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Genetics

Cloning of chromosomal genes in *Streptococcus pneumoniae*

(plasmid transformation/recombinant DNA/genetic recombination/amylomaltase/sulfonamide resistance)

DIANE L. STASSI, PALOMA LOPEZ*, MANUEL ESPINOSA*, AND SANFORD A. LACKS

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

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ABSTRACT **A system for molecular cloning in *Streptococcus pneumoniae* was developed. The multicopy plasmids pMV158 (5.4 kilobases) and pLS1 (4.3 kilobases), which confer tetracycline resistance, were used as vectors to clone chromosomal genes of *S. pneumoniae* in host cells of this species. A 3.3-kilobase restriction fragment containing the *malM* gene, which codes for amylomaltase, was cloned in a deletion mutant lacking chromosomal homology with the fragment. The recombinant plasmid, pLS70, could transform over 50% of a recipient population to maltose utilization. Amylomaltase constituted up to 10% of the protein of cells containing pLS70. A derivative with a deletion, pLS69, appeared to gain a selective advantage by producing less enzyme. A 10-kilobase restriction fragment containing the *sul-d* gene for sulfonamide resistance was cloned in the presence of the homologous chromosomal gene. *De novo* establishment of a recombinant plasmid was just as frequent as transformation in an endogenous plasmid. Despite the processing of DNA during uptake in the transformation of *S. pneumoniae*, recombinant plasmids can be introduced. Models for the reconstruction of recombinant DNA in cells of *S. pneumoniae* and *Bacillus subtilis* are considered and compared.**

Despite the fact that bacterial transformation was discovered in *Streptococcus pneumoniae*, this species has not until now been used to clone recombinant DNA. In the bacterium commonly used for cloning, *Escherichia coli*, the artificial method of transformation allows uptake of DNA without alteration, whereas the naturally evolved DNA uptake systems of *S. pneumoniae* and *Bacillus subtilis* process the incoming DNA (1). The cutting of DNA during its binding and entry and the necessity to reconstruct a plasmid from single-stranded fragments are impediments to cloning in these Gram-positive bacteria. Plasmid introduction in *B. subtilis* requires a multimeric donor molecule (2), although monomers have been shown to be active in *S. pneumoniae* (3, 4). Nevertheless, cloning of chromosomal genes by introduction of a recombinant plasmid has been achieved in *B. subtilis* (5, 6). However, a more promising approach may be the transformation of an endogenous plasmid by recombinant DNA (7). The latter process can be called *plasmid transformation*, as opposed to the former process of *plasmid transfer*, in which a new plasmid replicon is established in the cell. The recent introduction of multicopy plasmids into *S. pneumoniae* (4, 8) has made cloning in this species practicable.

Our approach to cloning in *S. pneumoniae* depended on previous analysis of the maltose-utilization system (9, 10). The *mal* region—a cluster of genes concerned with maltosaccharide utilization—includes the *malM* gene, which specifies amylomaltase, and a regulatory gene. Restriction fragments, either unfractionated or enriched for the *malM* gene, were ligated to the plasmid pMV158 (11) and used to transform recipient cells with and without endogenous plasmid.

Successful cloning of the wild-type allele of the *malM* gene was achieved. A recombinant plasmid with a deletion in the *mal* region that apparently reduced *malM* transcription was also obtained. The behavior of recombinant plasmids in transformation provided insight into the mechanism of reconstruction of plasmid replicons after the degradative process of entry. There appears to be a significant difference between *S. pneumoniae* and *B. subtilis* in the reconstruction process.

MATERIALS AND METHODS

Bacterial Culture and Transformation. Strains of *S. pneumoniae* were derived from R6 (9, 10, 12); *mal* mutations are mapped in Fig. 3*C*. Media and procedures for culture growth, chromosomal DNA preparation, and transformation have been described (9). Cultures at 5×10^6 colony-forming units per ml were treated with DNA at 0.1–1.0 μg/ml for 40 min at 30°C, then at 37°C for 2 hr, before transfer to selective media. Selection for tetracycline resistance conferred by pMV158 was according to Smith *et al.* (8).

Plasmid DNA Preparation. C. Saunders kindly provided pMV158. Cleared lysates were prepared and fractionated in CsCl/ethidium bromide gradients by published procedures (13). Alkaline lysates were prepared by modifying the method of Birnboim and Doly (14). Lysozyme was omitted and cells were lysed with 0.1% sodium deoxycholate for 5 min at 37°C, after which 0.175 M NaOH and 1% $NaDodSO_4$ were added.

