Exclusion of long heterologous insertions and deletions from the pairing synapsis in pneumococcal transformation

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We have studied the mode of recombination of six insertions during genetic transformation of *Streptococcus pneumoniae*. The six heterologous insertions are located at the same site in the *ami* locus of the pneumococcal chromosome; insertion sizes range from 4 to 1374 bp. With respect to single-point markers we found that the number of transformants in one-point crosses is reduced, while the number of wild-type transformants in two-point crosses is drastically increased, what we call hyper-recombination. The magnitude of the shift is correlated with the size of the insert. This effect could result either from a special repair pathway of multibase heteroduplexes or from the exclusion of multibase heterologous insertions out of the pairing synapsis. To test these hypotheses we have used insertions in two kinds of three-point crosses. The repair model predicts that the excess of wild-type transformants remains in one set of crosses but is suppressed in the second set. The results we obtained are reversed, ruling out the hypothesis of a repair process, but in agreement with predictions based on the exclusion model. Moreover, we have re-examined the situation of deletions, our previous results suggesting that deletions were likely to be converted at the heteroduplex step. Genetic evidence we obtained in this work no longer supports this hypothesis. Thus, long heterologous insertions are partly excluded at the pairing step.

Keywords: *Streptococcus pneumoniae*, recombination, insertions, deletions, heteroduplexes

INTRODUCTION

During pneumococcal transformation, one DNA strand enters the cell and recombines with the chromosome thereby inducing the formation of a donor/recipient heteroduplex joint (for a review, see Lacks, 1988). Once replicated, such heterozygotes generate one transformant and one recipient genotype. However, mismatches may undergo repair prior to replication. The origin of this hypothesis was the discovery that several mutations are poorly transformable in wild-type *Streptococcus pneumoniae* strains (Ephrussi-Taylor & Gray, 1966). Subsequently, mutants were found, called *hex*, which integrate all markers with the same high efficiency (Lacks, 1970; Tiraby & Sicard, 1973a). Further studies showed that donor/recipient mismatches may trigger a complete excision of the donor DNA from the heteroduplex structure, followed by a resynthesis of the recipient strand

(Méjean & Claverys, 1984). This mismatch repair system, which is absent in *hex* mutants, has been called Hex. The mutator phenotype of *hex* strains strongly suggests that it also acts on mismatches spontaneously arising in DNA (Tiraby & Sicard, 1973b; Tiraby & Fox, 1973). Heteroduplexes A/G and C/T corresponding to transition mutations are efficiently corrected by Hex. Those resulting from transversions are partly repaired, except C/C and A/G (for a review, see Claverys & Lacks, 1986). Mispairs corresponding to small heterologous insertions, i.e. from 1 to 4 bases, are also processed by Hex (Gasc *et al.*, 1987). Due to the multiplicity of mispairs processed, Hex is considered as a generalized mismatch repair system.

Heteroduplexes corresponding to heterologous insertions longer than 4 bases escape the action of Hex (Gasc *et al.*, 1987). Moreover several studies showed that long deletions do not behave as point mutations during transformation. Ghei & Lacks (1967) found that the kinetics of integration were much slower for deletions than for point mutations. Also, the transformation efficiency of deletions is reduced (Lacks, 1966; Lefevre *et al.*, 1989). Moreover,

Abbreviations: *ami*-ins, *ami*-insertion sequence; RF, replicative form.

in two-point crosses, when donor deletions are used to transform a strain containing a linked point mutation, wild-type recombinants occur in very large excess relative to the physical distance separating the two mutations (Lefèvre *et al.*, 1989). This hyper-recombination could have two origins. First, the heteroduplex forms a multi-base loop which might be corrected to the wild-type. Second, recombination might frequently stop at the region of heterology, excluding it from the pairing synapsis. Genetic evidence that we previously obtained for long deletions argues for the first possibility (Lefèvre *et al.*, 1989).

In order to define more precisely how long heterologous insertions recombine, we have carried out a study using a set of chromosomal insertions of different sizes, located at the same site on the pneumococcal chromosome. We must point out that the formation of donor/recipient heteroduplexes generated by either donor deletions or by donor insertions should differ in more aspects than just the symmetry of donor/recipient heteroduplexes. During DNA entry, natural breaks will occur within an insertion, generating a loss of flanking homology, while deletions escape entry nicks just as point mutations. In addition, pairing of donor fragments containing insertions certainly requires the single-stranded heterologous insertion for folding, while recombination of deleted fragments should involve the bypass of the double-stranded chromosomal region. Both processes are likely to be different, and it was not directly possible to extrapolate previous results obtained with deletions to the behaviour of insertions in genetic crosses. This work was therefore carried out in order to clarify this.

