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Pathway of Plasmid Transformation in Pneumococcus: Open Circular and Linear Molecules Are Active

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We have extended the analysis of plasmid transformation in *Streptococcus pneumoniae* by finding that monomeric and dimeric open circular and linear forms of pMV158 were active in transformation. Their efficiencies were at least 35-fold lower than those of the corresponding closed circular forms. The evidence came largely from analysis of S1 nuclease-digested plasmid deoxyribonucleic acid by combinations of dye-buoyancy, gel electrophoresis, and sedimentation velocity methods. As with closed circular forms, monomer open circular forms gave second-order kinetics and dimer forms gave first-order kinetics. Unique linear products of digestion by either of two restriction enzymes were inactive, but a mixture of the two digests was active, as was the mixture of linear monomer deoxyribonucleic acids produced by S1 nuclease. Absolute efficiencies of transformation were low even for closed circular donors. All of the results, including the low efficiencies, were consistent with the interpretation that plasmid replicons were assembled in the recipient cell by pairing of fragments of single strands that had entered the cell separately from duplex donors that had been cut on the cell surface.

In pneumococcus, both monomeric and dimeric covalently closed (CC) forms of plasmid pMV158 are active in transformation, the former with second-order kinetics and the latter with first-order kinetics (21). Requirements for competence and for a nuclease essential for uptake of chromosomal transforming DNA suggested that plasmid transformation used the same entry pathway as chromosomal DNA, and that plasmids were assembled in the recipient cell from single-strand fragments that had been cut from duplex donors on the cell surface before entry (20, 21). The fact that a variable small fraction of plasmid transformants arose from the non-CC region of dye-buoyancy gradients of crude or of cleared lysates (20, 23) suggested that linear or open circular (OC) forms might also be active in transformation, as would be expected from the above model. To test this possibility, we examined S1 nuclease-digested pMV158 DNA by the multiple separation methods used previously. The results showed rigorously that several OC and linear forms of plasmid DNA were active in transforming pneumococcus. Their activities were 35 to 40 times lower than those of the corresponding CC forms, which were themselves shown to be very inefficient. The overall results, including the low absolute efficiency, were consistent with the general model proposed previously (21).

MATERIALS AND METHODS

Plasmids and bacterial strains. pMV158 is a tetracycline-resistance plasmid isolated from a group B streptococcus by Burdett (2). Its size is now estimated to be 3.4 to 3.5 megadaltons (2) instead of the 3.6 megadalton figure used previously (20, 21). Strain DP3271 contains the *ery-2* chromosomal marker and pMV158. Rx1 cells were used as recipients for transformation.

Growth, transformation, and scoring. Media, growth, preparation of competent cells, and transformation procedures were as described (9, 25). Competent Rx1 cells were exposed to DNA for 30 min at 37°C, and the reaction was terminated by addition of pancreatic DNase to 10 μg/ml in 10 mM $MgSO_4$. Scoring of transformants was done on 10-cm plates by the agar overlay method as described (25).

Analytical gel electrophoresis. DNA samples were electrophoresed in TBE buffer in horizontal 0.5% or 1.0% agarose gels that were stained with 1 μg/ml of ethidium bromide (EtBr) and photographed as described (25).

Preparative gel electrophoresis. The methods and equipment described by Polsky et al. (18) were used to collect fractions from a large 1% agarose gel (Bio-Rad Laboratories) with the modifications of Saunders and Guild (21), except that a cleared lysate containing ~1.5 μg of DNA was loaded, the sample and collection chambers were 4.3 cm apart, and the cycles were 30 min of electrophoresis at 1.0 V/cm, 2.0 min for pumping out the collection chamber, and 1.3 min for refilling the chamber with TBE.

