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Ectopic Integration of Chromosomal Genes in *Streptococcus pneumoniae*

BRUNO M. MANNARELLI AND SANFORD A. LACKS*

Biology Department, Brookhaven National Laboratory, Upton, New York 11973

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When a DNA fragment containing a marker gene was ligated to random chromosomal fragments of *Streptococcus pneumoniae* and used to transform a recipient strain lacking that gene, the gene was integrated at various locations in the chromosome. Such ectopic integration was demonstrated for the *malM* gene, and its molecular basis was analyzed with defined donor molecules consisting of ligated fragments containing the *malM* and *sul* genes of *S. pneumoniae*. In a recipient strain deleted in the *mal* region of its chromosome, these constructs gave Mal$^+$ transformants in which the *malM* and *sul* genes were now linked, with *malM* located between duplicate *sul* segments. Ectopic integration was unstable under nonselective conditions; *mal(sul)* ectopic insertions were lost at a rate of 0.05% per generation. Several possible mechanisms of ectopic integration were examined. The donor molecule is most likely to be a circular form of ligated homologous and nonhomologous fragments that, after entry into the cell, undergoes circular synapsis with the recipient chromosome at the site of homology, followed by repair and additive integration.

Ectopic integration is defined as a process in which chromosomal genes are inserted into the chromosome at places other than their normal location. Such integration was encountered during attempts to establish recombinant plasmids in *Streptococcus pneumoniae* (20). When mixtures of plasmids and wild-type (*mal*$^+$) chromosomal DNA were cut with restriction enzymes, ligated, and used to transform a strain of *S. pneumoniae* deleted in its chromosomal *mal* region, many Mal$^+$ transformants contained no recombinant plasmids. It was hypothesized that in these transformants *mal*-containing fragments had been ligated to other chromosomal fragments and were then inserted into the chromosome at sites homologous to those fragments. The ultimate location of the *mal* gene in the chromosome would then depend on the particular chromosomal fragment in the donor DNA to which it had been ligated.

In the present work we explored the mechanism of ectopic integration. Our approach was to ligate defined DNA fragments containing the pneumococcal *mal* and *sul* genes, which previously had been cloned (20), for use as donor DNA. Analysis of the novel linkage produced between the *mal* and *sul* genes, of the stability of the ectopic insertion, and of the structure of the chromosomal integration products revealed by Southern hybridization has illuminated various facets of the process. On the basis of these results, we have examined several different models for the mechanism of ectopic integration. The ability to insert DNA fragments into the chromosome at various locations by ectopic integration may be generally useful.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. pneumoniae* 193 (*malDXMP581*) and 642 (*ery-2*) were derived from strain R6 (24). Plasmids pLS70 and pLS80, which contain the *malM*$^+$ and *sul-d* genes, respectively, were prepared from strains 808 and 878.

Growth and transformation of cultures. Media and procedures for culture growth and transformation have been described previously (9). Unless otherwise stated, transformation was carried out at a DNA concentration of 1 μg/ml. Selection for Tc^r, Sul^r, and Mal$^+$ was previously described (13).

DNA preparation, restriction cutting, and ligation. Crude cell lysates (9), purified chromosomal DNA (2), crude plasmid extracts (20), and purified plasmids (4) were prepared as described previously. Restriction enzymes were from either New England BioLabs or Boehringer Mannheim Biochemicals and were used as specified by the supplier.

Ligation mixtures generally contained DNA at ~0.25 mg/ml in 50 mM Tris (pH 7.6)–10 mM $MgCl_2$. They were heated at 65°C for 5 min and cooled stepwise by holding at 37, 20, 5, and 0°C for 10-min intervals. To the mixtures were added 1 mM ATP, 10 mM dithiothreitol, 1 mM spermidine, bovine serum albumin at 100 μg/ml, and T4 DNA ligase at 40,000 U/ml (New England BioLabs), and the mixtures were held at 0°C for 17 h. To produce the limited ligation shown in Fig. 1, lanes 2 and 3, the ligase concentration was reduced to 800 U/ml. In the ligation shown in Fig. 1, lane 8, the DNA concentration was only 0.5 μg/ml.

Recovery of DNA from agarose gels. Fragments of DNA were separated by electrophoresis in 0.6% gels of low-melting-point agarose (Ultra Pure; Bethesda Research Laboratories). The electrophoresis buffer (21) contained ethidium bromide at a concentration of 1 μg/ml, and DNA fragments were directly visualized by their fluorescence in UV light. Gel fragments were cut out, mixed with two volumes of TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA), heated at 65°C for 10 min to melt the gel, and cooled to 30°C. This mixture was extracted first at 30°C and then at room temperature with equal volumes of Tris (pH 8.0)-buffered phenol. To the supernatant fluid was added 0.2 volume of 10 M ammonium acetate (also carrier tRNA [*Escherichia coli*; Sigma Chemical Co.] if the concentration of DNA was <20 μg/ml), and DNA was precipitated with two volumes of ethanol at −70°C. The precipitate was dissolved in 0.3 ml of TE buffer, 0.1 ml of 10 M ammonium acetate was added, and the

* Corresponding author.

