*GENETIC HETEROZYGOSITY IN PNEUMOCOCCAL TRANSFORMATION**

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Newly integrated fragments of transforming DNA have been shown to be associated with the DNA of the recipient bacteria in the form of a physical hybrid of complementary polynucleotide chains.[1-3] It has been proposed[4] that this structure carries the genetic information of both the donor and the recipient alleles in the respective complementary polynucleotide chains. It has further been proposed that these structures replicate in a semiconservative fashion giving rise to progeny of each genotype, without the intervention, at any significant level, of events that might convert heterozygous heteroduplex structures into homozygous structures prior to replication. The results that will be described, of a genetic analysis of the segregation products of bacteria transformed for a single marker, lend substantial support to these proposals.

Clones arising from bacteria that have been transformed for a pair of linked markers have also been examined. These bacteria only very rarely give rise to clones that contain, in addition to bacteria transformed with respect to both members of the linkage group, bacteria transformed with respect to only one member of that linkage group. This observation sets stringent limits on the extent to which a fragment of transforming DNA can participate repeatedly in a transformation event, as well as the extent to which partial conversion by excision and repair may occur spontaneously in transformed bacteria. Furthermore, the rarity of clones that contain both bacteria transformed for only one member of the linkage group and bacteria transformed for only the other member sets stringent limits on the extent to which both strands of a fragment of transforming DNA may participate in a single event by affecting adjacent genetic regions on alternate polynucleotide strands of the recipient chromosome.

Method.—The strains of pneumococcus employed were R 6-12 and mutant strains carrying the *a* and *d* markers conferring sulfanilamide (SA) resistance.[5] These two markers are linked to the extent that they are transformed jointly with a frequency of about 50%. DNA isolation and transformation were carried out in a manner already described.[1, 6, 7]

Transformed bacteria were prepared by exposing a competent wild-type population to the appropriate DNA (about 2 μg/ml), carrying one or both markers concerned, for 20 min at 30°C. DNase (10 μg/ml) was then added and the culture was further incubated for 3 min at 35°C. The transformation frequency for the *d* marker, correcting for the number of bacteria per chain, was about 1 or 2% under these conditions.

B medium (double strength): One liter H_2O; 10 gm NaCl; 5 gm Difco Neopeptone; 9.2 gm Difco Brain-Heart infusion; 34 mg thymine; 15 mg shikimic acid; 16 mg adenosine; 230 mg "N–Z Case" (Sheffield Chem. Corp., Norwich, N.Y.); 750 mg sucrose; 2.1 gm K_2HPO_4; 6.6 gm glucose; 1.2 gm bovine serum albumin fraction V (Pentex, Inc., Kankakee, Ill.). The first four ingredients were sterilized together by autoclaving. The second four were autoclaved together in 50 ml of water. The other ingredients were sterilized separately by autoclaving or filtration. The medium was assembled from these components shortly before use.

Ultrasound inactivation was carried out in an MSE disintegrator with $^3/_8$-in. probe, immersed to a depth of about 1 cm in a 15-mm heavy-wall tube containing 3 ml of culture, and maintained in an ice bath.

Mixed clones were detected by plating in B medium with 0.75% agar buffered with a 1:4 dilution of 0.1 *M* phosphate, pH 6.8, in the presence of an SA concentration that allowed normal plating efficiency of wild-type bacteria as colonies that were distinctly smaller than those arising from any of the sulfonamide-resistant mutant strains. This SA concentration was somewhat variable, between 6 and 10 μg/ml, from one batch of medium to another. Whole colonies submerged in agar were collected, after 18 hr of growth, with capillary pipettes under a dissecting microscope. They were suspended in B medium and incubated for about 6 hr at 37°C, reaching a titer of about 10^7 colony-forming units/ml, and either tested directly or frozen in 10% glycerol at −20°C for later examination. Colonies were collected only when the numbers of wild-type (small) and transformant-containing (large) colonies were consistent with the numbers observed on parallel plates prepared with and without the selective drug. The total number of colonies on collection plates was kept under 2×10^3. Since the pneumococcus colonies are small under these conditions, it is easy to avoid mechanical contamination during collection.