Centrifuge experiments. All runs were in 5-ml polyallomer tubes in an SW50.1 rotor at 20°C. (See legend to Fig. 3 for description of runs of sucrose gradients). For dye-buoyancy gradients, DNA samples were gently mixed with CsCl and EtBr to a density of 1.56 g/ml and 70 μg of EtBr per ml. Mineral oil was added to fill the tubes. Fractions were collected from the bottom, and 5-μl samples were assayed for transforming activity.

DNA preparations. Cleared lysates of 1.2 or 2 liters of DP3271 culture were made in 500- to 600-ml batches by scaling up the procedure described for 10-ml cultures (20). This included lysis with deoxycholate and sodium dodecyl sulfate, precipitation with 1 M NaCl, deproteinization with chloroform-isoamyl alcohol or phenol and ethanol precipitation. The batches of a given preparation were pooled after the ethanol precipitation step. Preparation A, from 1.2 liters, was redissolved in 1 ml of saline-citrate, passed through a Sepharose 6B column to remove residual sodium dodecyl sulfate or other inhibitors, and dialyzed against 120 mM NaCl. The final volume was 3 ml and contained 2 to 3 μg each of plasmid and chromosomal DNA, as estimated from gel and transformation data.

Because removal of chromosomal DNA was much less complete in large-scale batches than in the small batches previously described (20), the procedure was altered to prepare DNA for restriction analysis. Preparation B, from 2 liters, was deproteinized three times with chloroform-isoamyl alcohol and once with phenol before ethanol precipitation and resuspension, and banded to equilibrium in EtBr-CsCl. After the CC and non-CC regions were located by transformation assay, the fractions from the CC region were pooled, passed through Dowex-50 to remove EtBr, and dialyzed against 6 mM Tris (pH 7.5).

The preparation of ^{3}H-labeled ColE1 has been described (21).

Digestion with S1 nuclease. A 1.3-ml portion of lysate A containing about 1 μg each of plasmid and chromosomal DNA (see above) was mixed with an equal volume of buffer to give a final concentration of 30 mM sodium acetate (pH 4.6), 100 mM NaCl, 1 mM $ZnCl_2$, and 5% glycerol. S1 nuclease (1,500 U; Bethesda Research Labs) was added. After 8 min at 37°C, sodium citrate was added to 15 mM and the mixture was dialyzed against TBE. DNA forms purified by preparative gel electrophoresis (200 μl) were dialyzed against 120 mM NaCl. Portions (50 μl) of each were placed in three tubes, to two of which was added 0.1 volume each of concentrated buffer to give a composition equivalent to that above. To one of the two tubes, 900 U of S1 nuclease was added. After 5 min at 37°C, 0.1 volume of 10× TBE was added, and the samples were assayed for transforming activity.

Digestion with restriction nucleases. *Hin*dIII was purchased from Bethesda Research Labs. *Bgl*I was a gift from Gary Wilson. For single digests, the buffers used were those recommended by Bethesda Research Labs. For the double digest, the buffer was 20 mM Tris (pH 7.5)–7 mM $MgCl_2$–30 mM NaCl–3.5 mM betamercaptoethanol. DNA (0.8 μg) from preparation B was digested with 50 U of *Hin*dIII or 60 U of *Bgl*I or both, at 37°C for 2 h in a total volume of 150 μl.

Estimation of low DNA concentrations. To concentrate preparative gel fractions to levels allowing detection of separated monomer forms on analytical gels, fractions containing monomer CC (1 ml), OC (4 ml), or linear DNA (4 ml) were precipitated with ethanol and carrier RNA. Each precipitate was dissolved in 50 μl of TBE containing RNase, and 20-μl samples were run on analytical gels along with lambda DNA standards of known amount. After being stained with 1 μg EtBr per ml, the gels were photographed on Kodak TriX film. DNA concentrations were estimated from densitometer tracings of the negatives, using a correction factor of 1.5 to allow for greater binding of EtBr by CC DNA at this concentration (1).

