUMOCOCCAL MERODIPLOIDS AT THE MOLECULAR LEVEL

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ABSTRACT

The DNA of a sulfonamide-resistant Pneumococcal strain (heterozygous for *sul^r-c*) and that of three highly resistant and persistently heterozygous *cd* transformants, derived by introducing *sul^r-c* marker into a stable sulfonamide resistant strain (*sul^r-d*), were studied to analyze the genetic basis of their merodiploidy. The physical properties of the native and denatured DNA from the heterozygotes and the nonheterozygous strains were not distinguishable. The denaturability and the renaturability of biological activity for the heterozygous markers were essentially identical to those of the normal markers. The heterozygosity extends to the closely linked locus giving rise to four different configurations of *cd* and *cd⁺* transformants, characterized by their frequencies of segregation and donor-marker activities. The marker-activity ratios and the frequency of co-transfer of heterozygous markers were found to remain the same in each when the donor DNA was native, denatured or reannealed without fractionation or reannealed after remixing of resolved strands. Possible models were weighed against these observations and these considerations led to the suggestion that tandem duplication of a gene region may be responsible for the heterozygosity and instability of this region. A more detailed examination of this model will be presented in an accompanying paper.

THE sulfonamide-resistant mutant, *sul^r-c* of *Pneumococcus* (HOTCHKISS and EVANS 1958) was found to be "unstable", segregating a few percent of wild-type progeny as it is propagated in culture. This *c* marker retains this characteristic when introduced into other genotypes, including *sul^r-a*, *-b* and *-d* but with the last-mentioned a new association is produced (HOTCHKISS 1965). The *sul^r-cd* pair become closely linked, and the *d* marker may partake in the heterozygosity, *cd/c⁺d⁺*, (HOTCHKISS, ABE and LANE 1971). The linkage of *sul^r-c* with *sul^r-d*, and also with *str^r* was taken as evidence (HOTCHKISS 1965, LEDBETTER and HOTCHKISS 1975a) that *sul^r-c* occupies a chromosomal site. Segregation of the resistance markers was not accelerated by Acridine Orange which often causes loss of episomal markers; the segregation appeared to have the properties of an intracellular recombination (LEDBETTER and HOTCHKISS 1975a and b). On the other hand the gene dosages of *c* and *d* markers suggested a triplication of these chromosomal sites, for which a triple-stranded, or branched DNA would be one possibility (HOTCHKISS, ABE and LANE 1967, 1971).

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Seeking a critical test of these formulations, we have explored quantitatively the denaturability and renaturation of the biological activities of DNA of several merodiploid strains bearing several different configurations of these markers.

MATERIALS AND METHODS

Pneumococcal strains.

Two frequently described competent strains of *Pneumococcus* were used as recipients. Growth of the wild-type strain RI-26 (GABOR and HOTCHKISS 1966) is inhibited in media containing low concentrations of sulfanilamide (SA) and p-aminosalicylic acid (PAS) and resistant to p-nitrobenzoic acid (NOB). Strain RF6-7 (a wild type transformed by the *sul^r-d* of HOTCHKISS and EVANS 1958) is resistant to SA but sensitive toward both PAS and NOB. The latter drug suppresses growth of all strains carrying the *d* marker.

The standard heterozygotic *c* strain, RF3-7, had been isolated after transformation of wild-type cells with the *sul^r-c* marker of a spontaneous resistant mutant RF_c (HOTCHKISS and EVANS 1958). Strains bearing this marker are resistant to SA and PAS and sensitive to NOB. The derivative RF3SK-7 was prepared for this work by introducing micrococcal resistance (K) and streptomycin resistance, S, representing the *str^r* used in this laboratory and now designated *str^r-61* (M. ROTHEIM, private communication).

The *cd* strains RF63SK-10, -11 and -13 were obtained by HOTCHKISS and LANE (unpublished work) by introducing *sul^r-c* marker into *sul^r-d* recipient and then selecting single colonies from the transformants in growth medium containing 600 γ SA per ml. The markers *S* and *K* were introduced into these highly resistant strains, either in stepwise fashion or by simultaneous introduction of these markers carried on different particles of donor DNA. All these *cd* strains are resistant to more than 1 mg of SA per ml. These *cd* types differ in the pattern and the frequency of their segregation and the characteristic dosages of the markers *c*, *d* and *d⁺* in relation to *str^r* (HOTCHKISS, ABE and LANE 1971). Summary of the strain properties is given in Table 1.