DNA Cleavage and Ligation. Restriction enzymes and phage T4 DNA ligase were from New England BioLabs except for *Eco*RI (Miles) and *Dpn* II (15). Ligation mixtures containing $\approx$10 μg of DNA in 15 μl of 30 mM Tris·HCl (pH 7.6)/10 mM $MgCl_2$ were heated to 65°C and cooled stepwise to 0°C. After addition of 1 mM ATP and 300 units of ligase, the mixtures were held at 20°C for 15 min, then at 5°C for 15 hr. Samples were diluted 1:15 in Tris/$MgCl_2$/ATP at the same concentration, 300 units of ligase was added, and incubation was continued at 5°C for 24 hr.

Gel Electrophoresis. Restriction fragments were separated and plasmids were analyzed by electrophoresis in 1% agarose, with phage T7 DNA fragments serving as standards (16). Bands were revealed by staining with ethidium bromide. Small amounts of DNA were recovered from macerated segments of agarose incubated in buffer. To obtain larger amounts suitable for ligation, the agarose was dissolved in NaI and DNA was absorbed to glass powder (17).

DNA and Enzyme Assays. DNA concentrations were estimated from fluorescence of gel bands or determined in purified preparations from A_{260} and in crude extracts by fluorescence enhancement of 4′,6′-diamidino-2-phenylindole (18). Amylomaltase and phosphorylase were assayed as before (10).

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Abbreviations: kb, kilobases; Mal$^+$, maltose-utilizing; Str^r, streptomycin-resistant; Tc^r, tetracycline-resistant.

* Permanent address: Instituto de Inmunologia y Biologia Microbiana, C.S.I.C., Velazquez, 144, Madrid-6, Spain.

RESULTS

Cloning of Chromosomal *mal* DNA. Restriction analysis of pMV158 showed no cuts with *Bam*HI or *Bgl* II, single cuts with *Bgl* I and *Hin*dIII, and double cuts with *Pst* I and *Eco*RI. The *Bgl* I cut is unsuitable for cross-ligation, and *Hin*dIII destroyed the chromosomal *mal* transforming activity, but plasmids partially digested with *Pst* I or fully cut with *Eco*RI proved suitable for cloning.

Transforming activity for several chromosomal markers was measured before and after restriction cutting. The size of a DNA fragment containing a marker was determined by gel electrophoretic migration of the transforming activity. Approximate size and residual transforming activity for *Eco*RI fragments were: *nov-1*, 18.7 kilobases (kb), 76%; *sul-d*, 11.7 kb, 56%; *str-41*, 8.5 kb, 28%; *malM594*$^+$, 4.8 kb, 17%; *pyr-c*, 4.0 kb, 6%; *ery-2*, 2.4 kb, 0.5%. For *Pst* I fragments residual activities were *sul-d*, 34%; *malM594*$^+$, 11%; *ery-2*, 0.3%; *str-41*, 0.2%; *nov-1*, 0.1%. The *Pst* I *mal* fragment was 3.3 kb long. Neither *Eco*RI- nor *Pst* I-cut DNA could transform the *mal* deletion strains used. Size fractions corresponding to the *Eco*RI *sul* and the *Pst* I *mal* fragments were purified by gel electrophoresis.

Results of an experiment in which recipient strains, with *mal* deletions of different extent, and with or without endogenous plasmid, were transformed by various ligated DNA mixtures are shown in Table 1. Total cut chromosomal DNA or purified fragments were ligated with fully or partially digested plasmid DNA at a ratio of 10:1. The Str^r transformation indicated the relative competence of the recipient cultures.

Complete cutting of pMV158 with *Pst* I prior to ligation prevented transfer of Tc^r. Either Tc^r or plasmid replication was destroyed by removal of one of the two *Pst* I fragments. Complete cutting with *Eco*RI, however, permitted Tc^r transformation, and analysis of the plasmids contained in the transformants showed them to be generally reduced in size, having lost the smaller *Eco*RI fragment (Fig. 1*A*). The resulting miniplasmid, pLS1, became the vector for recombinants found among the Tc^r transformants: 5 among 15 Tc^r examined with strain 193 as recipient, and 10 among 50 with strain 217. With unfractionated DNA, inserts of various sizes were found (Fig. 1*B*). A different experiment using the *sul*-containing fraction of *Eco*RI fragments gave more uniform inserts of the expected 10- to 12-kb size (Fig. 1*C*).