RESULTS

To examine transforming activities of non-CC forms of pMV158 DNA, we treated a cleared lysate of strain DP3271 with S1 nuclease and examined it by several criteria before and after fractionation by preparative gel electrophoresis. Before treatment, only monomer CC and OC pMV158 could be detected readily in analytical gel photographs of this preparation. After treatment, the CC form was not detectable and monomer OC and linear forms were present in about equal amounts (Fig. 1).

Dye-buoyancy analysis. Plasmid transformation was reduced about sevenfold by S1 nuclease treatment, and most of this loss reflected removal of activity associated with CC forms (Fig. 2, Table 1). Before treatment, 90% of the plasmid activity banded in the CC region of an EtBr-CsCl gradient. Less than 1% of this activity remained in the CC region after treatment, and the activity banding with the chromosomal DNA marker (non-CC), representing 94% of the surviving activity, increased in absolute level. The fact that the increase was not large suggested that conversion of CC to non-CC forms was associated with substantial loss of activity. Because the residual activity in the CC region appeared to have slightly greater density than the bulk of the CC DNA before treatment, it may be that this material was less supercoiled and therefore less susceptible to S1 nuclease (24).

Sedimentation velocity. Figure 3 shows sucrose gradient velocity profiles of transforming activities in the preparation before and after S1 nuclease treatment and in several fractions from the preparative electrophoresis run (see below). Across the top of the figure are shown the positions predicted for various forms of pMV158, relative to the position of an ^{3}H-labeled ColE1 internal standard. Before treatment (Fig. 3A), the activity was largely in two peaks corresponding to monomer CC and dimer CC (20), with little activity at the position expected for mon-

omer OC, which represented about half the plasmid DNA in the gradient (Fig. 1). The S1 nuclease treatment shifted the velocity distribution

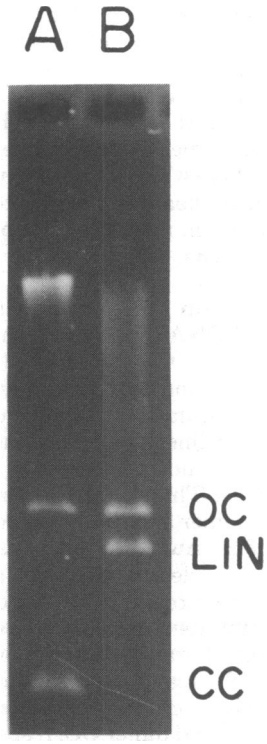

FIG. 1. *Electrophoresis in 0.5% agarose of a cleared lysate of DP3271 before (A) and after (B) digestion with S1 nuclease. LIN, Linear DNA.*

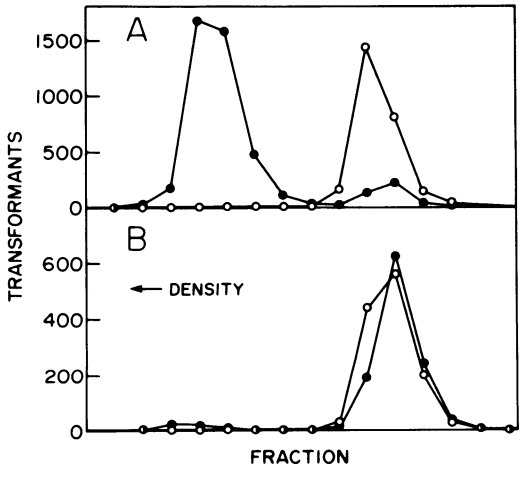

FIG. 2. *Distribution of transforming activity in EtBr-CsCl gradients before (A) and after (B) S1 nuclease digestion of a cleared lysate of DP3271. The input samples were equivalent to 0.1 (A) and 0.2 ml (B) of lysate. Symbols: ●, pMV158; ○, ery2 chromosomal marker.*

TABLE 1. *Effect of S1 nuclease on recovery and distribution of plasmid transforming activity in a dye-buoyancy gradient*[a]

Region of gradient	No. of transformants counted[b]		Survival (%)
	Before S1 nuclease	After S1 nuclease[c]	
CC	4,092	35	0.9
non-CC	430	562	131
Total	4,422	597	13.5

[a] Data are from the experiment shown in Fig. 2.
[b] 0.2 ml was plated per fraction.
[c] Entries are one-half of the observed numbers to correct for the fact that twice as much of the treated DNA was put into the gradient.