METHODS

Strains, media and transformation procedures. *Streptococcus pneumoniae* strains used and constructed in this study derive from strain R36A of Avery (Avery *et al.*, 1944). They have a R801 genetic background (Lefèvre *et al.*, 1979) i.e. wild-type except for a mutation in the *hexB* gene. Thus they transform all point mutations with the same high efficiency (Tiraby & Sicard, 1973a). The *str-41* marker, conferring resistance to streptomycin, was present in strain R119 (Tiraby & Fox, 1973). *polA* mutations were characterized in *S. pneumoniae* (Martinez *et al.*, 1986) and transferred into the R801 background as described by Pasta & Sicard (1994). Culture media, preparation of chromosomal DNA and pneumococcal transformation methods were as described by Claverys *et al.* (1980). Routinely for a transformation involving 1 ml diluted competent cells, we used about 0.2 µg chromosome and 0.1 µg plasmid, i.e. saturating

amounts of DNA. Plasmid- and M13-derived recombinant vectors were amplified in the *Escherichia coli* strain JM101 [$\Delta(lac-pro)thi supE F' traD36 proAB lacI^qZAM15$] (Messing *et al.*, 1981). Double-stranded and single-stranded DNAs were prepared as described by Maniatis *et al.* (1982).

Pneumococcal crosses involving the *ami* locus. The *ami* locus is an operon involved in oligopeptide transport (Alloing *et al.*, 1990). *ami* strains are resistant to 0.02 mM methotrexate, but their growth is inhibited on synthetic medium containing excess isoleucine (Sicard, 1964). Conversely, *ami*⁺ bacteria are sensitive to methotrexate, but they grow on synthetic medium containing excess isoleucine. One-point crosses can therefore be carried out

in both directions: transformation of an *ami*⁺ recipient with *ami* DNA or vice versa. The transformation efficiency of the donor site is then measured as the number of *ami* or *ami*⁺ transformant colonies divided by the number of transformants for the reference point mutation *str-41*, also carried on the donor DNA and conferring resistance to streptomycin. The possible screening of *ami*⁺ bacteria allows one to perform two-point crosses. In such crosses, a strain mutated within the *ami* locus is transformed with another *ami* mutation, together with the reference marker *str-41*. *ami*⁺ bacteria, generated by recombination between the two sites involved in the cross, are selected on synthetic medium. Their number is divided by the number of *Str*^r transformants and multiplied by 100 to give the recombination index. In *hex* backgrounds, the recombination index obtained with point *ami* mutations depends mainly on the distance separating the two sites. On average, 27 bp lead to a recombination index of 1% (Claverys *et al.*, 1979).

Insertion of heterologous insertions at the *Bam*HI site of the *ami* locus.

The *Eco*RI A fragment of the *ami* locus is approximately 1800 bp in size, with a *Bam*HI site at approximately 1100 bp from the *Eco*RI right end (Fig. 1). The recombinant plasmid pR6 carries almost all of the wild-type *ami* locus on a 5800 bp insert (Méjean *et al.*, 1981). We have subcloned the wild-type allele of the A fragment from pR6 into the *Eco*RI site of a M13mp11 replicative form (RF), from which we previously deleted the *Sma*I–*Hinc*II fragment, containing the only *Bam*HI site in M13mp11 (Messing & Vieira, 1982). The resulting recombinant vector, RV15, carries the wild-type A fragment with the unique *Bam*HI site. RV15 was linearized with *Bam*HI to insert heterologous insertions. A small insertion of 4 bp was introduced by filling in the recessed *Bam*HI ends with 'Klenow fragment' (Maniatis *et al.*, 1982) and ligating with T4 DNA ligase. Among clones resistant to *Bam*HI digestion, two were sequenced (Sanger *et al.*, 1977); both carry the 4 bp insertion at the *Bam*HI site. Longer insertions correspond to *Sau*3A fragments of the pBR322 plasmid (Rodriguez *et al.*, 1977). A *Sau*3A digest of pBR322 generates 22 fragments from 8 to 1374 bp. The two longer ones, 1374 and 665 bp, well separated on an agarose gel, were directly purified and ligated to RV15, linearized with *Bam*HI and 5'-dephosphorylated (Maniatis *et al.*, 1982). Smaller *Sau*3A fragments from pBR322 are located within a smear. To define more precisely the *Sau*3A sequences to be cloned, we first generated prefragments that separate well on a gel by hydrolysing pBR322 with enzymes that cut rarely. Two such prefragments were purified. One is *Ase*I–*Hinc*II, of 368 bp. It contains three *Sau*3A sites generating four fragments, including 18 bp and 46 bp *Sau*3A sequences that can be ligated to *Bam*HI termini. The second prefragment is *Bam*HI–*Nru*I, of 598 bp. When hydrolysed with *Sau*3A it generates 92 and 359 bp fragments that can be cloned into *Bam*HI. *Sau*3A digests of prefragments were phenol-extracted, ethanol-precipitated and ligated to RV15, hydrolysed with *Bam*HI and dephosphorylated. All ligation mixtures were used to transform separately the *E. coli* strain JM101. RF and viral DNAs were prepared from individual plaques. Recombinant RV15-pBR322 vectors were checked by sequencing and comparing the inserted fragment to the expected sequence of pBR322 (from the EMBL database). We have isolated clones containing the expected sequences of 1374, 665, 359 and 92 bp. Three clones, generated by ligating the *Sau*3A digest of the *Ase*I–*Hinc*II prefragment into RV15, were sequenced. None carried the 18 bp or the 46 bp sequence, but they carried the complete 64 bp sequence instead. We kept six recombinant clones, each carrying a defined insertion in the *Bam*HI site of the *ami* locus (of 4, 64, 92, 359, 665 and 1374 bp).

