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Mismatch Excision and Possible Polarity Effects Result in Preferred Deoxyribonucleic Acid Strand of Integration in Pneumococcal Transformation

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Heteroduplex deoxyribonucleic acid molecules having a drug resistance marker on one strand and its wild-type allele on the other have been used as donors in pneumococcal transformation. Opposite strands are not equally effective in producing transformants, and this strand bias is not the same, either in direction or magnitude, for various different genetic markers. Selective excision of mismatched base pairs is probably responsible for the large differences in strand efficiency seen with discriminating (*hex*$^+$) strains, for when the recipient is nondiscriminating (*hex*$^-$), and therefore presumably lacking an excision enzyme system, strand bias is drastically reduced or altered. The evidence also indicates that excision occurs after integration, as it is provoked by specific donor-recipient mismatch and not by the same mismatch when introduced within donor heteroduplex molecules. Excision can extend to include a neighboring linked marker which would otherwise not be excised, thus altering its intrinsic strand bias as well as its efficiency in transformation. There is a small bias in relative strand efficiency for some markers, not caused by mismatch excision, which perhaps is due to polarity in the integration process itself.

A discriminating strain of pneumococcus (*hex*$^+$) is transformed for various genetic markers with widely different efficiency. Ephrussi-Taylor et al. (2) described two efficiency classes for a series of aminopterin resistance (*ami*-r) mutants, referring to these as high efficiency (HE) and low efficiency (LE). Lacks (12) observed four different efficiency classes with many mutants at the amylomaltase (*mal*) locus. The *sul*-rd marker used as an HE reference for the *mal* mutants (12) is twice as efficient as the HE *str*-41 marker used as reference for the *ami*-r mutants. Thus, the term very high efficiency (VHE) has been used to describe markers that fit in this category (16, 17). Excision induced by mismatched base pairs of donor-recipient complexes was proposed by Ephrussi-Taylor and Gray (1) to account for the low efficiency of some markers, and nondiscriminating mutant strains (*hex*$^-$), which are transformed for most of these markers with equally high efficiency, have provided much support for this hypothesis (13, 16, 17).

It has also been observed, with donor deoxyribonucleic acid (DNA) that is heteroduplex for certain HE markers, that one strand is integrated with greater efficiency than the other. These results have been taken to indicate that double-strand marker efficiencies reflect the sum of the individual single-strand efficiencies (5, 6). The present study was undertaken to see whether the process that gives rise to LE markers (presumably mismatch excision) is also responsible for the low efficiency observed with one strand only for HE markers. The data to be presented will show that this is the case. In addition, evidence will be presented for a preferred strand of integration that is not caused by mismatch excision but can be explained in terms of a polarity in the integration process.

MATERIALS AND METHODS

Preparation of native and heteroduplex DNAs. Native pneumococcal DNAs were purified by methods customarily used in this laboratory (7). The chloroform-isoamyl alcohol emulsion technique was used to remove proteins. Resolution of the strands of denatured DNA, either by means of methylated albumin-coated kieselguhr columns or by interaction with poly(uridylate, guanylate) [poly(U,G)] and subsequent preparation of annealed heteroduplex molecules, has also been described (14). The complementary resolved strands will be referred to as light or heavy. Recovery of activity in the annealed heteroduplexes is very high when strands are resolved on methylated albumin-coated kieselguhr columns: 50 to 100% native activity for non-excised strands. Heteroduplexes prepared with poly(U,G)-resolved strands are not more than half as active as this.

Transformation. Transformation of pneumococcal cells was accomplished by methods described previously (4). In most cases competent cells were exposed to DNA for 30 min at 30°C before termination of the reaction with deoxyribonuclease. Variations in this procedure have included shorter exposure times and different exposure temperatures. Expression of phenotype before addition of drugs was accomplished in one of two ways: 90 min at 37°C in liquid medium or 2 h at 37°C in solid medium. The latter method was principally relied upon whenever comparison of marker efficiencies or relative strand efficiencies were made.