Two kinds of tests were used in characterizing the contents of the clones selected. Bacteria that are wild type for the *d* allele (d^+) are resistant to *p*-nitrobenzoic acid (NOB) and sensitive to SA, and *d* transformants resistant to SA are sensitive to NOB. It is possible, therefore, to distinguish d^+ bacteria even in the presence of a large number of transformants. Test tubes, 25-mm OD, were prepared in the following manner: 5 ml of growth medium containing 1.5% agar and 140 μg/ml SA was allowed to harden on the bottom, then 30 ml of growth medium containing 1.5% agar and 5×10^3 to 5×10^4 bacteria derived from an isolated colony were added and again allowed to harden. Finally, the agar column was topped with 5 ml of growth medium containing 1.5% agar and 80 μg/ml of NOB, again allowed to harden, and incubated for 20 hr at 37°C. Under these conditions the growth of bacteria is challenged only in the proximity of the layers containing the drugs. Standard strains behave in the following manner. The SA-containing layer inhibits wild-type bacteria for a distance of about 2.5 cm from the drug interface and *a* transformants for a distance of about 0.5 cm. Bacteria transformed with respect to the *d* marker or with respect to both *a* and *d* are not affected by the SA. The NOB layer inhibits all the bacteria transformed with respect to the *d* marker whether or not they have been transformed for *a*, for a distance of 2.5 cm from the agar interface, whereas bacteria carrying the d^+ allele are unaffected. This method distinguishes transformant colonies that have ad^+ from those that have a^+d or *ad* bacteria, and, for colonies with *d* bacteria, it detects the presence or absence of any d^+ bacteria to a resolution of about 10^{-3} or better.

In mixed clones analyzed by this test the frequency of wild-type bacteria is between 1/10 and 1/100 of the number of SA-resistant bacteria, probably due to differential growth rates on the plates from which the colonies have been collected. Because of the low frequency of wild-type bacteria in mixed clones, it is also possible to detect colonies that contain both ad^+ and a^+d bacteria. A clone including these two types would have none of its members selected against during the clonal isolation and would therefore contain them in comparable numbers, giving rise to continuous growth through the tube from the NOB interface to the SA interface, with no more than a twofold reduction in colony density at either boundary. Artificial mixtures behave in precisely this manner.

On the basis of the description by Hotchkiss and Evans[5-7] of the properties of the sulfonamide-resistant strains, the contents of clones containing transformants have been further characterized. The properties of bacteria of various genetic constitutions with respect to growth inhibition by various analogues of *p*-aminobenzoic acid (PAB) under various conditions are described in Table 1. An aliquot of a culture, derived from an isolated colony and containing about 10^4 to 10^5 bacteria per milliliter, is distributed into a number of cups in a sterile disposable tray containing a grid of 96 cups, each of 2.5-ml capacity. Each cup receives 1 ml of double-strength growth medium containing 5 ×

TABLE 1. *Patterns of genetic resistance to PAB analogues in pneumococcus.*

	Concentration (μg/ml)										
	SA			NOB			PAS			NOB + PAS	
Genotype	40	80	200	1	5	10	1	2.5	5	2.5	5
++	−	−	−	+	+	+	±	−	−	−	
a+	+	−	−	+	+	+	+	+	+	+	
+*d*	+	+	−	−	−	−	−	−	−	−	
ad	+	+	+	−	−	−	+	±	−	−	

+ = Growth; ± = partial growth; − = no growth.

10^{-5} *M* phenol red, the appropriate concentration of drug or drugs, and 1 ml of 1.5% agar buffered with 0.05 *M* phosphate at pH 7.6. The tray is covered and incubated for 14 hr at 37°C. Absence of inhibition is clearly indicated by conversion of the dye to the yellow acid form. Inhibited bacteria fail to manifest the color change if their inoculum does not substantially exceed 10^5 colony-forming units per cup. In tests with pure cultures or artificial mixtures, uninhibited bacteria are detected even when present at frequencies as low as 10^{-3}. Thus all of the genotypes can be distinguished. The possible pairs of genotypes that might be represented in a colony are indicated as follows:

$\left\{\begin{matrix} ad/ad \\ ad/a^+d \end{matrix}\right.$	a^+d/a^+d	$\left\{\begin{matrix} ad^+/ad^+ \\ ad^+/a^+d^+ \end{matrix}\right.$
ad/ad^+	a^+d/a^+d^+	
ad/a^+d^+	a^+d/ad^+	

Most of these pairs can be characterized. The two sets of classes marked with braces comprise pairs of arrangements that cannot be distinguished. Clones containing mixtures of three types cannot be resolved by this method and would be assigned to one of the classes of mixed clones.