Fig. 1. Physical and genetic map of the A fragment. The *EcoRI* A fragment of the *ami* locus is about 1800 bp. The unique *BamHI* site divides the A fragment into two subfragments: A2 (*EcoRI*-*BamHI*, about 700 bp) and A1 (*BamHI*-*EcoRI*, about 1100 bp). The complete wild-type locus and most mutations of the A1 fragment have been sequenced (Alloing *et al.*, 1990; Gasc *et al.*, 1989). *ami-6*, *ami-22* (point mutations) and *ami-109* (395 bp deletion) are mapped precisely; positions are in parentheses. Genetic analysis showed that *ami-42* is a point mutation located between 30 and 50 bases to the left of the *BamHI* site on the A2 fragment.

Isolation of pneumococcal strains carrying the *ami* insertions.

RF DNA corresponding to a recombinant clone for a given size of insert was used to transform strain R801. Methotrexate-resistant transformants were selected on plates. Such transformants correspond mainly to the desired allelic replacement of the recipient *ami*⁺ locus by the donor *ami*-inserted DNA. However, 'insertion-duplication' events integrating the full vector into the chromosome (Vasseghi *et al.*, 1981) also lead to methotrexate resistance, by disrupting the *ami* locus. The first class of mutations almost never reverts to *ami*⁺, while the second class should easily revert by homologous recombination excising the plasmid. For each size of insertion, three *ami* transformant colonies were picked, subcultured three times in rich medium and plated on synthetic medium to score for *ami*⁺ revertants. Out of 18 *ami* transformants, 2 gave *ami*⁺ colonies. For each of the six *ami*-insertion constructs, one nonreverting mutant was kept for further analysis. One set of similar mutants carrying the streptomycin-resistance gene was also constructed by transformation.

Construction of double *ami* mutant strains. Five double *ami* mutants have been constructed for the purpose of this study: *ami-42 ami-6*; *ami-ins-1374 ami-6*; *ami-ins-665 ami-6*; *ami-ins-359 ami-6*; *ami-42 ami-109* (see Fig. 1 for mutations). Double mutants were constructed by transformation of a defined mutant with a second mutation. To increase transformation frequency at a genetic site, we used cloned *ami* mutations as donor DNAs. The point mutant *ami-42* and insertion mutants *ami-ins-1374*, *ami-ins-665* and *ami-ins-359* were transformed with the recombinant plasmid pR32, which carries the *ami-6* point mutation on a 5800 bp pneumococcal fragment (Méjean *et al.*, 1981), to yield, respectively, the first four mutants indicated above. The recombinant vector RF824 (from A. M. Gasc, this laboratory) is derived from M13mp11. It carries the *ami-109* deletion on the A1 fragment (Fig. 1). We have transformed *ami-42* with this plasmid to construct the last double mutant indicated above. Double *ami* mutants are not more resistant to methotrexate than single ones and therefore must be screened by back-crosses. After each transformation, 50–100 colonies were picked on methotrexate plates and cultured in rich medium. Chromosomal DNA was extracted and used to transform both parental strains. *ami*⁺ transformants were scored by plating on synthetic medium. Only double mutants cannot produce any wild-type recombinants in both crosses. Among strains giving no *ami*⁺ transformants, i.e. 5–25% of the picked colonies, one from each class was selected for further analysis. Double-mutant genotypes were checked by transformation with the two appropriate parental DNAs and by observing that no *ami*⁺ transformants were produced.

RESULTS

Transformation efficiencies of insertions

Chromosomal DNA from the six strains containing a heterologous insertion within the *ami* locus was used to transform R801, which is a *hex* strain. This study is therefore free of any interference from *Hex*, the generalized mismatch repair system of *S. pneumoniae*.

In agreement with previous findings (Gasc *et al.*, 1987), the smallest insertion of 4 bp transforms as efficiently as a point mutation, since its transformation efficiency is approximately 1 (Table 1). With respect to this reference value, we observe that longer insertions have a reduced transformation efficiency. This is particularly noteworthy for the longest insertion, which displays an efficiency of 0.55 (Table 1). Insertions therefore exhibit a decreased efficiency of transformation, inversely related to their size.