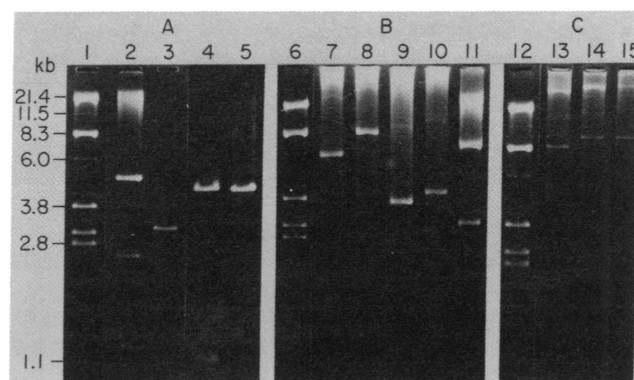

FIG. 1. Chromosomal *Eco*RI-fragment inserts in the pLS1 miniplasmid. (*A*) Vector plasmids: pMV158 (CsCl/ethidium bromide purified), lane 3, uncut, and lane 4, cut with *Eco*RI; pLS1 (cleared lysate), lane 2, uncut, and lane 5, cut. (*B*) Lanes 7–10, recombinant plasmids (alkaline lysates) from ligation of unfractionated cut chromosomal DNA and pMV158; lane 11, pLS1. (*C*) Lanes 13–15, recombinants from ligation of 10- to 12-kb fraction with linear pLS1 fragment. Lanes 1, 6, and 12, *Dpn* II-cut T7 DNA reference fragments. In lanes 2, 3, 7–11, and 13–15 lower bands correspond to covalently closed circular forms, upper bands (predominant in cleared lysates) to open circular forms of plasmid; smudge below wells, to chromosomal DNA. In lanes 13 and 14 linearized plasmids are also visible.

From Table 1 it is evident that Mal$^+$ transformants were obtained with all of the ligated DNA mixtures. Their frequency did not depend on the presence of an endogenous plasmid in the recipient. No recombinant plasmids were observed among 96 Mal$^+$ clones tested, which represented all four recipients transformed with either the EcoRI-cut or the enriched *Pst* I-*mal* donor. We believe that these Mal$^+$ transformants result from *mal* DNA ligated to other chromosomal fragments and inserted into the chromosome at sites homologous to those fragments, a process we propose to call *ectopic insertion*.

The frequency of ectopic insertions prevented the use of transformation to Mal$^+$ to directly select *mal* plasmid recom-

Table 1. Transformation by ligated chromosomal and plasmid DNA

Recipient strain (genotype, plasmid)	Transformed phenotype*	Transformed cells per 0.5 ml with donor DNA†: Chromosomal, uncut, or (pMV158)	*Eco*RI-cut ligated to fully cut pMV158	*Pst* I-cut ligated to fully cut pMV158	Purified *Pst* I-*mal* ligated to partly cut pMV158
217	Tc^r	(6,500)	1100	0	2000
(Δ*mal591*)	Mal$^+$	290	11	100	13
	Tc^r Mal$^+$	—	0	0	0
	Str^r	190,000	—	—	—
(Δ*mal591*, pMV158)	Mal$^+$	100	0	16	35
	Str^r	31,000	—	—	—
193	Tc^r	(1,700)	150	0	200
(Δ*mal581*)	Mal$^+$	160	0	5	5
	Tc^r Mal$^+$	—	0	0	2
	Str^r	4,200	—	—	—
(Δ*mal581*, pMV158)	Mal$^+$	6,500	19	35	18
	Str^r	150,000	—	—	—

* Tc^r, tetracycline-resistant; Mal$^+$, maltose-utilizing; Str^r, streptomycin-resistant.

† Plasmid DNA at 0.1 μg/ml, chromosomal DNA at 1.0 μg/ml.