Strains and markers. The pneumococcal strains from which the donor DNAs were prepared contain various combinations of the following markers (the drug levels at which transformants were selected are given in parentheses): *str*-r61 (200 μg of streptomycin per ml), *ery*-r2 (0.1 μg of erythromycin per ml), *ami*-r (0.5 μg of aminopterin per ml), *mic*-r (0.075 μg of micrococcin per ml), *can*-r (500 μg of canavanine per ml), *sul*-ra (30 μg of *p*-aminosalicylate or 100 μg of 3,4-diaminobenzoic acid per ml), *sul*-rd (120 μg of sulfanilamide per ml), the pair *sul*-rad (400 μg of sulfanilamide per ml), *sul*-sd (d^+) (30 μg of nitrobenzoic acid per ml). Details regarding the *sul*-r markers, which are believed to be intracistronic, have been described (9, 10). The recipient pneumococcal strains were usually wild type for the above-mentioned donor markers and either *hex*$^+$ (R1) or *hex*$^-$ (R6x). R1 is the recipient strain routinely used in this laboratory and is a substrain of R6. The strain R6x was derived from R6 by transformation to *hex*$^-$ and was obtained from Gérard Tiraby. Its properties are fully described elsewhere (16). The recipient strains *sul*-ra and *sul*-rd are both hex$^+$. With the exception of the strain carrying the *ery*-r2 marker, obtained from Arnold Ravin, all of the drug resistance markers were isolated in this laboratory and are spontaneous in origin.

RESULTS

Marker efficiencies are equalized with nondiscriminating *hex*$^-$ recipient. In Table 1 relative efficiencies are given for the various genetic markers used in this study when native DNAs are donors in transformation for either the *hex*$^+$ or *hex*$^-$ recipient strains. Of the seven markers utilized here, none is in the category usually referred to as LE, which would show about one-tenth the yield of the *str*-r marker on the *hex*$^+$ recipient. Four (*str*-r61, *sul*-ra, *ami*-r, and *mic*-r) are HE markers. Two markers (*ery*-r2 and *can*-r) are in a category intermediate between LE and HE. One (*sul*-rd) is a VHE marker, as has already been observed by others (12, 16). The differences in marker yield occur only with the discriminating *hex*$^+$ recipient. When the recipient is the nondiscriminating *hex*$^-$ strain, all seven markers are equal in transforming efficiency. These results are similar to those described by Lacks (12) and Tiraby and Fox (16) with other genetic markers, as well as with the VHE *sul*-rd marker described here, and are presented to show that the markers utilized in this study, which include six in the high or intermediate class, are subject to the *hex* function. *hex* function has been fairly well identified with the loss of newly integrated DNA segments, probably by excision, after the recognition of certain mismatched base pairs formed as intermediates in transformation (16). It has usually been invoked to account for the LE markers.

TABLE 1. *Marker efficiency and strand efficiency with hex*$^+$ *and hex*$^-$ *recipient strains*

Marker	Marker efficiency[a]		Relative strand efficiency[b]	
	hex$^+$ recipient	*hex*$^-$ recipient	*hex*$^+$ recipient (L/H)	*hex*$^-$ recipient (L/H)
str-r61	1.0	1.0	0.15	0.9
sul-ra	1.0	1.0	0.20	2.0
sul-rd				
From *sul*-rd donor	1.8	1.2	1.8	1.8
From *sul*-rad donor	1.0	1.2	1.0	1.8
ery-r2	0.5	1.0	5.0	0.6
can-r	0.5	1.2	2.5	0.9
mic-r	0.7	0.7	0.4	2.0
ami-r	1.2	1.2	1.0	1.0

[a] Marker efficiencies are expressed relative to *str*-r61 transformants with the same native donor DNA.

[b] Relative strand efficiency (L/H) represents the number of transformants obtained with the light (L) strand divided by the number of transformants obtained with the heavy (H) strand of two complementary heteroduplex molecules when the same culture is recipient.

Relative strand efficiency varies greatly with different genetic markers and tends to be equalized with *hex*$^-$ recipient. Since it appears from the equalization of all marker efficiencies that HE markers are subject to excision of the type previously associated only with LE markers, the question arises: is the low efficiency observed with individual DNA strands of HE markers due to the same selective process? To answer this question, relative strand efficiency was compared with discriminating and nondiscriminating strains as transformation recipients.