To understand more completely the efficiencies of transformation, we performed the reciprocal crosses. The six strains containing an insertion were used as recipients. Donor DNA was *ami*⁺ and Str^r. We must point out that this situation, i.e. wild-type locus on the donor and insertion on the recipient, is physically identical to crosses with deletions on the donor. Thus, these reciprocal crosses allow the measurement of the transformability of a set of donor deletions of increasing size, flanked by the same DNA sequences. We still observe a decrease in the efficiency, ranging from 1 for the smallest insertions down to 0.36 for the longest ones. Comparing these efficiencies to those with donor insertions, it appears that deletions transform slightly less efficiently than donor insertions.

Recombination of insertions in two-point crosses

In the two-point crosses between linked *ami* mutations, one was located on the donor DNA, the other on the recipient chromosome. The recombination index depends on breaks and recombination events occurring between the two *ami* sites and is therefore mostly proportional to the physical distance separating them. On average, for point mutations in a *hex* background, a recombination index of 1% corresponds to a distance of 27 bp between

Table 1. Effect of insertion length on transformation efficiency

Efficiency is measured as the ratio of either *ami* to *Str*^r transformants (donor insertions, wild-type recipient; central column), or *ami*⁺ to *Str*^r transformants (wild-type donor, recipient insertions; right column). Each value is the mean efficiency measured from three independent transformations with two platings, each time screening for *ami* or *ami*⁺ and *Str*^r colonies.

Insertion length (bp)	Transformation efficiency	
	<i>ami</i> -ins donor × <i>ami</i> ⁺ recipient	<i>ami</i> ⁺ donor × <i>ami</i> -ins recipient
1374	0.55	0.37
665	0.63	0.36
359	0.64	0.51
92	0.78	0.80
64	0.80	1.03
4	1.03	0.98

Table 2. Effect of insertion length on recombination index in two-point crosses involving donor insertions

Chromosomal DNAs carrying both an *ami* insertion and the *Str*^r marker were used to transform either the *ami*-22 or the *ami*-42 mutant. *ami*⁺ recombinants and *Str*^r transformants were counted from three platings and the mean values are indicated below.

Insertion length (bp)	Mean recombination index observed	
	<i>ami</i> -22 recipient	<i>ami</i> -42 recipient
1374	22	13.5
665	23.4	16.2
359	16	8.6
92	7.3	3.4
64	8.8	4.9
4	4.8	1.2

the mutations (Claverys *et al.*, 1979). One set of crosses was carried out with insertions located on the donor DNA. The recipient mutation was either *ami*-42 or *ami*-22. These two point mutations are located, respectively, on the left and on the right of the insertion site (*Bam*HI) and very close to it (Fig. 1). Crosses involving the 4 bp insertion gave the recombination indices expected for a point mutation, based on the locations of *ami*-22 and *ami*-42 relative to the insertion site, i.e. 4.8 and 1.2%, respectively (Table 2). When using longer insertions, experimental indices of *ami*⁺ recombinants exceed these reference values, i.e. insertions trigger hyper-recombination. This behaviour amplifies with the length of the heterologous insertion and appears to reach a plateau for the two longest insertions (Table 2). We also observed that hyper-recombination was higher when crosses were performed with *ami*-22 than with *ami*-42. For instance, the 665 bp insert generates about 19% *ami*⁺ transformants above the 4.8% expected with *ami*-22, but 15% above the 1.2% expected with *ami*-42. This differential hyper-recombination between the proximal and the distal recipient marker remains for each insertion (Table 2) and

was previously observed in crosses involving long donor deletions (Lefèvre *et al.*, 1989). Whatever the mechanism responsible for hyper-recombination, the close vicinity of a long heterologous insertion apparently affects recombination at a genetic site. Similar crosses were also performed for each donor insertion in an R801 *pol*A background with either *ami*-22 or *ami*-42. The lack of DNA polymerase I had no effect on hyper-recombination (data not shown).

A second set of crosses was performed, using insertions in the recipient. Donor DNA was *ami*-22 *str*-41 or *ami*-42 *str*-41 (Table 3). Crosses involving the 4 bp insertion generate numbers of *ami*⁺ colonies very close to those obtained above, still in agreement with the distances between genetic sites. For longer insertions, we no longer observe hyper-recombination. The numbers of *ami*⁺ transformants are even below the reference value obtained with the 4 bp insertion, decreasing slightly as the length of the heterologous insertion increases. This decrease mostly disappears when dividing the recombination index for each insert by the corresponding efficiency obtained in crosses

Table 3. Effect of insertion length on recombination index in two-point crosses involving recipient insertions

Chromosomal DNA carrying the point mutations *ami-22* or *ami-42*, together with the *Str^r* marker, was used to transform *ami*-insertion recipient strains. *ami*⁺ recombinants and *Str^r* transformants were counted on three platings and the mean values are indicated below.