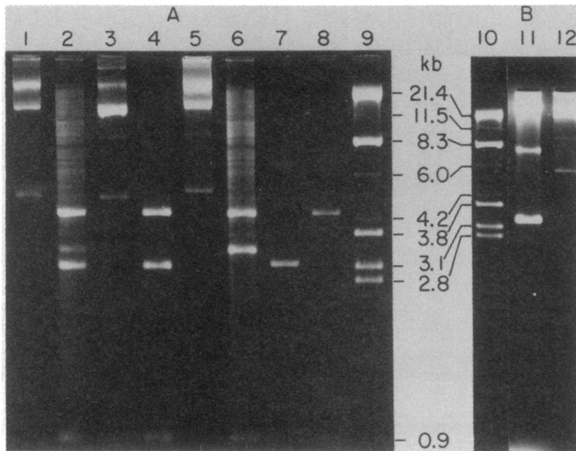

FIG. 2. (*A*) Size and composition of *mal* recombinant plasmids in cleared lysates. Lanes 1 and 2, original clone 69; lanes 3 and 4, purified pLS69; lanes 5 and 6, pLS70, lanes 7 and 8, pMV158. Lanes 1, 3, 5, and 7, uncut; lanes 2, 4, 6, and 8, cut with *Pst* I. Lane 9, *Dpn* II-cut T7 DNA. Multiple light bands in lanes 2 (except at 3.3 kb) and 6 are chromosomal DNA restriction fragments. (*B*) Minority recombinant plasmid in transformation of a recipient that contains pMV158. Lane 10, as lane 9; lane 11, alkaline lysate of strain 803 (Δ*mal581*/pMV158) transformed to Mal$^+$ with *Eco*RI/*Bgl* I-cut pLS70; lane 12, pLS70.

binants. However, selection of Tc^r Mal$^+$ transformants proved suitable. Although unfractionated *Eco*RI-cut DNA gave no Tc^r Mal$^+$ transformants, use of the purified *Pst* I-*mal* fragment in the ligation mixture gave two such transformants, clones 69 and 70, which contained plasmids with chromosomal *mal* DNA inserts.

Composition of *mal* Recombinant Plasmids. Cleared lysates of clones 69 and 70 were analyzed by gel electrophoresis with and without cutting by *Pst* I (Fig. 2*A*). Both contained plasmids, the covalently closed circular forms of which (lowest bands in lanes 1 and 5) migrated more slowly than pMV158 (lane 7). Clone 70 contained a single plasmid, pLS70, which on cutting with *Pst* I gave in addition to the two vector fragments of 4.5 and 0.9 kb (lane 8) an insert of 3.3 kb that corresponded to the chromosomal *Pst* I-*mal* fragment (lane 6). Clone 69 contained a mixed plasmid population: a minor component identical to pLS70 and a major component (≈90%) with a 2.9-kb insert (lane 2). The latter plasmid was purified by transfer to the *mal591* strain (lanes 3 and 4) and called pLS69.

The vector and *mal* recombinant plasmids were analyzed by restriction cutting with *Pst* I, *Eco*RI, *Bgl* I and *Hin*dIII (data not

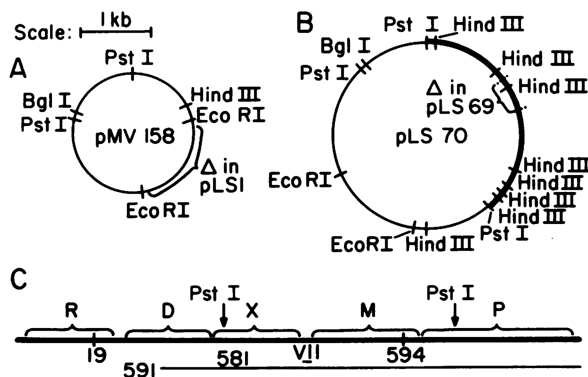

FIG. 3. Maps. (*A*) Vector plasmids. (*B*) *mal* recombinant plasmids. (*C*) Chromosomal *mal* region (letters refer to gene segments on chromosome, numbers refer to mutations); location of *Pst* I sites is approximate.

Table 2. Transforming activity of recombinant plasmid pLS70

		Transformants per ml with recipient strain*		
Donor DNA	Marker	193 (Δ*mal581*)	803 (pMV158, Δ*mal581*)	708 (*malM594*)
Crude lysate	Tc^r	4,100	—	24,000
(strain 810)	Mal$^+$	1,900	5,500	1,100,000
Cleared lysate	Tc^r	41,000	—	170,000
(strain 808)	Mal$^+$	13,000	50,000	12,000,000
Pst I-cut	Mal$^+$	<10	<10	610,000
*Eco*RI-cut	Mal$^+$	<10	640	2,500,000
*Eco*RI/*Bgl* I-cut	Mal$^+$	<10	95	4,600,000
Chromosomal	Mal$^+$	6,200	26,000	270,000
(strain 533)	Str^r	37,000	320,000	210,000

* Donor DNA at 1.0 μg/ml. Actual values for Str^r; values for Mal$^+$ and Tc^r adjusted to level of competence of strain 708 on the basis of Str^r yields.

shown). This analysis showed that pLS69 differs from pLS70 by a 0.4-kb deletion in the interior of the chromosomal insert. Maps of the vector plasmids pMV158 and pLS1 are shown in Fig. 3*A*. Preliminary maps of pLS69 and pLS70 are presented in Fig. 3*B*. Seven *Hin*dIII sites in the chromosomal fragment are arbitrarily drawn. A genetic map of the chromosomal *mal* region (10, 19) is shown in Fig. 3*C*.