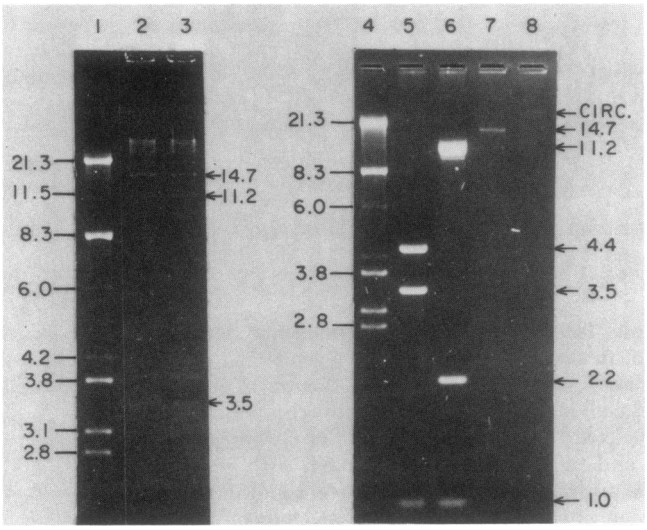

FIG. 1. Ligation of *mal* and *sul* fragments to construct defined donor molecules. DNA fragments and ligation mixtures were subjected to electrophoresis in 1% agarose gels. Lanes 1 and 4, *Dpn*II-cut phage T7 DNA fragments used as size reference markers. Lanes 2 and 3, products of limited ligation of the 3.5-kb *mal* and the 11.2-kb *sul Pst*I fragments. Lanes 5 and 6, pLS70 and pLS80, respectively, digested with *Pst*I. Lane 7, the 14.7-kb fragment (linear *mal-sul* monomer) purified from the ligation products shown in lanes 2 and 3. Lane 8, products of ligation of the 14.7-kb fragment at low concentration. Sizes of generated fragments are indicated with arrows; CIRC, circular *mal-sul* monomer.

solution was reprecipitated with ethanol. The final solution did not contain impurities to hinder further restriction cutting or ligation. Recovery of DNA was >80%.

Southern hybridization analysis. The blotting procedure used was a modification of the Southern procedure described by Thomashow et al. (22). Nick translation of probes was performed by the method of Sciaky and Thomashow (16). Specific activities of the probes were $\sim 10^8$ cpm/μg. Filters were exposed to XAR-5 film (Eastman Kodak Co.) at −70°C with Lightning—Plus intensifying screens (Du Pont Co.).

Reversion of Mal$^+$ clones. Cultures of Mal$^+$ clones grown to 2×10^8 CFU/ml in the presence of maltose were diluted to 2×10^2 CFU/ml in sucrose medium and grown to 2×10^8 CFU/ml. The cultures were diluted and plated on maltose medium containing limiting sucrose (9) to give ~100 CFU per plate. On this medium revertant Mal$^-$ clones can be distinguished as small colonies compared with the large colonies of nonrevertant Mal$^+$ clones.

RESULTS

Ectopic integration with total chromosomal DNA. Chromosomal DNA that had been cut with restriction enzymes and ligated in the absence of plasmid DNA gave rise to ectopic integration when recipient cells were treated with the ligated DNA. In the experiment shown in Table 1, total chromosomal DNA from strain 642 was used as the donor to transform the *mal* deletion mutant strain 193 to Mal$^+$. Samples of the donor DNA were either untreated, cut with restriction enzymes *Eco*RI or *Pst*I, or cut and then ligated. The untreated DNA transformed strain 193, but DNA cut with either *Eco*RI or *Pst*I gave no Mal$^+$ transformants. With uncut DNA the entering strands were large enough to bridge the deletion in the *mal* locus and become integrated into the chromosome. Upon cutting with restriction enzymes, the donor *mal*$^+$ molecules were cleaved at sites embraced by the deletion (20). Entering strands derived from these fragments, therefore, could not be integrated and did not give rise to Mal$^+$ transformants. However, after ligation of the cut fragments, Mal$^+$ transformants again were obtained. Ligation then must have produced some DNA structures that allowed integration and expression of the *mal* gene. The *mal* fragment presumably was ligated to some other fragment or fragments to form a construct that enabled the *mal* gene to become inserted together with the other fragment(s).

Construction of defined donor molecules. To determine the types of molecules that give rise to ectopic integration, and the location and structure of ectopically integrated genes, we constructed molecules with defined genetic fragments. Recombinant plasmids pLS70 and pLS80 (20), which contain chromosomal inserts that include the *malM* and *sul* genes, respectively, served as our source of defined fragments. Plasmid pLS70 was cut with *Pst*I to yield a 3.5-kilobase (kb) chromosomal fragment containing the *malM* gene and a large part of the *malX* gene (Fig. 2). Plasmid pLS80 was cut with *Pst*I to yield an 11.2-kb fragment composed of a 9.0-kb chromosomal segment containing the *sul-d* marker and a 2.2-kb vector segment containing the *tet* gene (Fig. 2). These two fragments were purified, mixed, and ligated together (Fig. 1). The resultant ligation products were separated on an agarose gel (Fig. 1, lanes 2 and 3), and the 14.7-kb fragment that corresponds to the ligation product containing one of each of the original fragments, which we call the linear *mal-sul* monomer, was excised and purified (Fig. 1, lane 7). This linear monomer was then religated under dilute conditions to produce a ligation mixture (Fig. 1, lane 8) containing circular monomers, some remaining linear monomers, and probably some multimers that were not visible as bands.