Insertion length (bp)	Mean recombination index observed	
	<i>ami-22</i> donor	<i>ami-42</i> donor
1374	2	0.8
665	2	0.5
359	2.6	0.34
92	3.5	0.94
64	4	0.84
4	5.8	2.3

Fig. 2. Models accounting for the reduced transformation efficiency and hyper-recombination of insertions. (a) Pairing is completed successfully, generating a single-stranded loop which may be repaired to wild-type. (b, c) Exclusion or one-sided pairings: recombination stops because heterology is a strong barrier and/or because homology on one side is absent or very short. (d) Both flanking homologies are too short; there is no recombination at all. Situations (a), (b), (c) and (d) account for the reduced efficiency of insertions. Situations (a) and (b) also explain hyper-recombination in the two-point cross symbolized in this figure. Black line, donor strand; grey lines, recipient strands; dashed line, heterologous *ami*-insertion.

between donor *ami*⁺ and recipient *ami*-insertions (calculation not shown). Thus, the reduced efficiencies and the slight hyporecombination observed when using recipient insertions are likely to have the same cause.

Testing for a loop repair or one-sided pairings

The results described above show that, with respect to point mutations, long donor insertions have a reduced transformation efficiency in one-point crosses and induce hyper-recombination in two-point crosses. We propose two models to account for this behaviour (Fig. 2). The same models may also account for hyporecombination due to recipient insertions.

First, recombination of a fragment carrying an insertion should generate a single-stranded heteroduplex loop. Such structures might undergo correction biased to the wild-type sequence and be closely localized around the loop to prevent co-conversion, as shown in Fig. 2(a). In

addition, this correction would not be dependent on DNA polymerase I, since *polA* mutants allow hyper-recombination to occur. The second model, the exclusion model, is based on the fact that a long insert is a barrier which might inhibit recombination of donor DNA. Moreover, donor fragments broken within the insertion cannot fully recombine. Such one-sided pairings, shown in Fig. 2(b, c), will exclude the long insertion, broken or not, from the pairing synapsis, thus reducing their efficiency. These events will also produce hyper-recombination (Fig. 2b).

In order to distinguish between the two possibilities, we have carried out two kinds of three-point crosses. In a first set of crosses (three-point cross of type I, Fig. 3), the double mutant strain *ami-42 ami-6* was used as recipient. *ami-42* and *ami-6* flank the insertion site *Bam*HI (Fig. 1). *ami*-insertions were located on the donor DNA. In this situation a loop repair event should generate a wild-type transformant when the central insertion and both flanking

Fig. 3. Three-point cross of type I. This class of cross was used to test the loop repair model in a positive way. *ami*-insertions were used to transform the *ami-42 ami-6* double mutant. A localized loop-repair event (a) should mostly generate a wild-type transformant, as in the two-point cross of Fig. 2(a). One-sided pairings (b), which exclude the heterologous insertion and flanking DNA, should lead to a mutant genotype, while they generate an *ami*⁺ genotype in the two-point crosses of Fig. 2(b). Line symbols are as in Fig. 2.

Table 4. Effect of insertion length on recombination indices in three-point crosses of type I

The double mutant *ami-42 ami-6* was transformed with the *ami*-insertions, as shown in Fig. 3.

Insertion length (bp)	Mean recombination index values		
	Observed*	Predicted by a loop repair model†	Predicted by double recombinations‡
1374	0.68	10.5	0.4
665	0.62	12.5	0.4
359	0.53	6.7	0.4
92	0.37	2.7	0.4
64	0.19	3.7	0.4
4	0.38	—	0.4

* Observed indices were calculated as described in Methods.

† These are the expected indices if repair is the cause of hyper-recombination. With respect to the situation of Fig. 2(a), the number of loop repairs leading to a wild-type strand should be reduced by about 23 %, i.e. the frequency of events integrating *ami-42*⁺ and *ami*-ins, without integrating *ami-6*⁺. Values observed in two-point crosses (Table 2, *ami-42* recipient) were multiplied by 77 % to give the predicted indices. This calculation is not relevant for the control insertion of 4 bp which does not induce hyper-recombination.