Transformation by the *mal* Plasmids. The transforming activities of plasmid pLS70 with three kinds of recipient strains—a *mal* deletion mutant without a plasmid, the deletion mutant containing a partly homologous plasmid, and a single-site *mal* mutant—are summarized in Table 2. Essentially similar results were obtained with *mal591* strains as recipients and with pLS69 as donor (data not shown). With the deletion mutants, Mal$^+$ and Tc^r transforming activities were generally equal and similar to Tc^r transformation by pMV158. These transformants result from plasmid transfer. They contained plasmids identical in size to the donor.

Specific transforming activities in all cases increased with purification of the plasmid, reaching a maximum of 3.3×10^7 per μg (30 times the crude lysate) for *mal594* transformation with the pure covalently closed circular plasmid. Both this observation and the equivalence of Mal$^+$ and Tc^r transfer noted above attest to the plasmid localization of the *mal* gene. Because the *mal* plasmid DNA is homogenous, very high levels of transformation of a chromosomal single-site mutation, such as *malM594*, can be achieved. The maximum observed frequency, with covalently closed circular pLS70 at 1.4 μg/ml, was 49.7% of the recipient genomes. This was based on the proportion of transformants in the population after growth for 2 hr, which allowed segregation of transformed strands and cells in the 4-cell pneumococcal chains (20).

Plasmids cut with restriction enzymes cannot effect plasmid transfer in Gram-positive bacteria (4, 21, 22), as demonstrated with strain 193 in Table 2. The linear fragments can all transform the chromosomal *malM594* mutant. The *Pst* I-*mal* fragment, unlike the others, retains no part of pMV158 and for this reason cannot transform the *mal581* strain that carries the plasmid. The *Eco*RI-*mal*-containing fragment of pLS70 can transform this strain to Mal$^+$, presumably by transforming the endogenous plasmid, to which it is homologous at both ends, by a mechanism akin to chromosomal transformation, as proposed for *B. subtilis* (21). The lower activity obtained with the *Eco*RI/*Bgl* I-cut fragment indicates that the frequency of integration depends on the

Table 3. Amylomaltase in strains with recombinant plasmids

Strain			Amylomaltase, units/mg protein	
Number	Genotype	Plasmid	Sucrose-grown	Maltose-grown
R6	Wild type	None	44	1020
807	Δ*malDMP581*	pLS69	112	1400
808	Δ*malDMP581*	pLS70	965	9790
809	Δ*malRDMP591*	pLS69	509	1210
810	Δ*malRDMP591*	pLS70	5230	9820

length of homologous region adjacent to the insert. The low content of recombinant plasmids in the transformants (Fig. 2*B*) indicated either that few resident plasmids were transformed per cell or that the recombinant was incompatible with the resident plasmid.

Transformation of pMV158-containing *mal* deletion mutants with intact pLS69 and pLS70 also gave a small proportion of recombinant transformants. Here, the mechanism can be either plasmid transfer or plasmid transformation. The frequency was considerably higher than was obtained with the *Eco*RI fragment but not much greater than was obtained by plasmid transfer into a plasmid-free strain.

Enzyme Synthesis in Strains with *mal* Plasmids. The *mal* region (Fig. 3*C*) contains five genes involved with maltosaccharide utilization (10). Genes *malM* and *malP* code for amylomaltase and phosphorylase, *malX* for a membrane protein (19), and *malD* for maltotetraose permease. Growth in maltose induces all of these functions; mutations in the repressor gene *malR* render them constitutive. The *Pst* I-*mal* fragment was presumed to contain *malM*, the only gene in the cluster known to be absolutely essential for maltose utilization, and an adjacent promoter/operator region susceptible to repression.