(Fig. 3B) so that substantial activity sedimented as though it might be due to monomer forms. However, most activity still sedimented faster than expected for monomers, suggesting that at these DNA concentrations the majority of the transformants arose from traces of multimeric DNA forms in the S1 nuclease treated preparation, as in untreated preparations (Fig. 3A and reference 20).

Preparative electrophoresis. For further analysis, 1.5 μg of the S1 nuclease-treated pMV158 DNA was fractionated on the preparative apparatus as described above. Fractions (12 ml) were collected, and 0.1 ml of alternate fractions used as donor to determine the profile of transforming activity. The result (Fig. 4) shows a number of well-resolved peaks between fractions 40 and 95 and a broad peak near fraction 150. The dye front was centered in fraction 13, and the scale of mobilities plotted at the top was derived from the equation $R_f = 13 \div$ (fraction no.).

It is particularly useful to compare the results with mobilities determined from analytical gels of various DNAs run at the same agarose concentration and voltage gradient. Figure 5 summarizes several such analyses and predicts the elution positions for various forms of pMV158 DNA, including those not detectable on the analytical gels. For those forms that were optically detectable, analytical gels on single fractions confirmed that linear monomers peaked in fraction 41 and OC monomers peaked in fraction 51, within one fraction of the predicted positions (Fig. 6). In a similar run on untreated DNA (see below), monomer CC DNA was found to elute at fraction 28 and 29.

The results in Fig. 4 show that transforming activity coeluted with linear monomer DNA at fraction 41, with OC monomer DNA at fraction 51, and very near the R_f values expected for dimer linear ($R_f \sim 0.18$; fraction 68) and dimer OC ($R_f \sim 0.085$; fraction 152).