Possible structures at each stage of the construction are shown in Fig. 2. Ligation of the 3.5-kb *mal* and 11.2-kb *sul* fragments can produce four possible 14.7-kb linear *mal-sul* monomers (Fig. 2B). Circularization of the linear monomers, however, will produce only two different circular monomers (Fig. 2C). Ligation of the linear monomers to form dimers can result in 48 possible structures. However, only with those in which the *sul* fragments were oriented in the same direction could the intervening material be integrated into the chromosome. Furthermore, only if a *mal* segment were located between the *sul* segments, could it be integrated. The orientation of an external *mal* segment would similarly be irrelevant. With respect to the orientation of *mal* segments that could be integrated into the chromosome by homologous pairing, therefore, consideration was narrowed down to the six possible dimer types shown in Fig. 2D.

In addition to the *sul* gene segment, the 11.2-kb fragment

TABLE 1. Recovery of transforming activity after ligation of DNA cleaved with restriction endonucleases

DNA treatment	Mal$^+$ transformants per ml[a]
Untreated	112,000
*Eco*RI cut	<10
*Eco*RI cut and ligated	1,405
*Pst*I cut	<10
*Pst*I cut and ligated	1,315

[a] After transforming recipient strain 193 with donor DNA from strain 642.

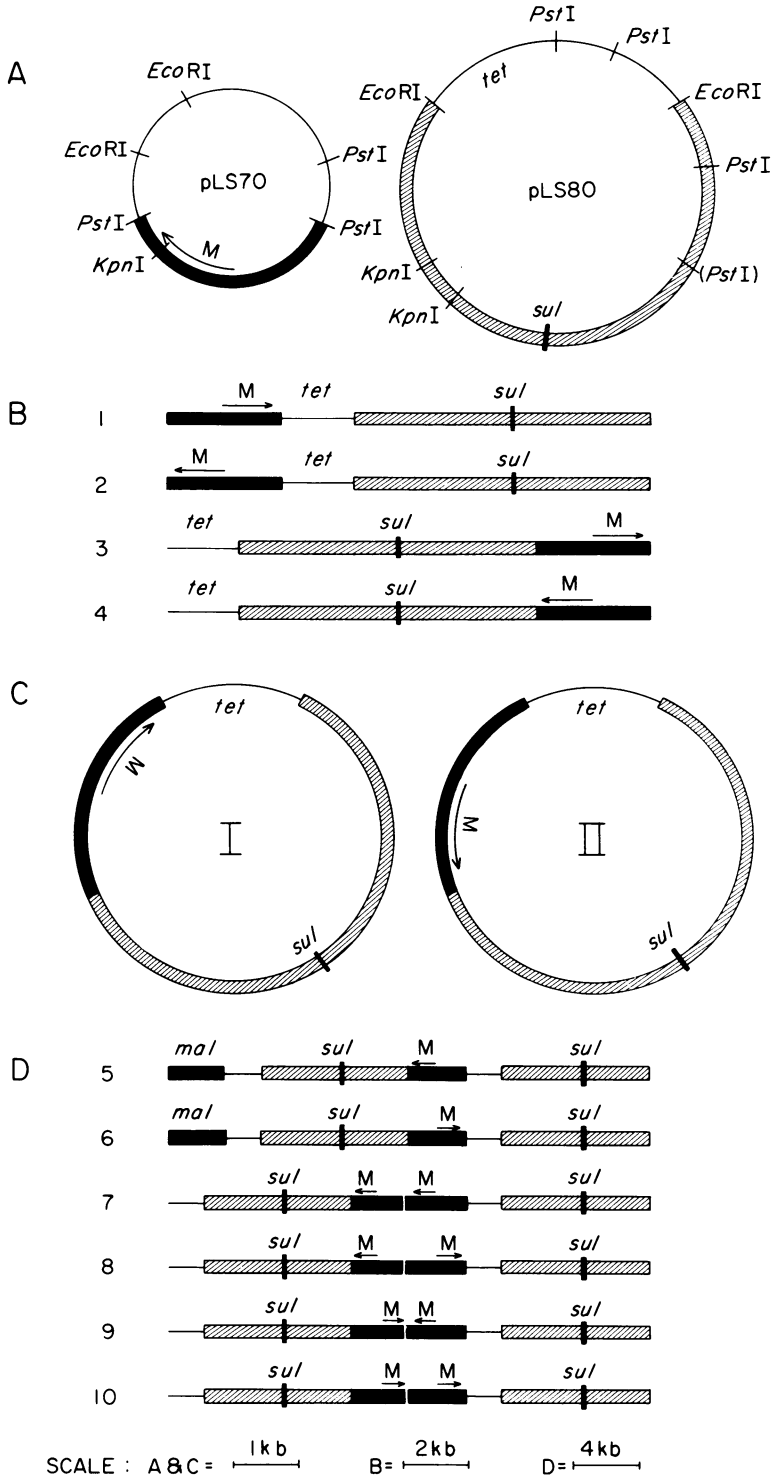

FIG. 2. Possible structures of *mal-sul* donor molecules. (A) Restriction maps of plasmids pLS70 and pLS80. The *tet* gene is located in the 2.2-kb *Pst*I-*Eco*RI segment of the plasmid. *Pst*I fragments containing the *mal* and *sul* genes were cut out and purified. The *mal* fragment contains the entire *malM* gene which is transcribed as indicated by the arrow, and part of the *malX* gene (not identified in the diagram) which is located at the other end of the fragment and transcribed in the opposite direction from the *malM* gene. (B) Linear *mal-sul* monomers: four possible linear monomers that can result from the ligation of the *mal* and *sul* fragments. (C) Circular *mal-sul* monomers. The four linear monomers can form two different circular monomers. (D) Linear *mal-sul* dimers. The linear monomers can form 48 possible linear dimers. Shown are six types in which the *sul* genes are oriented in the same direction, which would allow integration of the intervening *mal* genes into the chromosome. Segments are as follows: solid shading, *mal*; diagonal shading, *sul*; simple line, vector. In the *sul* segment a solid vertical bar indicates the *sul-d* marker.

TABLE 2. Transformation with DNA constructs[a]

Expt	*mal-sul* construct DNA	Mal$^+$ transformants per ml	Phenotype of Mal$^+$ transformants	
			Sul^r	Tet^r
I	Linear monomer	1	0/1	0/1
II	Linear monomer	3	2/3	2/3
II	Ligation mixture	106	42/45	39/45

[a] Recipient strain 193 was treated with *mal-sul* construct DNA at 10 ng/ml.