Pairs of complementary heteroduplex DNA molecules, bearing mutant resistance markers on one strand and wild-type alleles on the other, were tested for their ability to donate markers from either the light strand or the

heavy strand. Relative strand efficiencies for the various markers are given in Table 1 for the *hex*$^+$, as well as the *hex*$^-$, recipient strain. Ratios are reported as the relative number of transformants obtained when either the light (L) or the heavy (H) strand is the only donor for samples of the same recipient culture (L/H). With the *hex*$^+$ strain, heavy and light donor strands differ from each other by as much as five- to sevenfold in ability to integrate the *str*-r61, *sul*-ra, and *ery*-r2 markers; other markers, such as *sul*-rd and *ami*-r can be equally effective coming from either DNA strand, and *can*-r and *mic*-r differ about twofold in relative strand efficiency. It is seen that either the light or the heavy strand can be the one preferentially integrated. In contrast to this behavior, when the nondiscriminating *hex*$^-$ strain is recipient for the same heteroduplex donor DNAs, the bias in strand efficiency is eliminated, or in some cases is reduced to no more than a factor of 2.

HE markers occurring at different closely linked sites within a gene locus also show reversal of the strand that is successfully integrated (J. P. Claverys, M. Roger, and A. M. Sicard, manuscript in preparation). This leads one to conclude that insertion into either strand of a particular chromosomal segment can provoke excision, depending primarily upon the nature of the resulting mismatch.

Thus, when a *hex*$^-$ strain is recipient, not only are overall marker efficiencies equalized, as has been described by others (12, 16), but the relative strand efficiencies of HE markers are also either equalized or completely altered, in fact, in some cases they are even reversed for the strand of preference (e.g., *ery*-r2 and *mic*-r in Table 1). These new observations definitely establish that *hex* function determines strand preference or lack of it, as well as overall marker efficiency, confirming the previous suggestion that marker efficiency results from the individual single-strand efficiencies (6). It is clear that HE markers, previously considered to be immune to the excision process, can contain component strands which are excised.

Each ratio given in Table 1 summarizes an extensive amount of data obtained with many different DNA preparations, with strands resolved by different means [i.e., methylated albumin-coated kieselguhr column chromatography or poly(UG) resolution], and with different batches of competent cells and different conditions for the interaction of cells with donor DNA and expression of phenotype. None of these factors appears to influence strand selection significantly.

An additional point emerges from the data of Table 1. The *sul*-ra and *sul*-rd markers, which are intracistronic and cotransferred 50% of the time (9, 10), do not parallel each other with respect to relative strand efficiency. With the *hex*$^+$ strain as recipient *sul*-ra is always strongly biased in favor of the heavy strand, whereas *sul*-rd shows no apparent integration bias when integrated from a *sul*-ad donor and a bias favoring the light strand when coming from a *sul*-rd donor. If excision of mismatched base pairs accounts for these effects, it is reasonable to expect that markers situated within the same locus would differ in strand selection. They are at different sites and are likely to result from different alterations in base sequence. The dependence of *sul*-rd strand bias on the particular neighboring *sul*-ra allele associated with it is described below.

Mismatch recognition and excision can affect a neighboring marker. Excision of *sul*-ra frequently occurs when it is integrated from a light strand into a wild-type *hex*$^+$ recipient (L/H = 0.20) (Table 1). The data in Table 2 show that this excision removes the neighboring *sul*-rd marker with it most of the time, although *sul*-rd is otherwise not recognized by the *hex* function when mismatched. *sul*-rd is intrinsically a VHE marker. However, when it is associated with *sul*-ra as a donor in transformation, its efficiency is almost halved and becomes equal to that of the HE markers (see Table 1).

Heteroduplex DNAs, all mismatched for the *sul*-rd marker (*d*/+) and either mismatched (*a*/+) or matched (*a*/*a*) for the closely linked *sul*-ra marker, were used as donors for the wild-type *hex*$^+$ or *hex*$^-$ recipients (Table 2, column 1). The resulting intermediate donor-recipient heteroduplex configurations are given in column 2 (Table 2). When the total number of transformants to *sul*-rd are assayed in 120 μg of sulfanilamide per ml, +*d* donors show a relative strand efficiency of 1.5 to 2.0 on both *hex*$^+$ and *hex*$^-$ recipients, whereas *ad* donors show a relative efficiency of 1.0 on *hex*$^+$ and 1.8 on *hex*$^-$. The total *d* transformants from the *ad* donors are made up of those that have also received the *sul*-ra allele (*ad*) and those that have not (+*d*). In transformations with native homoduplex DNAs these two classes are usually equal in number. With the heteroduplex DNAs, the strand bias of the *ad* transformants with the *hex*$^+$ recipient is very close to the value of 0.20 observed for *sul*-ra. That for +*d* is 1.5 to 2.0, as though it were coming from a pure +*d* donor. Thus, the total *sul*-rd bias with a *hex*$^+$ recipient and *sul*-rad as donor results from the average of 0.2 for the *ad* transformants and 1.8 for the +*d*