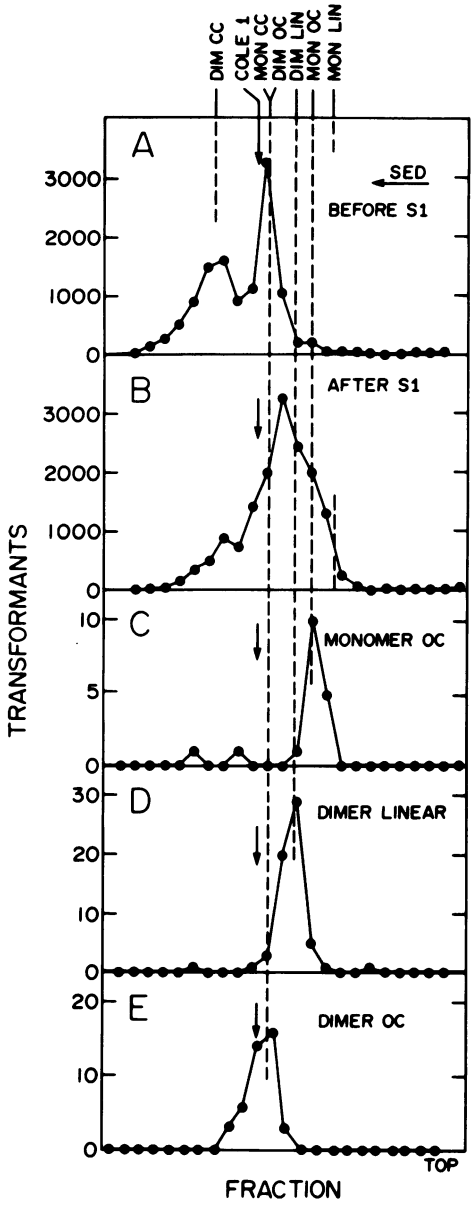

FIG. 3. *Sedimentation velocity of pMV158 transforming activity in a cleared lysate before (A) and after (B) digestion by S1 nuclease, and in fractions 51 (C), 68 (D), and 152 (E) from the preparative electrophoresis run shown in Fig. 4. The input plasmid DNA amounts were about 50 ng (A) and 100 ng (B). DNA (330 μl) in TBE, 30 μl of ^{3}H-labeled ColE1 (50,000 cpm), and 40 μl of 1.5 M NaCl–0.15 M sodium citrate were mixed, layered on 5% to 20% sucrose gradients in TBE–1.5 M NaCl–0.15 M sodium citrate, and centrifuged for 5 h at 33,000 rpm. Fractions (200 μl) were collected from the bottom. A 40-μl portion of each was counted to determine the peak of ^{3}H-labeled ColE1, which an analytical gel showed was over 95% in the 4.2-megadalton CC monomer form. Each fraction [50 μl from (A and B) or 100 μl from (C, D, and E)] was scored for transforming activity. The ordinates show transformants counted in 0.2-ml (A, B) or 0.5-ml (C, D. E) portions of plated culture. The positions indicated across the top are those expected for the various forms obtained by using the relations of Clowes (4).*

Because two forms might coelute either as expected (see Fig. 5 and reference 21, in which monomer OC and dimer CC comigrated) or by artifact, we used sedimentation velocity as a second criterion for identification of the form carrying the transforming activity. Portions of fractions 51, 68, and 152 were sedimented in sucrose, and the results (Fig. 3C through E) confirmed that the activities behaved as expected for monomer OC, dimer linear, and dimer OC forms. There was too little activity coeluting with monomer linear (fraction 41) to be analyzed by sedimentation, nor were the apparent peaks at fractions 80, 85, 95, and beyond 160 analyzed further.

Concentration response kinetics. The monomer OC DNA (fraction 51) showed two-hit kinetics, whereas the dimer OC (fraction 152) and linear (fraction 68) forms gave one-hit responses (Fig. 7). In each case, the chromosomal marker gave a linear response, indicating that the assays were not perturbed by inhibitors or other artifacts. These kinetics paralleled those seen for monomer and dimer CC forms (21).

Effect of S1 nuclease on separated plasmid forms. A cleared lysate of pMV158 was fractionated by preparative electrophoresis under nominally identical conditions to those in Fig. 4. Peaks of transforming activity centered at fractions 29, 54, and 158 were shown by velocity and buoyancy criteria to be due to monomer CC, dimer CC, and dimer OC, respectively (data not shown). Allowing for the fact that the dye front was centered about half a fraction behind that in Fig. 4, the agreement with the results in Fig. 4 and the predictions from Fig. 5 was excellent. (The run described in reference 21 used different conditions and was not strictly comparable for R_f values).

These three forms of pMV158 were each digested with S1 nuclease, which reduced the activities of both CC forms 35- to 40-fold but had only a small effect on the dimer OC and the chromosomal activities (Table 2).

A direct comparison of the activities of monomer CC, OC, and linear species at a single DNA concentration, done to eliminate the complication of the second-order kinetics, suggested results similar to those in Table 2, with the CC, OC, and linear forms giving approximately 930, 18, and 11 transformants per ml at donor con-