In certain applications, these strains are given shorthand designations suggesting their phenotypes (as donor of markers) rather than the genotype. Thus, the strain identified as RF63-10 (10 being its isolation number) would be described as *cd₁*, indicating the empirical fact that the relative *sul^r-d* marker activity is 1 and *sul^r-d⁺* is 2). In this system the strains are identified by the "dosage" of *d* genes and the presence or absence of heterozygosity (*c*) or other drug resistance (*S*=*str^r*), as shown in Table 1 under "symbol".

DNA preparation, denaturation, strand fractionation and reannealing

Purified DNA was prepared from *Pneumococcal* cells by a standard method previously described (HOTCHKISS 1957). The native DNA content was estimated from the degree of hyperchromicity at 260 $m\mu$. Denaturation of DNA was performed at room temperature using 0.1N NaOH. After 5 min at alkaline pH, the solution was neutralized by adding two equivalents of NaH₂PO₄ per equivalent of NaOH. The complementary strands of DNA were resolved by taking advantage of their differential interaction with poly UG (ROGER 1972, OPARA-KUBINSKA, KUBINSKI and SZYBALKSI 1964). Equal amounts (2 OD units per ml of each) of alkali-denatured DNA and poly UG (1:1 Copolymer, lot #m2 Biopolymers Inc.) were placed in 0.2M Sodium Phosphate solution at pH 7. The mixture was allowed to stand at room temperature for 90 min before solid cesium chloride and water were added to a final molarity of 7.8. Aliquots of 5 ml containing approximately 10 OD units of DNA were centrifuged in a SW 39 rotor at 35,000 rpm for 72 hours. Fractions of 4 drops were collected after puncturing the bottom of the tube, and diluted with 0.6 ml of water.

DNA was reannealed according to the method of MARMUR, SCHILDKRAUT and DOTY (1962) as modified by M. ROGER in this laboratory. A mixture of equal amounts of resolved complementary strands or the unfractionated denatured DNA was adjusted to 0.5M sodium ion concentration. The stoppered tubes containing the DNA were immersed in a 65° refluxing methanol bath for 1 hour followed by chilling in ice.

TABLE 1
Description of the Strains of Pneumococcus Used

Strain		Concentration of drugs permitting near maximal growth				Segregants		Marker activity ratios	
Stock	Symbol	Pheno- type	S/A (mg/ml)	PAS (mg/ml)	NOB (mg/ml)	type	frequency %	d ⁺ /S	d/S
RI-26	wild	c ⁺ d ⁺	2	1	20	—	—	—	—
RF3SK-7	cd ₀ S	cd ⁺	150	400	2	c ⁺ d ⁺	3	3	0
RF63SK-10	cd ₁ S	cd	1000	400	< 0.5	c ⁺ d ⁺	6 (low)	2	1
RF63SK-13	cd ₂ S	cd	1000	400	< 0.5	c ⁺ d ⁺	0.4 (medium)	1	2
RF63SK-11	cd ₃ S	cd	1000	400	< 0.5	c ⁺ d	1	0	3
RF6-7	d	c ⁺ d	80	0.5	< 0.5	c ⁺ d	—	—	—

Transformation and scoring

Transformation was carried out by a method routinely used in this laboratory (e.g. Fox and HOTCHKISS 1957; GABOR and HOTCHKISS 1966) and described only in outline here. Cells are grown to competence, held at 25° for 15 min for "phasing", frozen in 10% glycerol and stored in a freezer. These cultures, thawed immediately before use, are pre-incubated at 30° for 10 min before and 10 min after appropriate dilution, exposed to saturating amounts of DNA (0.45 µg/ml) at 30° and treated 15 min later with excess DNase, followed by incubation at 37° for 90 min. The mixture was diluted into molten neopeptone agar medium containing appropriate drugs and then poured into plates. The plates were incubated at 37° for 18 hours before scoring the transformants. The concentration of drugs used for scoring transformants for different markers were as follows: 150 µg of dihydrostreptomycin/ml (for *str^r*); 20 µg of NOB/ml (wild type, *c⁺d⁺*); 50 µg of SA/ml (*sul^r-d*, *-c* and *-cd*); 200 µg of PAS/ml (*sul^r-c* and *-cd*); 1000 µg of SA/ml (*sul^r-cd*).

The marker ratios were calculated by dividing the total transforming activity for any particular marker (or combination of linked markers) by the total number of *S* transformants obtained in the same transformed culture. The linkage of *c* and *d* or *d⁺* is expressed by the ratio of double transformants *cd* or *cd⁺* to the total number of *c* transformants.