TABLE 2. *Strand efficiency of sul-rd is influenced by proximity of sul-ra (with hex⁺ recipient only)*

Donor DNA heteroduplex	Donor-recipient heteroduplex[a]	Marker assayed[b]	Strand bias (light/heavy) ++/++ hex⁺[c]	Strand bias (light/heavy) ++/++ hex⁻[c]
+d / ++	+(d) / ++	Total *d*	1.5	1.5
+d / a+	+(d) / ++	Total *d*	2.0	2.0
ad / ++	+(d) / ++ and (ad) / ++	Total *d*	1.0	1.8
		ad	0.14	1.7
		+*d*	2.0	1.8
ad / a+	+(d) / ++ and (ad) / ++	Total *d*	1.0	1.9
		ad	0.3	2.2
		+*d*	1.5	1.6

[a] Parentheses designate marker or markers donated to recipient.

[b] Total *d* includes all *d* transformants, both +*d* and *ad*, and is assayed at 120 μg of sulfanilamide per ml. *ad* is assayed at 400 μg of sulfanilamide per ml. +*d* is determined by the difference between total *d* and *ad* when *ad* is donor. It is the same as total *d* when the donor is +*d*.

[c] Recipient strain.

transformants. These differences in strand efficiency do not occur with the *hex*⁻ strain.

That excision of *sul*-ra on the light strand also eliminates the *ad* pair is obvious. However, if *sul*-ra were eliminated without *sul*-rd, and then repaired to wild type, total *d* would remain the same, *ad* would decrease, and +*d* would increase, accordingly, in number. This does not happen. There is, in fact, a significant total loss in *sul*-rd transformants coming from the light strand whenever it is associated with *sul*-ra in donor DNA.

Mismatch recognition and excision occurs with donor-recipient heteroduplex and not with donor heteroduplex. When heteroduplex DNAs are donors in transformation, two different mismatched heteroduplex situations exist: the initial donor mismatch and the transient donor-recipient mismatch. To distinguish between mismatch excision of heteroduplexes at the two possible levels, different configurations of the *sul*-ra and *sul*-rd markers and their wild-type alleles were used as donors with wild-type, *sul*-ra, and *sul*-rd recipients. In this way the same mismatch could occur with either incoming donor DNA or the donor-recipient complex, or with both. Particular donor-recipients could also result from totally different donors. Table 3 summarizes the results obtained in this way. The data are again presented as the relative strand efficiency for the particular marker or markers assayed. Strand bias resulting from the same mismatched donor-recipient configuration can be compared for totally different mismatched donor configurations (horizontal rows). Also, strand bias resulting from the same mismatched donors can be compared for different final donor-recipients (vertical columns). The vertical columns show that the same donor can give rise to widely different strand bias for donated (*a*), (*d*), or (*ad*). The horizontal rows show that the same donor-recipient configuration results in similar strand bias coming from widely differing mismatched donors. Thus, it is clearly the donor-recipient mismatch and not the donor mismatch that determines whether excision will occur for this pair of markers. The horizontal rows also indicate that only when *sul*-ra is both mismatched and *cis* to *sul*-rd in a donor-recipient heteroduplex does the presence of *sul*-ra determine an integration bias strongly favoring the heavy strand for the neighboring *sul*-rd marker.

Strand bias independent of mismatch exci-

TABLE 3. *Configuration of donor-recipient heteroduplex determines strand bias (light/heavy) with hex⁺ recipient strains*

Donor-recipient heteroduplex configurations	Marker assayed[a]	Donor heteroduplex DNA configurations +d/++	+d/a+	ad/++	ad/a+	a+/++
+(d)[b] / ++	+*d*	1.5	2.0	2.0	1.5	
(+d) / a+	+*d*	3.0	4.0			
(ad) / ++	*ad*			0.14	0.3	
a(d) / a+	*ad*	1.6	1.9	1.5	1.6	
(a)d / +d	*ad*		0.17	0.18		0.22

[a] For assay of markers, see footnote *a*, Table 2.