Results.—Selection of mixed clones: The growth of pneumococcus in chains, so that any transformed bacterium is nearly always attached to another bacterium, permits the design of a model experiment that demonstrates our ability to detect all mixed clones independently of any proposed mechanism of transformation. Given a newly transformed bacterium, it is unlikely that all of its neighbors are transformed. Hence, colonies containing such a transformant should always contain bacteria that are wild type for the marker concerned.

To test this assumption a bacterial population, transformed with a DNA carrying the *ad* markers, was plated in B medium with various concentrations of SA. The conditions desired would yield small colonies of wild-type cells as a consequence of slight inhibition by SA without a reduction in plating efficiency, and colonies of normal size for those clones containing transformants. As indicated in Table 2, the efficiency of recognition of colonies containing transformants was very high even in the presence of low doses of SA. Furthermore, the clones containing *d* cells derived from plates where the SA concentration ranged between 5 and 10 μg/ml all (194/194) contained d^+ cells as well, while those derived from plates with higher sulfanilamide concentrations were more and more rarely found to be mixed. The plating efficiency of wild-type bacteria was constant between 0 and 10 μg/ml of SA, although the wild-type colonies were very small at 10 μg/ml. At higher concentrations, wild-type bacteria no longer yielded macroscopic colonies.

Production of mixed clones from transformed single viable cells: Treatment of transformed populations with ultrasounds appears to be a useful procedure for

TABLE 2. *Genetic composition of clones from transformed bacterial chains.*

SA concentration on collection plates	Colonies collected	Transformants	Genotypes			Colonies with d and d^+ bacteria
			ad^+	a^+d*	ad*	
0	10	0				
5	50	48	16	16	16	32
6	50	50	21	12	17	29
7	50	47	19	18	10	28
8	50	50	16	17	17	34
9	50	50	8	22	20	42
10	50	50	21	16	13	29
15	20	20	9	4	7	10
20	20	20	4	8	8	12
30	20	20	2	10	8	6
40	20	20	0	12	8	0
50	20	20	0	13	7	0

* Clones containing d transformants examined for the presence of d^+ bacteria.

dissociating bacterial chains. Microscopic examination of bacterial populations that have been sonicated suggests that this treatment, besides causing bacterial killing, results in breakage of chains; an ultrasound dose that reduces the viable count to $^1/_2$ or $^1/_3$ is sufficient to reduce the number of cells per chain from 4.6 to 1.3. Bacteria surviving various exposures to ultrasound treatment were further treated with ultraviolet light. The UV inactivation curves, described in Figure 1, manifest a smaller and smaller shoulder with increasing ultrasound treatment, in agreement with the expectation that an increasing fraction of the surviving colony-forming units are single viable bacteria.

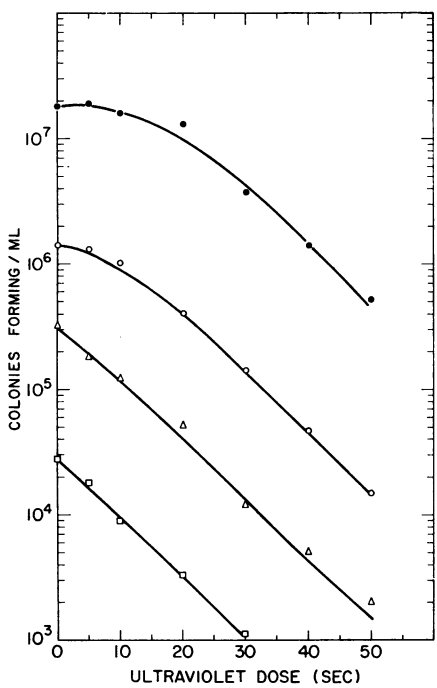

FIG. 1.—Inactivation by ultraviolet light of bacteria that had survived increasing exposure to ultrasound. (●) No ultrasound treatment; (○) 2 min; (△) 4 min; (□) 6 min. UV exposure at about 6 ergs/mm²/sec.

Several experiments describing the composition of clones arising from survivors of ultrasound-treated populations that had been transformed with DNA carrying both the *a* and *d* markers are described in Table 3, experiments 1, 2, and 3; in experiments 4, 5, and 6, the transforming DNA carried only the *d* marker.