RESULTS

Strand fractionation of DNA from heterozygotes

If the mode of segregation and the marker abundance of the sulfonamide-resistant heterozygote of *Pneumococcus* were to be attributed to an anomalous physical structure of its genome, such physical anomaly might reveal itself in the complementary strands of its DNA. Illustrated in Figure 1 is a typical profile of strand resolution of DNA from a *c* strain. Resolution, on banding in CsCl density gradients, is based on difference in buoyant density of the two strands due to their differential binding with poly UG. The pattern shown is typical of profiles from our merodiploids and shows no discernible difference from those of DNA from stable *Pneumococcal* strains (ROGER 1972 and unpublished results). No enrichment of any of the markers, including those involved in heterozygosity, was ever detected in any fraction. Neither could enrichment of the heterozygous markers be achieved in any fraction when denatured DNA bound on MAK columns (ROGER 1968) was eluted by salt gradient or in stepwise fashion (data not shown here). Fractionation of native DNA by sucrose density gradient centrifugation also failed to enrich any fraction in *c* marker (ABE, LANE and HOTCHKISS, unpublished results).

Residual activity of denatured DNA and recovery upon reannealing

In Table 2 there is a summary of the residual transforming activity of the markers in denatured DNA and the recovery of activity upon reannealing either the unfractionated denatured DNA or the re-mixed resolved complementary fractions. The heterozygous marker *c* is inactivated, on denaturation with alkali, to the same extent as the markers *d* and *d⁺*, or the loosely linked marker *str^r*. This last marker remains unaffected by the presence of *c* marker in the same strain, but the *d* and *d⁺* markers have been previously found to take part in the merodiploidy.

FIGURE 1.—Optical density profile of complementary strand resolution of merodiploid DNA. Denatured DNA of Pneumococcal strain RF63SK-13 (*cd₂S*) centrifuged with polyribo-UG in a cesium chloride gradient.

As is commonly found with biological activity of various normal markers and DNA's in this and other laboratories, unfractionated (heat or alkali) denatured DNA can be reactivated to about 50% of native activity. Likewise, the lower (35%) reactivation of mixed fractions obtained by the polyribotide fractionation is similar to all experience in this laboratory (ROGER 1972 and unpublished observations). It may be pointed out that a significant part of the optical density of the complementary strand fractions is due to bound poly UG, more with the heavy strand than with the lighter complement. An inhibitory effect of poly UG, on annealing, could be postulated from our (unpublished) findings that heavy-strand fractions self-anneal very little (ca. 1% of native activity) and less than half as much as light-strand fractions.

The data show that the recovery of *c* activity upon reannealing is not drastically lower than that of the other markers, *str*, *d* and *d⁺* for either way of reannealing. A recovery for *c* possibly 10% lower than that of *str^r* even if significant is not simply interpretable in structural terms. Lower than normal re-naturability (ca. 60%) is characteristic of an otherwise normal marker, *mic^r* (micrococccin resistance) (ROGER, BECKMANN and HOTCHKISS 1966; GABOR and HOTCHKISS 1966; ROGER 1972).

TABLE 2
Transforming Activity of Denatured and Renatured Merodiploid DNA

	Marker:	Percent of native activity on RI-26 recipient				Percent of native activity on RF6-7 recipient			
		<i>S</i>	<i>c</i>	<i>d</i>	<i>cd</i>	<i>S</i>	<i>c</i>	<i>d</i> ⁺	<i>cd</i> ⁺
RF3SK-7 (<i>cd₆S</i>)	Denatured	5.5	5.9	—	—	5.4	5.8	6.8	6.1
	Reannealed (unfractionated)	58	50	—	—	58	47	60	62
	Fractionated and annealed	34	31	—	—	36	30	47	30
	Denatured	4.9	6.0	5.4	5.0	5.5	6.5	5.3	—
RF63SK-10 (<i>cd₇S</i>)	Reannealed (unfractionated)	49	44	56	42	54	49	52	—
	Fractionated and annealed	33	36	41	32	34	29	36	—
	Denatured	3.7	5.1	4.5	2.6	4.3	4.8	4.9	5.9
	Reannealed (unfractionated)	48	52	40	32	45	39	50	46
RF63SK-13 (<i>cd₈S</i>)	Fractionated and annealed	35	37	35	21	37	32	43	30
	Denatured	4.5	5.1	4.9	6.0	4.8	5.7	—	—
	Reannealed (unfractionated)	49	50	51	48	41	34	—	—
	Fractionated and annealed	27	23	36	22	24	22	—	—
RF63SK-11 (<i>cd₉S</i>)	Denatured	4.5	5.1	4.9	6.0	4.8	5.7	—	—
	Reannealed (unfractionated)	49	50	51	48	41	34	—	—
	Fractionated and annealed	27	23	36	22	24	22	—	—
	Denatured	4.5	5.1	4.9	6.0	4.8	5.7	—	—