[b] Parentheses designate the marker or markers donated to the recipient strain.

sion. The *sul*-ra and *sul*-rd markers show a moderate strand bias (200) in the *hex*$^-$ recipient, which is presumably independent of mismatch excision. Both *ery*-r2 and *mic*-r also show a bias when introduced into the nondiscriminating *hex*$^-$ recipient in which the preferred strand of integration is the reverse of that observed with the discriminating strain (Table 1). Although a factor no greater than 2 is involved in this residual bias with the *hex*$^-$ recipient, either strand can be favored or both strands can be equivalent, so that trivial explanations tend to be ruled out. A low level of residual mismatch excision can also be ruled out, since the preferred strand of integration is reversed in three instances and overall marker efficiencies have been equalized (Table 1).

The *sul*-rd marker can be assayed for both the mutant (*sul*-rd) and sensitive wild-type alleles (*sul*-sd) on appropriate recipient strains, by virtue of a reversal in sensitivity of the two alleles to *p*-nitrobenzoic acid (10). *sul*-rd is more resistant to sulfanilamide and *sul*-sd is more resistant to *p*-nitrobenzoic acid. Thus, when heteroduplexes contain *sul*-rd on one strand and *sul*-sd on the other, the activity of both strands of each heteroduplex can be assayed. It has just been described that the *sul*-rd allele favors the light strand by a factor of 2 when mismatch excision is not operating. When the same heteroduplexes are assayed for *sul*-sd, the light strand is still favored by a factor of 2. Excision of mismatched bases would predict a reversal of the preferred strand. This means that the same donor strand is favored, regardless of which allele it contains, and therefore that the specific base mismatch of the donor-recipient probably has no influence (Table 4).

It is interesting that three different alleles within the *sul*-r locus, *sul*-ra, *sul*-rd, and *sul*-sd, show the very same strand preference when mismatch excision is not involved. This particular bias could then be locus specific rather than allele specific, suggesting that it could be caused by a polarity of integration. For example, markers situated close to molecular ends rather than near the centers could show such a bias if integration were started from a particular end and were directional (8). Overall marker yield need not be influenced, since the average lengths of inserted sequences could be about the same from either end and thus the average integration probabilities could be the same. A fairly uniform population of DNA molecules would be required to observe such an effect.

It is also observed (Table 4) that when *sul*-ra is in *cis* configuration to *sul*-sd on the light strand the integration efficiency of *sul*-sd is greatly decreased. Mismatched *sul*-ra excises *sul*-sd with it, as it does *sul*-rd.

TABLE 4. *Strand bias of sul-sd (sul-d$^+$) with hex$^+$ recipients*[a]

Donor-recipient heteroduplex configurations	Donor heteroduplex DNA configurations			
	$\frac{++}{+d}$	$\frac{a+}{+d}$	$\frac{++}{ad}$	$\frac{a+}{ad}$
$\frac{+(+)}{+d}$[b]	1.8		1.9	
Combined $\frac{(a+)}{+d}$ and $\frac{+(+)}{+d}$		0.90		0.90

[a] Ratio of light to heavy strand when d^+ allele is assayed.

[b] Parentheses designate the marker or markers donated to the recipient strain.

DISCUSSION

It was first suggested by Ephrussi-Taylor and Gray (1) that the properties of certain LE markers in transformation could be accounted for by an excision process that recognizes specific base pairing mismatches of a donor-recipient heteroduplex, permitting some to be normally integrated and others only infrequently. This was based upon relative marker frequencies obtained with many pneumococcus aminopterin resistance mutants (*ami*-r), which were believed to be point mutations and which fell into two efficiency classes, referred to as HE and LE. Evidence had already been presented by Lacks (11) and by Fox and Allen (3) indicating that only one strand of donor DNA is integrated into a recipient chromosome, thus providing the heteroduplex region required for recognition. With many mutants at the amylomaltase (*mal*) locus, Lacks (12) described four categories of marker efficiency and attributed the low efficiency of some markers to multisite mutation and of others to mismatched base pairs. Additional evidence that supports and extends the mismatch excision hypothesis has been accumulating (15–17), and mutant strains (*hex*$^-$) that do not discriminate between LE and HE markers have been identified, integrating all with the same high efficiency (13, 16). The data presented in this paper add additional support for excision of mismatched base pairs at the donor-recipient heteroduplex level and show

that it can occur, but with one strand only, for markers in the HE class.