from pLS80 contains a 2.2-kb vector segment. This segment contains the entire *tet* gene but does not contain functions essential for plasmid replication. The constructs shown in Fig. 2, therefore, could not be established as autonomous plasmids and could only transfer their markers by integration into the chromosome.

Transformation with *mal-sul* constructs. The ability of the defined donor molecules to produce Mal$^+$ transformants when introduced into strain 193 was examined. Both the *mal-sul* linear monomers and the ligation mixture containing circular monomers and possible multimers were tested. Mal$^+$ transformants were obtained in both cases, but the ligated material gave a 50-fold higher yield than the linear monomers (Table 2). The activity of the circular monomers was comparable to the level observed with ligated total chromosomal DNA fragments (Table 1). Two of the four Mal$^+$ clones derived from the linear monomers [called linear *mal*(*sul*) clones] and most of the clones derived from the ligation mixture [called simply *mal*(*sul*) clones] showed resistance to sulfanilamide and tetracycline.

In another experiment the presumed *mal-sul* circular monomer band was cut out of the gel, eluted, and characterized by cleavage with *Eco*RI. A single band of 14.7 kb was produced. This proved that the original band contained only the circular monomer, because linear multimers (or circular multimers which should include head-to-head forms) would give rise to *Eco*RI cleavage products of several sizes. When this eluted material was used to transform strain 193, it produced 40 times as many Mal$^+$ clones as did an equivalent amount of the linear monomer. In a rough measurement of the dependence of Mal$^+$ transformants on the concentration of this material, DNA concentrations of 2, 4, 6, and 8 ng/ml gave, respectively, 8, 9, 21, and 18 Mal$^+$ transformants per ml. Although they are not very precise, these results indicate a linear dependence on DNA concentration, and hence the ability of a single *mal-sul* circular monomer to produce an ectopic integration.

Linkage of *mal* and *sul* markers. Mal$^+$ transformants derived from the *mal-sul* donor constructs were examined for the possible presence of plasmids or extrachromosomal covalently closed circular DNA. Crude extracts of cells from 35 Mal$^+$ ectopic clones showed no evidence on electrophoretic gels of covalently closed circular material in the range from 1 to 30 kb. This method readily detects pDP1, which is present at one copy per chromosome (19). It is unlikely, therefore, that the markers were present in an independently replicating plasmid or in some persistent covalently closed circular monomer identical to the donor molecule. It appears, rather, that the marker genes were introduced into the chromosome.