Amylomaltase measurements of various strains grown with and without maltose are listed in Table 3. The wild type showed a 20-fold induction by maltose. Maltose-grown strains containing pLS70 showed 10 times this amount of amylomaltase, which, on the basis of the specific activity of the purified enzyme, corresponds to 10% of the total cellular protein. Without maltose, in the constitutive *mal591* background, pLS70 produced over half this level. In the repressor-containing *mal581* strain without maltose present, pLS70 still gave a level of amylomaltase 20-fold higher than the repressed wild-type level. Although pLS69 appeared to be present in as many copies per cell as pLS70, the level of amylomaltase it gave with and without induction was similar to the wild type. In the constitutive background it gave only 1/10th the amylomaltase activity of pLS70. The deletion in pLS69 thus behaved as a down promoter mutation that reduced transcription to 1/10th of its normal rate.

Phosphorylase was absent from the plasmid-containing strains. The *Pst* I-*mal* segment must not contain the entire *malP* gene. Analysis of cell extracts by polyacrylamide gel electrophoresis (not shown) confirmed that large amounts of the amylomaltase protein were produced in induced cells containing pLS70. Another polypeptide, possibly a fragment of the *malX* or the *malP* protein, was produced to a similar extent.

Cloning of the Sulfonamide-Resistance Gene. With the recombinant *mal* plasmids it was possible to investigate procedures for cloning genes in the presence of homologous chromosomal genes. When pLS70 transformed the *malM594* strain, all Tc^r Mal$^+$ transformants contained a plasmid the size of pLS70. Therefore, an attempt was made to clone the *sul-d* gene (23) by ligating the partially purified *Eco*RI *sul* fragment with *Eco*RI-cut pLS1, transforming the sulfonamide-sensitive strain 708, and selecting for Tc^r sulfonamide-resistant transformants. This approach was successful—a recombinant plasmid, pLS80, containing a 10-kb chromosomal insert with the *sul-d* gene was obtained. Thus, it should be possible to clone any pneumococcal gene for which selective pressure can be applied.

DISCUSSION

The properties of the recombinant plasmids examined indicate that they can serve as a good source of homogenous chromosomal DNA. Their multicopy nature was attested by plasmid DNA content (estimated on gels), transforming activity, and level of phenotypic expression; 10–30 copies appear to be present. The recombinants can be propagated equally well in strains with or without homology to the chromosomal segment. The cloned *mal* DNA, being homogenous, can transform as much as 50% of a recipient population. Such high levels of transformation have only been observed in *S. pneumoniae*—for the *amiA* gene cloned in *E. coli* by Claverys *et al.* (24) and for plasmid transformation by another plasmid marker (4).

The pLS69 plasmid appears to have been derived from a plasmid equivalent to pLS70, inasmuch as the originally transformed clone carried both plasmids. Presumably, a spontaneous deletion in the wild-type *mal* segment of the original recombinant occurred during its propagation. A selective advantage of the mutant, pLS69, could have caused it to become the predominant plasmid. The amylomaltase content of strains containing pLS69 and pLS70 suggests that the deletion acts as a down promoter mutation, which causes a reduction to 1/10th of the induced level of enzyme. The deletion in pLS69 thus behaves similarly to the previously reported *V11* mutation (10). With pLS70, amylomaltase accounts for 10% of the total cellular protein. It is possible that the requisite high levels of transcription interfere with plasmid replication. Such interference has been suggested in another host–vector system (25).

From the cloning experiences with *S. pneumoniae* and *B. subtilis* certain differences emerge with respect to their respective interaction with recombinant DNA. In *S. pneumoniae* recombinant plasmids were established *de novo* more readily than by plasmid transformation. The similarity of the uptake process—in both species DNA is cut on binding and converted to single strands on entry (1)—suggests that the difference in cloning behavior results from differences between the species in the subsequent reconstruction of plasmid DNA. A striking difference in the abilities of the two species to effect plasmid transfer is the activity of monomers in *S. pneumoniae* (3, 4), compared to the requirement in *B. subtilis* for multimers (2) or, at least, repeated sequences (26). Also, mixtures of linear monomers, cleaved by different restriction endonucleases, can give rise to plasmid transfer in *S. pneumoniae* (4), but not in *B. subtilis* (22). The model shown in Fig. 4 suggests how *S. pneumoniae* is able to reconstruct an intact plasmid from single-stranded fragments of two different donor molecules, whereas *B. subtilis* can reconstruct a plasmid only from a single molecule containing a redundant sequence.