A strong bias in relative integration efficiency of the complementary strands of heteroduplex molecules has been described by Gabor and Hotchkiss for a number of genetic markers (5, 6). These results were obtained with HE markers and suggested that overall marker efficiency could result from the sum of the individual strand efficiencies. Preferential integration of one strand of a heteroduplex donor DNA is obtained for these same markers in this study, and it is additionally shown that the nondiscriminating *hex*$^-$ strain, which equalizes marker efficiencies, either eliminates or drastically alters strand preference. Therefore, strand efficiency and marker efficiency are both controlled by the *hex* function and thus most likely by a mismatch excision process. If *hex* function can indeed be attributed to recognition and excision of some base pairing mismatches but not others, four different integration situations can be visualized. (i) Both donor strands are excised when heteroduplex ($x/+$ and $+/x$), giving rise to a LE marker; (ii) both donor strands are integrated without excision, giving rise to a VHE marker; (iii) and (iv) only one particular donor strand is excised (either $x/+$ or $+/x$, but not both), giving rise to the HE markers. Since totally different mispairings of bases occur for the two reciprocal donor-recipient heteroduplexes (iii and iv), recognition of one and not the other is predictable. In any event, it is clear that HE markers can be recognized by the mismatch correction process and that the steps leading to integration or rejection are probably similar for all classes of markers.

Two additional factors can contribute to a preferred strand of integration. One is a neighboring marker effect and the other is a relatively small integration bias not caused by excision processes. Both factors influence the behavior of the linked *sul*-ra and *sul*-rd markers. Integration of *sul*-ra strongly favors the heavy strand in the discriminating strain and integration of *sul*-rd without *sul*-ra favors the light strand by a factor of 2. When they are both inserted into a light recipient strand, the excision of *sul*-ra extends into the neighboring *sul*-rd region, which otherwise would not be subject to excision. It is not possible to estimate accurately the length of the tract of bases subject to this co-excision. Linkage, as measured by co-transfer of 50%, would put the estimate at hundreds of base pairs at least. Co-correction of HE markers with adjacent LE markers in the *ami*-rA locus has been postulated (1) to account for an overall deficiency of the former among transformants from homoduplex donor DNA. The excision proposed here would be similar in extent and is specifically demonstrated to occur in a particular donor strand. Also, it is generated by an HE marker (*sul*-ra) that co-excises a neighboring VHE marker (*sul*-rd), a situation that would not previously have been predicted. If excision were followed by a failure to repair, lethal events would ensue which would be equivalent to excision of the entire inserted piece of DNA. Were this the case, co-excision would extend for the length of an inserted segment. It is difficult to distinguish experimentally between these two mechanisms. Following these results, a prediction can be made that two closely linked HE markers of opposite strand preference could essentially annihilate each other during integration and thus both appear to be of low efficiency when the pair is inserted into a recipient genome.

When excision of an inserted marker does not occur, either because the particular mismatch is not recognized by the discriminating strain or because the recipient is a nondiscriminating strain, a preferred strand of integration can still remain. Four of the seven markers studied show this residual bias: three favor the light strand and one the heavy. With three of them the strand then preferred is precisely the one excised in the discriminating strain, so that residual excision cannot be invoked as an explanation. The data presented here suggest that a reversed polarity in the integration of opposite strands could be the basis for this excision-independent bias. Regardless of whether the wild-type allele or the resistant allele of *sul*-rd is donated to the light recipient strand, it is more favorably integrated than when donated to the heavy strand. This is opposite to the result predicted for excision of base-pairing mismatches but is compatible with a polarized integration process beginning at or near a molecular end. Those markers close to one strand end could be inserted earlier than those from the other. If the average length of the integrated segment is smaller than the incoming strand length, ends would show a strand bias and middles would not. The average efficiency of the two strands could still be equal for different markers, and this seems to be the case for these markers with the nondiscriminating strain. Hotchkiss (8) has proposed polarized strand integration or assimilation for recombination processes in general.

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