Inasmuch as the *mal* region was completely deleted in the recipient chromosome it would be expected that the donor material would integrate in the *sul* region of the chromosome and that the *mal* fragment should be found adjacent to the *sul* locus. Therefore, we examined the linkage of the *mal* and *sul* markers in transformation by chromosomal DNA from Mal$^+$ Sul^r clones. Normally, transformation by chromosomal

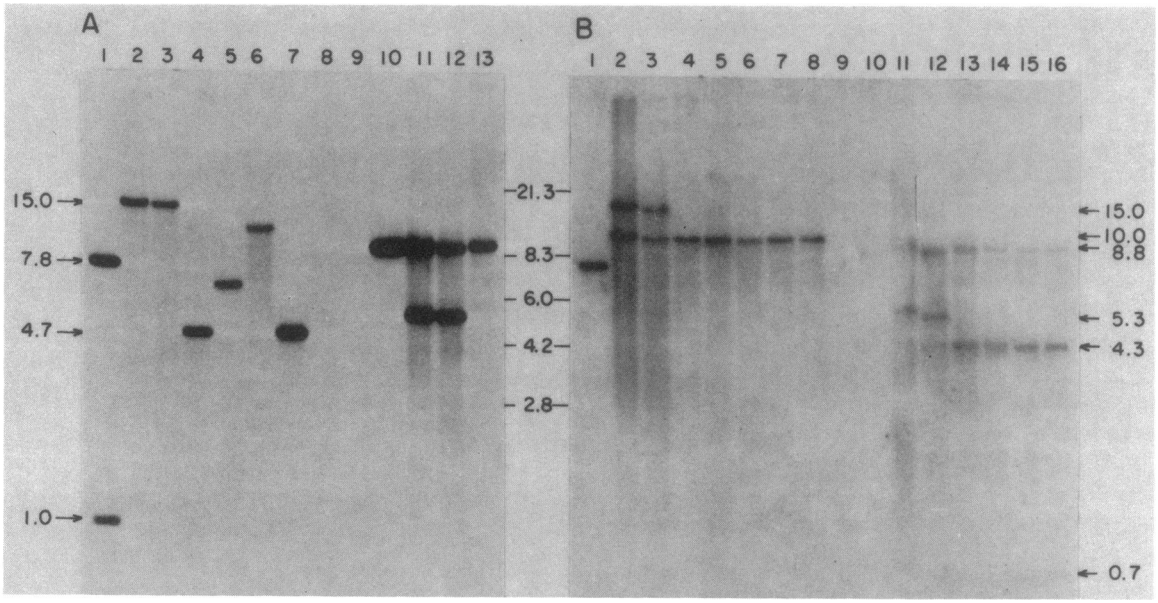

FIG. 3. Hybridization of chromosomal DNA from transformants showing ectopic integration. DNA digests were probed with ^{32}P-labeled pLS70 to detect *mal* (A) and with ^{32}P-labeled pLS80 to detect *sul* (B). DNA in lanes 1 to 8 was digested with *Eco*RI and in lanes 10 to 16 with *Kpn*I. Lanes: 1 and 10, plasmid pLS70; 2 and 11, *mal*(sul) clone M11; 3 and 12, *mal*(*sul*) clone M16; 4 and 15, random chromosomal clone R3. Lanes 5, 6, and 16 correspond to random chromosomal clones R13, R14, and R11, respectively. Lanes: 7, wild-type strain R6; 8 and 14, recipient strain 193; 13, random chromosomal clone R8. Lane 9 contains *Dpn*II-cut phage T7 DNA fragments used as visual reference markers after staining and, therefore, appears blank in the autoradiogram. Positions of the reference markers and their size in kb are indicated between A and B. Sizes of fragments produced are indicated by arrows. Some bands in B, lanes 10 to 12, were poorly labeled because a bubble hindered contact of the probe to the filter. Amounts of DNA digest applied to gels: plasmid, 0.015 μg per lane; chromosomal, 4.5 μg per lane.

DNA shows no linkage between *mal* and *sul* markers (6). When strain 193 was transformed by DNA from 10 different Mal$^+$ Sul^r ectopic clones, the mean ratio of Mal$^+$ to Sul^r transformants was 0.07, which reflected the lower transformation efficiency of the multisite (14.7-kb) marker relative to the single-site Sul^r marker (9). The mean ratio of Mal$^+$ to Mal$^+$ Sul^r transformants was 0.42, which indicated a rather close linkage. This linkage was demonstrated also by picking individual Mal$^+$ transformants and examining them for Sul^r; 10 Mal$^+$ colonies from each transformation were picked, and 44% of them were also Sul^r. Mal$^+$ Sul^s transformants result from recombination events between *mal* and the *sul-d* site in the donor DNA. These results are consistent with a separation of only a few kb of DNA between the *mal* and *sul* markers in the *mal*(*sul*) ectopic clones.