‡ Values correspond to independent recombination events: integration of *ami-42*⁺ without *ami*-ins (about 1.8 %) and integration of *ami-6*⁺ without *ami*-ins (about 23 %).

wild-type sites cointegrate into the chromosome, as in Fig. 3(a). Thus, if the hyper-recombination observed in crosses between the *ami-42* recipient and the long *ami*-insertion donors is due to loop correction, it will be mostly maintained in the present cross, as *ami-6* is linked to the insertion site. Predictive calculations are in Table 4. Conversely, if hyper-recombination results mainly from one-sided pairings, the number of wild-type transformants should be lowered considerably, as a point mutation still remains on the chromosome (Fig. 3b). Experimental values are very close to the number of wild-type transformants predicted by double recombination events (Table 4), i.e. independent integrations of *ami-42*⁺ and of *ami-6*⁺ without integration of the central heterologous insertion, as explained in the legend of Table 4. The results of these crosses do not support a loop repair model. On the other hand, they are compatible with a one-sided pairing model, although they do not directly prove it.

To obtain evidence directly supporting the latter possibility, i.e. the exclusion model, also called a one-sided pairing model, we have carried out a second kind of cross (three-point cross of type II, Fig. 4). Three double *ami* mutant strains were constructed, containing one of the larger *ami*-insertions and the *ami-6* mutation. The streptomycin-resistance marker was also present in these strains. Chromosomal DNA from these mutants was used to transform the *ami-42* recipient strain. The following is assumed: with respect to crosses between the *ami-42* recipient and the *ami*-insertion donors, the exceedingly large number of *ami*⁺ bacteria should be mostly unchanged in such three-point crosses provided that one-sided pairings are responsible for hyper-recombination. Indeed, according to this model the *ami-6* mutation which flanks the heterologous insertion should be excluded with it, or unlinked by breaks occurring within the insertion (Fig. 4a). In addition, one should note that in this kind of cross, loop repair events cannot generate a wild-type

Fig. 4. Three-point cross of type II. This class of cross was used to test the exclusion model in a positive way. (a) The insertion being excluded (the linked point mutation *ami-6*) should also be excluded from the pairing or should be unlinked by entry breaks within the insertion. This should lead to a wild-type recombinant as in the two-point cross of Fig. 2(b). (b) Loop corrections should not frequently lead to a wild-type transformant, with respect to the two-point cross of Fig. 2(a). Line symbols are as in Fig. 2.

Table 5. Effect of insertion length on recombination indices in three-point crosses of type II

The *ami-42* mutant was transformed with the doubly mutated DNA (*ami-ins-1374 ami-6*, *ami-ins-665 ami-6* or *ami-ins-359 ami-6*) as shown in Fig. 4.

Insertion length (bp)	Mean recombination index values		
	Observed*	Predicted (exclusion)†	Predicted (correction)‡
1374	16	13.5	3.1
665	14	16.2	3.7
359	7.6	8.6	2

* Observed indices are calculated as described in Methods.

† Expected values if exclusion is the cause of hyper-recombination. The prediction is as follows. As *ami-6* is excluded with the heterologous insertion (Fig. 4a), the situation is identical to that of Fig. 2(b) and should lead to the same indices. Thus values predicted from the exclusion model are those from Table 2 (*ami-42* recipient).

‡ Predicted *ami*⁺ indices, based on a loop correction model. The prediction is as follows. The situation becomes identical to Fig. 2(a) when *ami-ins* integrates without *ami-6*. As the disjunction frequency between *ami-6* and the insertion site is about 23%, *ami*⁺ numbers should be reduced to 23% of those observed in two-point crosses (Table 2, *ami-42* recipient), thus giving the numbers indicated.

genotype (Fig. 4b), unless a recombination occurs between *ami-6* and the right border of the insertion. Therefore, based on a multibase loop repair model, the number of *ami*⁺ transformants observed in crosses between the *ami-42* recipient and the *ami*-insertion donors should be drastically affected by the presence of the flanking *ami-6* mutation on the donor DNA. Experimental results and expected values according to one model or to the other are detailed in Table 5. We found that the numbers of *ami*⁺ transformants are identical to those obtained in a cross between the *ami*-insertion donors and the *ami-42* recipient. This result directly supports the exclusion model and, moreover, it does not substantiate a loop correction model.

Using the *ami-109* deletion in three-point crosses

The results with donor insertions indicate that reduced efficiency and hyper-recombination are not consistent with a loop correction model. Reduced transforming

efficiencies and hyper-recombination were also characteristic of donor deletions. In particular, results obtained with the 395-base deletion *ami-109* (Lefevre *et al.*, 1989) indicated about 20% hyper-recombination. However, this excess of *ami*⁺ recombinants was attributed to a loop correction pathway, not to an exclusion of the donor deletion. To determine whether completely different mechanisms for donor insertion and donor deletion account for similar results, i.e. reduced efficiency and hyper-recombination, we have reinvestigated the behaviour of a donor deletion. For this purpose we carried out the same three-point crosses as described above, but involving *ami-109* as a long heterologous insertion.