Marker activity ratios in native, denatured and reannealed DNA from heterozygotes

Marker activities, as explained, are compared as ratios of the yield of transformants for a particular marker (or combination of markers) to that of *str^r* transformants obtained in the same transformed culture. Transformation for the *d* markers and their combinations can be scored on one recipient (R1-26), while the *d⁺* alleles (or combinations) can be estimated on the RF6-7 recipients. However, *str^r* transformants are available in transformations using either of the recipients.

Comparing the marker-activity ratios of native DNA from different heterozygotes (Table 3), it becomes apparent that the dosages of *d* and *d⁺* in the *c* and *cd* strains are substantially different. Besides relating markers to a reference marker (*str^r* or *S*, here), it is necessary to take into account the intrinsic "efficiency" (EPHRUSSI-TAYLOR, SICARD and KAMEN 1965) of different markers in a recipient. Thus, *d* and *d⁺* are commonly transferred about 1.6 times as efficiently as *str^r-61*, and *c* marker is about 0.2 times as efficient. The relations of these efficiencies are explored and discussed by LEDBETTER and HOTCHKISS (1975a) who conclude that *c* marker is not an example of the LE low efficiency class (EPHRUSSI-TAYLOR, SICARD and KAMEN 1965; LACKS 1966).

These considerations permit us to normalize the marker activity ratios in the strains RF3SK-7, RF63SK-10, RF63SK-13 and RF63SK-11 into the "dosages" of 3, 2, 1, and 0 for *d⁺* and 0, 1, 2, and 3 for *d* respectively. The dosage of *c* marker in all these strains is more or less the same. These estimates were observed and reported earlier (HOTCHKISS, ABE and LANE 1967, 1971). It is important here to note the consistency of these characteristic marker-activity ratios from each heterozygote, regardless of the physical state of the donor DNA. The frequency of the transfer of *d⁺*, *d* and *c* markers (alone and in combinations *cd⁺* and *cd*) in comparison to the reference marker remains the same whether the donor DNA is native, denatured or after it is reannealed, either by mixing the resolved complementary strands or without fractionation.

An examination of Table 3 also reveals that the frequency of co-transfer of *cd⁺* (i.e. *cd⁺*/total *c*) in the *c* strain is about 75–80%. The linkage of *cd* in *cd-10* and *cd-11* is of the same magnitude. On the other hand, the frequencies of co-transfer of *cd* and *cd⁺* in *cd-13* are not of the same order. Instead, *c* and *d* transfer of *cd* and *cd⁺* in *cd-13* are not of the same order. Instead, *c* and *d* in this strain are about 30% linked and the linkage of *c* and *d⁺* is around 40%. The possible significance of this point will be discussed elsewhere (KASHMIRI and HOTCHKISS 1975). Particularly relevant here is the fact that these characteristic frequencies of co-transfer of *cd* and *cd⁺*, for each native merodiploid DNA, remain unaltered when the DNA is in the denatured or reannealed state.

DISCUSSION

Unstable heterozygotes have been occasionally described among the bacteria, and have usually been attributed to partial diploidy or merodiploidy resulting from occurrence of genes duplicated on episomes, plasmids, extra chromosome

TABLE 3
*Comparison of the Marker Activity Ratios and the Frequency of Co-transfer of Markers
 in Native, Denatured and Reannealed DNA*