Hybridization analysis of ectopic integrants. To affirm the presence of the *mal* and *sul* fragments on the chromosome and to determine their location thereon, hybridization was carried out between chromosomal DNA fragments and *mal* and *sul* probes. We performed Southern blot experiments in which purified chromosomal DNA from several ectopic clones was cut with *Eco*RI or *Kpn*I and probed with nick-translated ^{32}P-labeled pLS70 (Fig. 3A) and pLS80 (Fig. 3B). Lanes 1 through 13 are identical in Fig. 3A and B. In lanes 1 to 8, samples were cut with *Eco*RI; in the other lanes, samples were cut with *Kpn*I. Lane 1 shows that pLS70 was cut with *Eco*RI at two sites to yield a 7.8- and a 1.0-kb band when probed with pLS70 but only a 7.8-kb band when probed with pLS80. Plasmid pLS80 is missing the 1.0-kb *Eco*RI vector fragment so it does not hybridize to the smaller fragment of pLS70. Lane 7 shows DNA from the wild-type strain R6, which gave a 4.7-kb band with the *mal* (pLS70) probe and a 10.0-kb band with the *sul* (pLS80) probe. Lane 8 corresponds to the *mal* deletion mutant recipient strain 193, which gave the expected 10-kb fragment when probed with pLS80 but no band when probed with pLS70 because it is deleted in the *mal* region. Lanes 4, 5, and 6 represent random chromosomal ectopics (i.e., Mal$^+$ clones produced with total chromosomal DNA, which had been cut by *Pst*I and ligated, as donor). When probed with pLS70, a band of different size was obtained for each one, that is, 4.8, 6.8, and 11.0 kb, respectively. When probed with pLS80, only a single-size fragment, the 10-kb fragment, was obtained for all three. This pattern is consistent with *mal* gene integration in different places on the chromosome, and not in association with the *sul* gene in the random chromosomal ectopic clones. Lanes 2 and 3 show results for DNA from two *mal*(*sul*) ectopic clones, M11 and M16. For both, hybridization with the *mal* fragment (pLS70) gave a single band of 15.0 kb. Hybridization with the *sul* fragment gave two bands, one of 15 kb and the other of 10 kb. This pattern corresponds to an integration event giving duplicate *sul* genes with the *mal* and *tet* genes between them (Fig. 4).

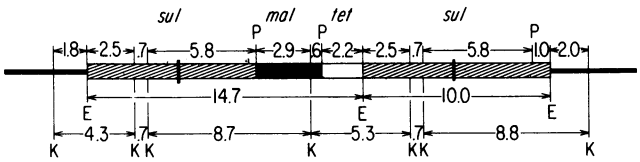

FIG. 4. Physical map of chromosomal DNA in clones showing ectopic integration. The gene sequence determined from Southern blot hybridization of DNA from clones M11 and M16 and the distances between restriction enzyme sites predicted from plasmid restriction maps are indicated. Gene segment markings are as follows: solid, *mal*; diagonal, *sul*; open, *tet*. Abbreviation of restriction enzyme sites: E, *Eco*RI; K, *Kpn*I; P, *Pst*I.

TABLE 3. Revertant frequency distribution of Mal$^+$ clones

Revertant frequency[a] (%)	*mal*(*sul*) ectopics[b]	Random chromosomal ectopics[b]
<1	0	2
1–10	26	5
11–20	14	0
21–30	1	1
31–40	1	2
41–50	1	0
51–60	1	1
61–70	0	0
71–80	0	1
81–90	1	1
91–99	0	1
>99	0	2

[a] Intervals of revertant frequency were determined as the ratio of Mal$^-$ to total cells after growth for 20 generations in the presence of sucrose.
[b] Number of clones that gave revertant frequencies within the indicated interval.

Because *Kpn*I cuts in both the *mal* and *sul* genes, hybridization of *Kpn*I fragments allowed the ordering of these genes in the integration product. In lane 10 pLS70 is shown cut with *Kpn*I at a single site to yield an 8.8-kb band when probed with either pLS70 or pLS80 (Fig. 3). Lanes 11 and 12 represent the *mal*(*sul*) clones M11 and M16. After *Kpn*I cutting both produced two bands, 8.8 and 5.3 kb, when probed for the *mal* fragment. They both produced four bands when probed with the *sul* probe: 8.8, 5.3, 4.3, and 0.7 kb. Lane 14 shows DNA from the *mal* deletion mutant strain 193, which gave three bands with the *sul* probe: 8.8, 4.3, and 0.7 kb. Lanes 15 and 16 (Fig. 3B) show two random chromosomal ectopics, which produced *sul* hybridization bands similar to strain 193. Lanes 14 to 16 therefore represent the normal chromosomal *sul* configuration. Chromosomal DNA cut wth *Eco*RI yields a 10-kb fragment that contains the *sul* marker (20). Within this fragment there are two internal *Kpn*I sites, 2.5 and 3.2 kb, from the left end of the fragment (13). For *Kpn*I to produce 4.3- and 8.8-kb fragments from chromosomal DNA, there must be a *Kpn*I site 1.8 kb from the left end of the fragment and another 2.0 kb from the right end (Fig. 4). In the 3.5-kb *mal* fragment there is an internal *Kpn*I site 0.6 kb from the *malM* end of the fragment (Fig. 2). Therefore, we can determine the order of the genes in the *mal*(*sul*) clones M11 and M16 from the banding patterns in Fig. 3, lanes 11 and 12, to be *sul-malXmalM-tet-sul* (Fig. 4). The mapping in Fig. 4 is consistent with the restriction maps of pLS70 (11) and pLS80 (12). Although five bands would be expected from *Kpn*I cutting, two of the bands (8.7 and 8.8 kb) would be indistinguishable, and only four distinct bands were observed. The observed order ot genes is consistent with integration of donor molecules CI or D6 (Fig. 2). Analysis of six more *mal*(*sul*) ectopic clones (data not shown) gave similar patterns of duplicative integration, with four clones showing *mal* in the same orientation as CI and two showing *mal* in the inverse orientation of CII (Fig. 2). One of the latter clones came from the experiment with purified circular monomer donor molecules.