In the same table, experiments 7 and 8, we describe a similar analysis of colonies isolated by plating bacteria, transformed with DNA carrying both the *a* and *d* markers, and then inactivated by heat. This kind of inactivation seemed to be another reasonable prospect for yielding a population of single, viable bacteria. The inactivation curve of bacteria heated for increasing times at 49°C or 52°C manifests a shoulder followed by exponential killing. Transformants present in such a population are inactivated with a reduced shoulder at 49°C, no shoulder at 52°C, and with the same exponential slope as the total population at each temperature. Because the survivors of heat treatment yield colonies that are extremely heterogeneous in size, it is more difficult to distinguish colonies containing transformants even in the presence of the appropriate sulfanilamide concentration.

Among 288 colonies that contained bacteria transformed for the *d* marker, all but four also contained bacteria with the d^+ allele. It should be further noted (Table 3, expts. 1–3, 7, 8) that among 78 colonies that had ad^+ transformants none contained transformants that were either a^+d or *ad*. Among the 72 colonies that contained transformants that were a^+d, none contained transformants that were ad^+. In addition, among 76 colonies that contained *ad* transformants none contained any ad^+ transformants. The presence of a^+d transformants in these clones would remain undetected. The rarity of transformant clones that are pure with respect to either of the donor markers is consistent with the proposal of single-strand displacement without conversion by excision and repair.

The model further demands that the proposed heterozygote should be destroyed by semiconservative replication. The technique described has been applied to transformed populations that had been allowed to grow for 30 or 60 minutes at 37°C following termination of the DNA exposure. These samples were then subjected to sonication and plated in the presence of a low SA concentration, and the composition of colonies containing transformants was determined. The results are presented in Table 4. All colonies that contained transformants from plating immediately after transformation also contained wild-type bacteria. Platings after 30 minutes' incubation, at which time about 40 per cent of the bacteria had doubled, as determined by counting in a Petroff-Hauser chamber, gave about 20 per cent pure colonies among the transformant-containing colonies. By 60 minutes, when the transformed population had undergone one doubling, about 90 per cent of the transformant-containing colonies were pure.

Discussion.—Analysis of the composition of transformant colonies presumably derived from single, viable cell survivors of ultrasound treatment has shown that nearly all the colonies that contain bacteria transformed with respect to the *d* marker also include bacteria that have the d^+ allele. The production of mixed clones, including progeny of both donor and recipient genotypes, agrees

TABLE 3. *Genetic composition of clones from transformed single viable cells.*

Expt.	Treatment	Survival of transformants	Colonies collected	Trans-formants	Types of Bacteria in Transformant Colonies						
					ad^+/a^+d^+* or ad^+ only	a^+d/a^+d^+	a^+d only	ad/a^+d^+	ad/ad* or ad/a^+d	a^+d/ad^+	ad/ad^+
1	Sonication	6×10^{-5}	69	49	24	14	0	11	0	0	0
2	Sonication	1.3×10^{-4}	100	90	29	21	0	40	0	0	0
3	Sonication	1.3×10^{-3}	50	48	13	21	0	14	0	0	0
4†	Sonication	2.5×10^{-3}	48	46	—	45	1	—	—	—	—
5†	Sonication	2.5×10^{-3}	48	48	—	47	1	—	—	—	—
6†	Sonication	8×10^{-3}	48	45	—	45	0	—	—	—	—
7	16 Min at 49°C	3.6×10^{-2}	40	8	2	3	1	2	0	0	0
8	4 Min at 52°C	8.5×10^{-3}	122	32	10	12	0	9	1	0	0
				Total:	*78*	*208*	*3*	*76*	*1*	*0*	*0*

* See text for explanation.
† In expts. 4–6, the donor DNA carried only the *d* marker.

TABLE 4. *Removal of transformant heterozygosity upon cell division.*

Sample taken after	Cell count per ml*	Colony on B†	Formers/ml on SA‡	Colonies collected	Trans-formants	Genotypes			Colonies containing *d* marker	Colonies containing *d* marker but not d^+ marker
						ad^+	a^+d	ad		
0 Min	1.6×10^7	4.5×10^6	3.8×10^5							
0 Min + sonication§		10^4	5×10^2	50	48	13	21	14	35	0
30 Min	2.2×10^7	4.6×10^6	2.8×10^5							
30 Min + sonication§		6.9×10^3	3×10^2	68	65	21	23	21	44	8
60 Min	3.2×10^7	6.2×10^6	2.6×10^5							
60 Min + sonication§		3.8×10^4	1.8×10^3	115	114	26	51	37	88	81

* Average of three independent Petroff-Hauser determinations.
† Determined on unselective B medium.
‡ Determined on 30 μg/ml of SA.
§ Samples were sonicated for 6 min under standard conditions.

with the prediction that the transformation event generates a heterozygous heteroduplex structure.