First, the double mutant strain *ami-42 ami-6* was transformed with donor DNA carrying *ami-109* (Fig. 5). This deletion is located approximately in the middle of the two sites (Fig. 1). A conversion of the deletion to the wild-type sequence suggests that hyper-recombination remains (Fig. 5a). Actually, the situation is identical to a cross between the *ami-109* deletion donor and the *ami-6* recipient,

Fig. 5. Testing the *ami-109* deletion in a three-point cross of type I. (a) Conversion of the deletion to wild-type leads to an *ami*⁺ strand. (b) Exclusion of the donor strand at the heterologous insertion allows one flanking mutation to be maintained on the chromosome. (b) Δ, *ami-109* deletion. Line symbols are as in Fig. 2.

Fig. 6. Testing the *ami-109* deletion in a three-point cross of type II. (a) A correction changing the deletion to wild-type has no effect on the *ami-42* donor mutation; the strain remains mutant. (b) Pairing stops at the deletion, thus preventing the *ami-42* mutation from being integrated into the recipient chromosome. Symbols are as in Fig. 5.

provided that the *ami-109* deleted site co-recombines with the *ami-42*⁺ site. According to the positions of *ami-42* and *ami-109*, disjunction between the two sites should be about 10 %. Consequently, according to a loop correction model, the *ami*⁺ index generated in the present three-point cross should be reduced by 10 % as compared with crosses between the *ami-109* donor and the *ami-6* recipient. Since this latter cross produces around 27 % *ami*⁺ colonies, the expected value based on a correction model should be approximately 24 % *ami*⁺ colonies. On the other hand, if one-sided pairings are responsible for the excess of *ami*⁺ transformants, this excess should disappear in this cross since one lateral mutation remains on the chromosome (Fig. 5b). We have obtained 0.71 % *ami*⁺ transformants, i.e. close to the expected number based on independent recombinations integrating both *ami-42*⁺ and *ami-6*⁺ sites without integrating the central deletion *ami-109*. Such events may be estimated at around 1 %, since the distance between *ami-109* and *ami-6* or *ami-42* is about 10 %.

In addition, we have constructed a double mutant *ami-109 ami-42*. Its DNA was used to transform the *ami-6* mutant strain (Fig. 6). Complete pairing and conversion of the deletion to the wild-type (Fig. 6a) should lead to a drastically reduced number of *ami*⁺ colonies relative to the cross between the *ami-109* donor and the *ami-6* recipient. Since recombination between the *ami-109* site and the *ami-42* site is approximately 10 %, the *ami*⁺ index in the present three-point cross should fall between 2 and 3 %. Conversely, if pairing stops at the heterologous insertion, the *ami-42* mutation is excluded (Fig. 6b), and the number of *ami*⁺ colonies should be mostly the same as in a cross

between the *ami-109* DNA and the *ami-6* recipient. We have observed an *ami*⁺ frequency identical to the one obtained in the cross between the *ami-109* donor and the *ami-6* recipient, i.e. between 25 % and 30 %. These results firmly support the prediction based on exclusion events to account for recombination of donor deletions. The conversion model, previously proposed (Lefevre *et al.*, 1989), is not confirmed by the present study; we have no satisfactory hypothesis to explain previous observations.

DISCUSSION

Several insertions and one deletion were used to elucidate the recombination behaviour of long heterologous insertions. By a combination of two- and three-point crosses, we have found that both donor insertions and donor deletions are not repaired at the heteroduplex level. Reduced transforming efficiency and hyper-recombination, induced by long deletions and insertions, are best explained by pairing inhibition excluding the donor heterologous insertion from the heteroduplex joint. Such exclusion may also account for the low efficiency and hyporecombination observed in crosses involving recipient insertions (Table 1, right column, Table 3). In that situation, recombination is identical to transformation involving donor deletions, i.e. the wild-type site should be frequently excluded from the pairing synapsis. In two-point crosses, while exclusion of donor insertions directly generates a wild-type transformant, exclusion of donor wild-type sites does not. Moreover, such exclusion events are 'lost' for recombining the wild-type site into the chromosome, thus explaining the slight hyporecombina-

tion observed in two-point crosses involving recipient insertions (Table 3).

Thus, effective pairing of homologous sequences would be initiated by the invading single-strand donor DNA, and the pairing process would be partly blocked at the level of long heterologous insertions. Inhibition is only partial, since the transformation efficiency of long heterologous insertions is reduced approximately to one-third or one-half of the efficiency of point mutations. This is consistent with *in vitro* studies showing that although RecA of *E. coli* is able to catalyse recombination of long heterologous insertions, the process is less efficient than for homologous DNA (Bianchi & Radding, 1983). In addition, regarding pneumococcal transformation, donor insertions must undergo nicks during entry. As the mean size for entering fragments is about 6000 bases (Morrison & Guild, 1972), insertions of a few hundred bases should be broken with frequencies close to 10% and more if longer. Such broken insertions cannot be efficient in one-point crosses whatever the situation, i.e. no recombination at all or recombination on one side only (Fig. 2). Natural breaks must explain, in the case of donor insertions, a significant part of the observed reduction of efficiency and hyper-recombination.