The *mal*(*sul*) ectopic clones M11 and M16 analyzed above were both Mal$^+$ Sul^r Tc^r in phenotype. Another clone, M8, that was Mal$^+$ Sul^r Tc^s was also examined by Southern hybridization. When its DNA was cut with *Eco*RI and probed with pLS70, hybridization occurred with a 14.0-kb

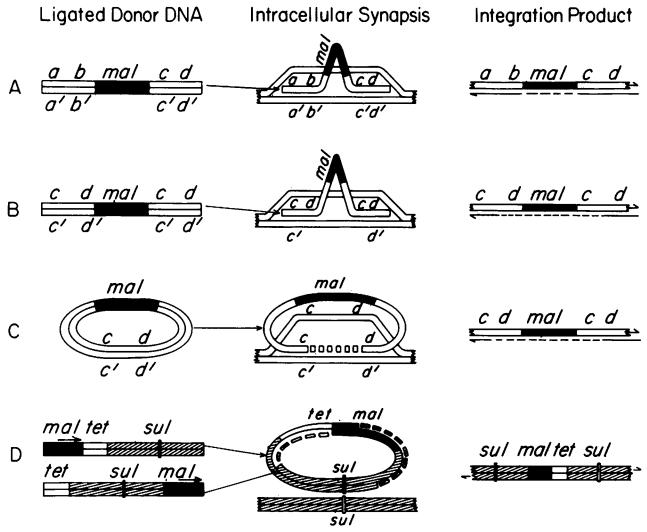

FIG. 5. Possible mechanisms of ectopic integration. A, B, and C depict possible pathways for ectopic integration by ligated chromosomal DNA mixtures (random chromosomal ectopics). B, C, and D depict pathways for ectopic integration by *mal-sul* donor constructs. (A) Two adjacent chromosomal fragments ligated to *mal*; linear synapsis; integration without duplication. (B) Two identical chromosomal fragments ligated to *mal* or *mal-sul* linear dimer; linear synapsis; duplicative integration. (C) Single fragment ligated to *mal* and circularized or *mal-sul* circular monomer; circular synapsis; duplicative integration. (D) Possible mechanism for ectopic integration by linear *mal-sul* monomers. Strands from two different 14.7-kb linear monomers reconstitute a duplex within the cell; integration by a duplex recombination event. Letters *a*, *b*, *c*, and *d* refer to specific sites, and *a'*, *b'*, *c'*, and *d'* are their complements. Gene segment markings are as follows: solid, *mal*; diagonal, *sul*; open, *tet* or random chromosomal DNA. In the *sul* segment a solid vertical bar indicates the *sul-d* marker, and an open bar indicated the *sul-s* marker. In B and C the *mal* segment represents heterologous *mal-tet* DNA, and *cd* represents homologous *sul* DNA in the *mal-sul* constructs.

fragment, which was approximately 1.0 kb smaller than the 15.0-kb fragment found with M11 and M16. When cut with *Kpn*I, M8 showed a single 8.8-kb fragment with the pLS70 probe and three bands, 8.8, 4.3, and 0.7 kb, when probed with pLS80. Thus M8 apparently contains a deletion of 1.0 kb within the ectopic insertion. The sequence of genes in M8 appears to be the same as in M11 and M16; the deletion in the middle of the *tet* gene would render the clone sensitive to tetracycline. The deletion reduced the size of the 5.3-kb *Kpn*I fragment in M11 and M16 to 4.3 kb in M8, so that the fragment now migrated to approximately the same position as the original 4.3-kb *Kpn*I fragment. The residual *mal* and *tet* segments in the new 4.3-kb *Kpn*I fragment were presumably not large enough (<0.6 kb) to hybridize to the pLS70 probe. pLS70 could only hybridize to the *mal* segment in the adjacent *Kpn*I fragment, giving a single 8.8-kb band. How or when the *tet* gene deletion occurred is not known.

Instability of ectopic integration. It was found that when Mal$^+$ ectopic clones were grown with sucrose rather than maltose, that is, without selection for the *mal*$^+$ gene, the Mal$^+$ marker was lost. The frequency of reversion to Mal$^-$ was examined for various ectopic clones. Thus, *mal*(*sul*) ectopics and random chromosomal ectopics were grown for 20 generations in medium containing sucrose and then plated in agar medium containing a low concentration of sucrose and a high concentration of maltose. This medium allows both detection of Mal$^-$ cells (as small colonies) and recovery of the revertant clones. Revertant frequencies were determined as the percentage of cells that were no longer able to grow with maltose.