Plasmid transfer in *S. pneumoniae* could result from interaction of fragments of two monomers (Fig. 4*A*) or of a single dimer (Fig. 4*D*), as proposed by Saunders and Guild (3). The reconstruction from a dimer is similar to that proposed by Dubnau *et al.* (27) for a trimer, except that some DNA replication is needed. In *B. subtilis* only route *D* appears to be available. Recombinants could be obtained by plasmid transformation (Fig. 4*C*) in either species. Here the donor strand recombines with the endogenous plasmid in the same manner that chromosomal transformation is effected. Successful introduction of a monomer recombinant plasmid (Fig. 4*B*) could occur only in *S. pneumoniae*; interaction with a fragment from an ordinary

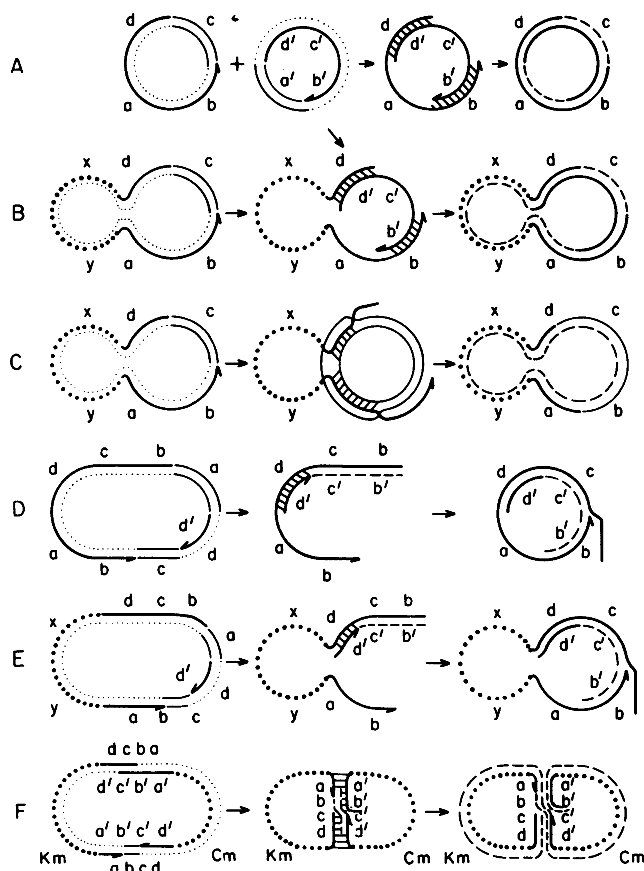

FIG. 4. Pathways of plasmid reconstruction. Modes *A–D* apply to *S. pneumoniae* and *C–F*, to *B. subtilis*. (*A*) Plasmid transfer by fragments of two monomers. (*B*) Recombinant plasmid transfer by monomers. (*C*) Plasmid transformation by recombinant. (*D*) Plasmid transfer by fragments of dimer. (*E*) Recombinant transfer with insert ligated to plasmid dimer. (*F*) Plasmid transfer with partial sequence repeats. Symbols: heavy line, donor strand segments introduced into cell, with half arrowheads showing initial entry; beaded line, recombinant insert in *B, C, E*, and nonrepeated plasmid in *F*; light line, donor segments not introduced into cell, also, in *C*, endogenous plasmid; dotted line, complementary strands degraded on DNA entry; broken line, new DNA synthesis; hatching, hydrogen bonding between complementary segments; *a–d, a′–d′*, plasmid markers and their complements; *x* and *y*, insert markers; *Km, Cm*, nonrepeated plasmid markers in *F*.

plasmid would be sufficient. Because ligation with a single plasmid molecule is adequate to obtain recombinants by routes *B* and *C*, ligation mixtures containing high ratios of chromosomal to plasmid DNA (10:1) are effective. Transfer of an *in vitro* recombinant plasmid into *B. subtilis* (Fig. 4*E*) requires either insertion into a dimer or, more generally, as proposed by Michel *et al.* (6), ligation of an insert with two plasmid monomers. Thus, low ratios of chromosomal to plasmid DNA (1:1) in the ligation mixtures are most effective (6). In such transfer, as in ordinary plasmid transfer from a dimer (Fig. 4*D*), a single-stranded fragment containing more than a full plasmid genome interacts with a complementary strand fragment from elsewhere in the same molecule. After partial replication, strand transfer gives a circular structure, which is completed by further replication and removal of the residual tail. Ehrlich and coworkers have shown that the presence of repeated DNA segments in a plasmid will allow its monomeric form to transform *B. subtilis* (26). The efficiency of the transfer was proportional to the second power of the repeated length. Although in many cases only one of the nonrepeated segments was retained (this could occur by route *E*), in some cases the entire plasmid was recovered. A plausible mechanism for the latter is shown in Fig. 4*F*. Two breakage/entry events must occur within the repeated sequence, hence the second-power dependence on its length. Complementary strands enter in opposite direction and overlap slightly at their ends due to their thermal separation prior to complete degradation. Annealing of complementary regions and replicative synthesis restores the double-strand configuration, which then circularizes by dint of the short repeated sequence at its ends. Modes *E* and *F* explain the findings of Ehrlich and coworkers (6, 26) as a consequence of the DNA uptake mechanism.