In this study we also found that insertions transform as well as or more efficiently than deletions of corresponding size. This observation was unexpected, because deletions have no physical size and should behave as single point mutations with regard to entry, while insertions undergo breaks. However, this observation is consistent with previous *in vitro* findings of Bianchi & Radding (1983): heteroduplexes are spanned more efficiently by RecA when the heterologous insertion is on the single-stranded partner rather than on the duplex DNA. Thus, we propose that the unwinding of a heterologous chromosomal region is the limiting step for the transformation of a donor deletion. It should be a more limiting step than single-stranded DNA folding is for the transformation of a donor insertion. Indeed, in spite of entry breaks destroying them, donor insertions appear to be more transformable markers than donor deletions, at least for the heterologous insertion sizes studied in this work. Lacks (1966) found that the transformation efficiency of the multisite mutation *malE9*, probably a very long deletion, is 0.4. Conversely, when transforming a *malE9* strain with *mal*⁺ DNA, i.e. in a situation identical to a donor insertion, the transformation efficiency of the wild-type locus decreased to 0.009. As the *malE9* deletion is very large, i.e. close to the mean size of an entering fragment, almost all *mal*⁺ entering fragments should be broken within the sequence deleted on the chromosome, thus explaining the very low efficiency. In our study, insertions are small compared to the mean length of entering DNAs. Interestingly, when carried on donor DNA, the *malE9* deletion had a transformation efficiency of 0.4 (Lacks, 1966). This value is very close to those observed in the present study when transforming strains containing the 665 or the 1374 bp insertions with *ami*⁺ DNA. This observation could indicate that, although a multibase chromosomal heterologous insertion partly

inhibits recombination of the donor single-stranded fragment, it can be bypassed *in vivo*, and this bypass could occur efficiently whatever the heterologous insertion size. This initial decrease of recombination for DNAs carrying heterologous insertions beyond a few hundred bases, followed by a plateau for longer heterologous insertions, is reminiscent of the findings of Bi & Liu (1994). They characterized two kinds of recombination in *E. coli*: *recA*-independent recombination, sensitive to the heterologous insertion length between two repeats, and *recA*-dependent recombination, sensitive to homology sizes.

The crosses carried out in this study suggest that no pathway specialized for correcting multibase loop heteroduplexes exists in *S. pneumoniae*. Nevertheless, such structures must be formed when recombination of a long donor heterologous insertion is completed successfully; this is likely to occur as long heterologous insertions are transformable markers. Thus, *S. pneumoniae* appears not to correct big looped-out heteroduplexes, while most point mismatches are repaired; regarding the respective bulks, this may appear paradoxical. However, it is likely that this situation is satisfactory for bacteria. Similarly one should recall, for example, that C/C mismatches appear to be very destabilizing for the DNA structure (Gasc *et al.*, 1989). Nevertheless C/C escapes correction by the Hex pathway (Claverys *et al.*, 1983) and is poorly repaired by the *E. coli* Mut system (Kramer *et al.*, 1984). Indeed, this mismatch should not be detrimental to bacteria: transversions, especially G → C, do not arise frequently because of the proofreading activity of DNA polymerase III (Wu *et al.*, 1990). Conversely, A/C and G/T, which are efficiently repaired by the Hex and Mut systems, result from transitions, which are not efficiently repaired by the proofreading activity of DNA polymerase III (Wu *et al.*, 1990). In the same perspective, we can imagine that looped-out heteroduplexes do not arise frequently in bacterial chromosomes and that specific counteracting systems have not evolved in bacteria. In addition, considering natural transformation, pneumococci could benefit by retrieving through recombination, long heterologous or heterospecific DNA fragments. Such horizontal transfers of blocks of foreign DNA have been proposed to explain the emergence of highly penicillin-resistant strains of *S. pneumoniae* among hospital isolates (Dowson *et al.*, 1989). Indeed, we can imagine that like heterologous insertions in this study, highly divergent regions could, sometimes, lead to the formation of heteroduplex bubbles and be integrated into a foreign chromosome, provided there is enough homology on flanking sides.

In *E. coli*, no repair system specializing in long multibase loop heteroduplexes was found (Carraway & Marinus, 1993). On the other hand, based on tetrad analysis, it was concluded that two long deletions undergo conversion in yeast (Fogel *et al.*, 1981). Correction mechanisms acting on single-strand bubble heteroduplexes have been demonstrated in mammals (Ayares *et al.*, 1987; Weiss & Wilson, 1987). The large size and high sequence redundancy of eukaryotic genomes may frequently induce polymerase slippage or interfere with meiotic recombinations. Subsequent looped-out structures could arise in high prop-

ortions and be toxic for cells unless they are repaired by specific pathways.

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