Random chromosomal ectopics displayed a spectrum of revertant frequencies ranging from less than 1% to more than 99% (Table 3). However, *mal*(*sul*) ectopics exhibited more uniform revertant frequencies. These *mal*(sul) ectopics were fairly stable, with a calculated reversion rate of ca. 0.05% per generation. Presumably, the reversion rate depends on the size of the fragment that was ligated to the *mal* fragment and, hence, duplicated in the ectopic integration. With random chromosomal ectopics the fragment could vary greatly in size, reflecting the size distribution of fragments produced by restriction cleavage of total chromosomal DNA. With the *mal*(sul) ectopics, the ligated fragment, *sul-tet*, was identical in every case. The known size of the *sul* segment, 9 kb, would give a direct repeat of this length in the chromosome of the ectopic integrant. If reversion results from genetic recombination between the direct repeats with the removal of the intervening DNA, it would be expected that reversion rate would depend on the size of the repeated segments.

DISCUSSION

From the foregoing it can be concluded that in random chromosomal ectopics the *mal* gene combined with different chromosomal fragments and was inserted into the chromosome in different locations. In *mal*(*sul*) ectopics, however, the *mal* gene was always inserted in the *sul* region. Such ectopic integration generally resulted in a duplication of the *sul* gene with the *mal* and *tet* genes inserted between the duplicated segments. This was indicated by the close linkage of the *sul* and *mal* genes, the instability of the integrants, and Southern hybridization analysis. Thus, the ectopic integration of chromosomal genes described here is similar to the chromosomal integration of foreign genes on nonreplicating plasmids that carry DNA homologous to the chromosome (5, 7, 15, 23).

Possible mechanisms of ectopic integration are depicted in Fig. 5. In every case the donor DNA will be converted to single-strand segments on entry into the cell, inasmuch as this has been shown to be the fate of transforming DNA in gram-positive species (10). Pathways A to C refer to random ectopic integration with ligated chromosomal DNA as the donor; pathways B to D refer to defined donor molecules (with *mal* in B and C representing the heterologous *mal-tet* DNA portion and *cd* representing the homologous *sul* DNA) (Fig. 5). We believe that these four classes of mechanism broadly represent all possible interactions based on homologous pairing that could lead to the observed results. Mechanism A (Fig. 5) could account for random chromosomal ectopic integrants that show no reversion (Table 3); such integration was demonstrated by Niaudet and Ehrlich (15). Mechanism B (Fig. 5), which would allow integration of dimer *mal-sul* molecules, was previously suggested by Vasseghi and co-workers (23). Only mechanism D (Fig. 5), in which separately entering single strands reassemble to form a duplex molecule that is integrated by a Campbell-like mechanism (3), would allow integration of *mal* from mixed *mal-sul* linear monomers; such integration was observed at very low frequency. Because random chromosomal ectopic integration by A or B (Fig. 5) requires a second ligation with a particular fragment in the mixture containing 1,000 different fragments, the probability of such interaction is low; this is not the case for C (Fig. 5) in which ligation circularizes the

binary structure. That a circular *mal-sul* monomer can give rise to ectopic integration was shown in the present work.

The mechanism shown in C (Fig. 5) is novel and interesting from several points of view. We call this interaction of the single-strand donor DNA with the chromosome circular synapsis, as opposed to the linear synapsis typical of chromosomal transformation (S. A. Lacks, Proc. Int. Congr. Genet. 15th, New Delhi, India, in press) or mechanisms A and B. Experiments on the chromosomal facilitation of plasmid transfer indicate that such circular synapsis between an entering plasmid strand and the chromosome does occur (8, 12, 13). The circularly synapsed single strand in C (Fig. 5) would undergo repair and be integrated by means of a reciprocal single-strand crossover with the corresponding strand of the chromosome to yield a duplication of the homologous region in one of the daughter cells after cell division. We believe that the mechanism suggested in C (Fig. 5) is more realistic than an earlier model for integration of a foreign plasmid monomer (23), which postulated two separate and sequential synapses and exchanges, because our model depends on circular synapsis and a single exchange event.

Aside from its use as a tool for genetic analysis and for discerning novel pathways of DNA recombination within cells, could ectopic integration have biological significance, for example, in the evolution of bacteria? The Omega factors of *S. pneumoniae* (17, 18) are sequences of DNA that carry drug-resistance genes and are at most only partly homologous with the wild-type pneumococcal chromosome. They may have originated by ectopic integration of a conjugative plasmid that could not replicate in *S. pneumoniae* but that carried partial, perhaps accidental, homology to the pneumococcal chromosome. These Omega factors can be transmitted by conjugation. Conceivably, they may recombine out of the chromosome, as did the unstable ectopic integrants that we observed, and the resultant circular plasmid, although unable to replicate vegetatively, would be transferable by conjugation. Two more systems in which the chromosomes of different strains of *S. pneumoniae* appear to differ by whole blocks of genes, the one determining capsular phenotype (1) and the other determining restriction phenotype (14), might similarly owe their origin to ectopic integration.

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