The foregoing considerations apply to plasmid transfer and transformation in the absence of homology between plasmid and chromosome. Findings in *S. pneumoniae* (unpublished) and in *B. subtilis* (28) indicate that when the plasmid is partially homologous with chromosomal DNA other mechanisms can intervene.

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1. Lacks, S. A. (1977) in *Microbial Interactions*, ed. Reissig, J. (Chapman and Hall, London), pp. 179–232.
2. Canosi, U., Morelli, G. & Trautner, T. A. (1978) *Mol. Gen. Genet.*. **166**, 259–267.
3. Saunders, C. W. & Guild, W. R. (1981) *Mol. Gen. Genet.* **181**, 57–62.
4. Barany, F. & Tomasz, A. (1980) *J. Bacteriol.* **144**, 698–709.
5. Keggins, K. M., Lovett, P. S. & Duvall, E. J. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 1423–1427.
6. Michel, B., Palla, E., Niaudet, B. & Ehrlich, S. E. (1980) *Gene* **12**, 147–154.
7. Gryczan, T., Contente, S. & Dubnau, D. (1980) *Mol. Gen. Genet.* **177**, 459–467.
8. Smith, M. D., Shoemaker, N. B., Burdett, V. & Guild, W. R. (1980) *Plasmid* **3**, 70–79.
9. Lacks, S. (1966) *Genetics* **53**, 207–235.
10. Lacks, S. (1968) *Genetics* **60**, 685–706.
11. Burdett, V. (1980) *Antimicrob. Agents Chemother.* **18**, 753–760.
12. Lacks, S. & Greenberg, B. (1975) *J. Biol. Chem.* **250**, 4060–4066.
13. Saunders, C. W. & Guild, W. R. (1980) *Mol. Gen. Genet.* **180**, 573–578.
14. Birnboim, H. C. & Doly, J. (1979) *Nucleic Acids Res.* **7**, 1513–1523.
15. Lacks, S. & Greenberg, B. (1977) *J. Mol. Biol.* **114**, 153–168.
16. McDonnell, M. W., Simon, M. N. & Studier, F. W. (1977) *J. Mol. Biol.* **110**, 119–146.
17. Vogelstein, B. & Gillespie, D. (1979) *Proc. Natl. Acad. Aci. USA* **76**, 615–619.
18. Kapuscinski, J. & Skoczylas, B. (1977) *Anal. Biochem.* **83**, 252–257.
19. Weinrauch, Y. & Lacks, S. A. (1981) *Mol. Gen. Genet.*, in press.
20. Lacks, S., Greenberg, B. & Carlson, K. (1967) *J. Mol. Biol.* **29**, 327–347.
21. Contente, S. & Dubnau, D. (1979) *Plasmid* **2**, 555–571.
22. Ehrlich, S. D. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1680–1682.
23. Hotchkiss, R. D. & Evans, A. H. (1958) *Cold Spring Harbor Symp. Quant. Biol.* **23**, 85–97.
24. Claverys, J. P., Lefevre, J. C. & Sicard, A. M. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3534–3538.
25. Raibaud, O. & Schwartz, M. (1980) *J. Bacteriol.* **143**, 761–771.
26. Michel, B., Palla, E. & Ehrlich, S. D. (1981) in *Transformation 1980*, ed. Polsinelli, M. (Cotswold, Oxford), in press.
27. Dubnau, D., Contente, S. & Gryczan, T. J. (1980) in *DNA: Recombination, Interactions and Repair*, eds. Zadrazil, S. & Sponer, J. (Pergamon, Oxford), pp. 365–386.
28. Canosi, U., Iglesias, A. & Trautner, T. A. (1981) *Mol. Gen. Genet.* **181**, 434–440.