	$\frac{c}{S}$	Ratio on RI-26 recipient $\frac{d}{S}$	Ratio on RI-26 recipient $\frac{cd}{S}$	$\frac{cd}{\text{Total } c}$	$\frac{c}{S}$	Ratio on RF6-7 recipient $\frac{d^+}{S}$	Ratio on RF6-7 recipient $\frac{cd^+}{S}$	$\frac{cd^+}{\text{Total } c}$
RF3SK-7 (cd_0S)	Native	0.15	—	—	0.13	2.38	0.10	0.76
	Denatured	0.17	—	—	0.15	2.61	0.11	0.76
	Reannealed	0.13	—	—	0.14	2.23	0.12	0.84
	(unfractionated)							
	Fractionated	0.14	—	—	0.13	2.53	0.09	0.74
	and annealed							
RF63SK-10 (cd_1S)	Native	0.18	0.87	0.12	0.12	1.30	0	—
	Denatured	0.22	0.95	0.13	0.14	1.26	0.02	0.13
	Reannealed	0.16	0.98	0.11	0.10	1.24	0.02	0.20
	(unfractionated)							
	Fractionated	0.19	1.06	0.12	0.10	1.39	0.02	0.17
	and annealed							
RF63SK-13 (cd_2S)	Native	0.14	1.18	0.04	0.10	0.71	0.04	0.40
	Denatured	0.20	1.42	0.03	0.12	0.80	0.06	0.49
	Reannealed	0.15	0.98	0.03	0.09	0.78	0.04	0.47
	(unfractionated)							
	Fractionated	0.15	1.17	0.03	0.09	0.84	0.03	0.37
	and annealed							
RF63SK-11 (cd_3S)	Native	0.14	2.27	0.10	0.09	—	—	—
	Denatured	0.16	2.43	0.14	0.09	—	—	—
	Reannealed	0.14	2.36	0.10	0.07	—	—	—
	(unfractionated)							
	Fractionated	0.12	2.95	0.08	0.06	—	—	—
	and annealed							

fragments or duplicated chromosomal segments. Merodiploids arising from transformation of a certain tryptophan marker of *Bacillus subtilis* (AUDIT and ANAGNASTOPOULOS 1973), specific *str^r* or erythromycin resistance markers (RAVIN and TAKAHASHI 1970) of *Pneumococcus* or a certain binary capsule transformant of the same species (BERNHEIMER and WERMUNDSEN 1969) were explained as gene duplications. In all cases except the first mentioned, the extra segment was pictured as a tandem duplication, usually without elaborate consideration of other alternatives.

The sulfonamide-resistant heterozygotes provide a system in which both the wild-type and the drug-resistant segregants of different marker combinations and equivalent transformants can be scored from mixed populations with relative ease. The existence of heterozygotes with four different configurations of alleles at two heterozygous loci provided opportunity for more detailed analysis.

The close association of *c* and *d* and their linkage to *str^r* is not consistent with an episomal configuration (HOTCHKISS 1965) neither is the stability of a *c* strain during growth in Acridine Orange (LEDBETTER and HOTCHKISS 1975a).

Three alternatives came under serious consideration: (1) the existence of a three-stranded structure for the heterozygous region, within which hydrogen bonds to one strand are shared between two alternative antiparallel strands in an overlap region (Figure 2) (HOTCHKISS, ABE and LANE 1971), (2) a tandem duplication that could occasionally produce a stable segregant by looping out between regions of homology, (3) a terminal redundant duplication (HOTCHKISS 1965), a model subsequently abandoned. The first alternative seemed a possible explanation for the association of gene dosages of 3, 2, 1 and 0 for the *d* marker with 0, 1, 2 and 3 respectively for its *d⁺* allele (Table 3). RAVIN and TAKAHASHI

FIGURE 2.—Formal model devised to account for the transfer ratios of 3 : 2 : 1 : 0 of *d* or *d⁺* markers from merodiploid DNA's. This model is not supported by the physical findings on the DNA's.

(1970) dismissed a somewhat similar "piggy-back" structure for their mero-diploid regions as unlikely to be maintained through the process of DNA extraction, and difficult to replicate. The particular structure postulated in Figure 2, however, would not be expected to be subject to spontaneous degradation during DNA extraction. Replication of this unorthodox structure might be pictured as proceeding by appropriate assembly of "Okazaki pieces" of DNA (OKAZAKI *et al.* 1968).

The essentially normal denaturability and renaturability of the heterozygous marker (Table 2) show that there is no gross structural abnormality of the DNA bearing them. Likewise, the normal banding of denatured merodiploid DNA with poly UG in a CsCl gradient (Figure 1) and normal heat stability, melting temperature profile, sedimentation rate and density of the native DNA (HOTCHKISS and ABE 1968) speak for the same conclusion.

Furthermore, the normal marker-activity ratios and frequency of cotransfer for *c* and all other markers on the same DNA are preserved unchanged as it goes through the rigor of denaturation, fractionation and renaturation. This could hardly be the case unless all of the markers share the normal type of relationship along the linear particles of DNA. For these reasons, the postulated triple-stranded structure now seems altogether unlikely.

The tandem duplication of a gene region bearing the *sul^r-c* and *sul^r-d* alleles, however, remains a reasonable explanation for the instability and heterozygosity of the several sulfonamide-resistant strains described. A more detailed examination of this model, together with the results of studies with artificial DNA heteroduplexes will be presented in an accompanying paper.

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