Transformed bacteria that have been permitted to grow before ultrasound treatment and clonal analysis yield increasing fractions of transformant-containing colonies that are pure. By the time the transformed population has undergone about one doubling, 90 per cent of the transformed bacteria have been purged of their capacity to yield wild-type progeny. This observation, besides supporting the proposed heterozygote model, also supports the conclusion that the colony formers that survive ultrasound treatment are predominantly single viable bacteria.

The loss of heterozygosity during the first doubling after transformation argues against a class of models involving transformation followed by conversion in one nucleus of a binucleate bacterium. Any larger multiplicity of nuclei is excluded.

Tomasz[8] has carried out an electron-microscopic examination of serial sections of pneumococcus, fixed at the competent state of an exponentially growing population. Among several hundred bacteria examined, none were observed to contain more than one distinct nuclear region. Bacteria apparently undergoing division at time of fixation contained dumbbell-shaped nuclear regions. These observations suggest that pneumococcus normally contains only one nucleus and that the interval between nuclear division and cell division is short. The loss of the capacity of transformants to yield mixed clones following one doubling would also require that the hypothetical binucleate cells exhibited a segregation pattern in which both daughters of a given parent nucleus always segregated together into a single daughter cell.

Assuming that the newly formed transformant is indeed heterozygous, the segregation patterns can be analyzed to yield further information concerning other properties of the transformation event. The results with labeled DNA[1] limit but do not exclude a class of double-strand insertion models, in which two single-stranded regions of opposite polarity would be inserted together with a very small region in which they overlap. In the case of transformation with a pair of linked markers like *a* and *d*, which became separated with a probability of 0.5 in transformation and are at least 2000 nucleotide pairs apart,[9] some bacteria might be expected to be transformed with respect to one of the markers in one strand and the other marker in the other. Such a bacterium would yield progeny consisting of a mixture of cells transformed for either of the two markers. In addition, if the extent of overlap were substantial, one might expect to find transformants whose mixed progeny includes cells transformed for both markers and cells transformed for one marker only. The rarity of such clones mixed with respect to two transformant genotypes argues strongly against the existence of two-strand events involving the complementary strands of a fragment of donor DNA.

The failure to observe mixed clones containing bacteria transformed for both linked markers and bacteria transformed for only one also argues against any significant persistence of a single fragment of transforming DNA capable of participating in further recombination events. This conclusion is in agreement

with that drawn on the basis of observations concerning the physical fate of transforming DNA in recipient bacteria.

Summary.—Evidence has been presented in support of the proposal that the product of bacterial transformation is genetically heterozygous and that this heterozygosity is rarely, if ever, converted to homozygosity prior to DNA replication.

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[1] Fox, M. S., and M. K. Allen, these PROCEEDINGS, **52**, 419 (1964).

[2] Bodmer, W. F., and A. T. Ganesan, *Genetics*, **52**, 412 (1964).

[3] Notani, N., and S. H. Goodgal, in *Proceedings of the Symposium on Macromolecular Metabolism, J. Gen. Physiol.* (Suppl.), **49**, 197–208 (1966).

[4] Fox, M. S., in *Proceedings of the Symposium on Macromolecular Metabolism, J. Gen. Physiol.* (Suppl.), **49**, 183–196 (1966).

[5] Hotchkiss, R. D., and A. H. Evans, in *Exchange of Genetic Material: Mechanisms and Consequences*, Cold Spring Harbor Symposia on Quantitative Biology, vol. 23 (1958), p. 85.

[6] Hotchkiss, R. D., and A. K. Evans, *Federation Proc.*, **19**, 912 (1960).

[7] Fox, M. S., and R. D. Hotchkiss, *Nature*, **179**, 1322 (1957).

[8] Tomasz, A., personal communication.

[9] Fox, M. S., *J. Mol. Biol.*, **5**, 643 (1962).