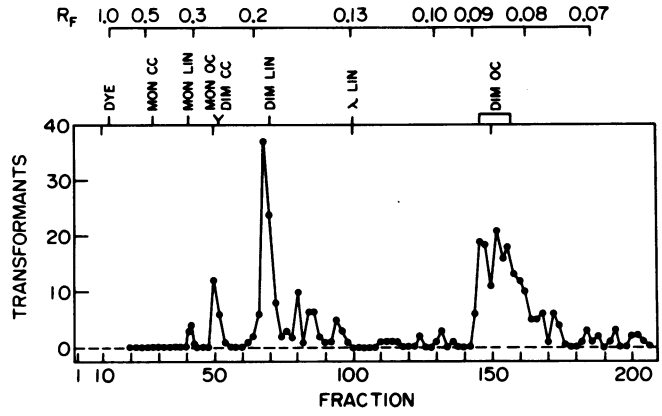

FIG. 4. *Plasmid transforming activity in fractions from preparative electrophoretic separation of the S1 nuclease-digested cleared lysate of DP3271. Samples (0.1 ml) of alternate fractions (12 ml) were assayed for transforming activity. The data plotted are transformants counted in 0.2-ml portions of plated culture, normalized for small variations in competence of the recipient cells. Below the R_f scale (R_f value = 13 ÷ fraction no.) are indicated the positions expected from the data shown in Fig. 5. MON, Monomer; LIN, linear; DIM, dimer.*

centrations near 1.0 ng/ml. However, the DNA concentrations were estimated indirectly (see above) and sufficiently uncertain to make these data only suggestive of the relative activities of the various monomer forms.

Effect of restriction nucleases. The product of S1 nuclease digestion is expected to include a mixture of full-length linear species cut at different places. Duncan et al. (6) found that when a phage DNA was digested with either of two restriction enzymes that cut once per genome, it could not transfect *Bacillus subtilis*, but that a mixture of the two digests was active. We asked whether pMV158 would show a similar result. pMV158 has a single recognition site for *Hin*dIII (V. Burdett, personal communication) and another one for *Bgl*I, well separated from the *Hin*dIII site (Fig. 8). After digestion of a cleared lysate with one or both of these enzymes, only a small fraction (~0.1%) of the initial activity remained, perhaps due to incomplete digestion (Table 3). However, when the separate digests were heated to inactivate the enzymes and then mixed, they gave substantial plasmid transformation.

Plasmids in transformants. Analytical gel electrophoresis patterns showed no obvious differences in the proportions of monomer and dimer species or in the size of the monomers among the donor strain DP3271 and transformants arising from monomer CC, dimer CC, or the mixed restriction digests (data not shown).

Proportion of dimers to monomers. By running higher amounts of small-scale cleared lysates at 3 V/cm in 0.5% agarose, we could resolve dimer CC from monomer OC and estimate its quantity from densitometer tracings of negatives of the gel photographs. Several such analyses (data not shown) gave values of 4 to 5% for the proportion of dimer to monomer forms. This estimate is consistent with earlier runs, in which it was often difficult to detect any dimer band. In the small-scale lysates, about 10% of the monomer was in OC form, whereas about half the monomer was OC (Fig. 1) in large-scale lysates. This suggests that even less of the dimer, perhaps a fourth, was in CC form in the large-scale lysates.

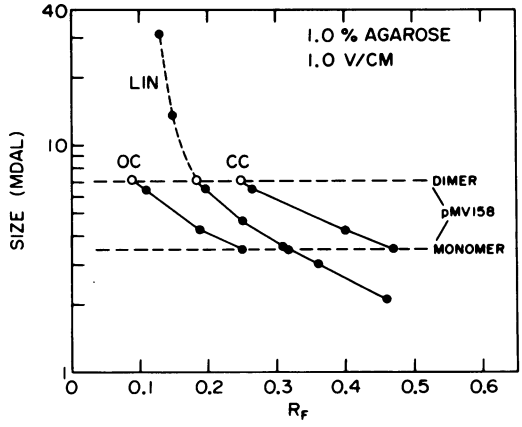

FIG. 5. *Mobilities of various DNA forms relative to bromocresol green in 1.0% agarose at 1.0 V/cm. Shown are mobilities observed (●) by EtBr staining of analytical gels for CC and OC pMV158, ColE1, and PM2; linear lambda and its EcoRI fragments; and linear pMV158 and PM2. Mobilities expected (○) for dimer pMV158 by extrapolation are also shown.*

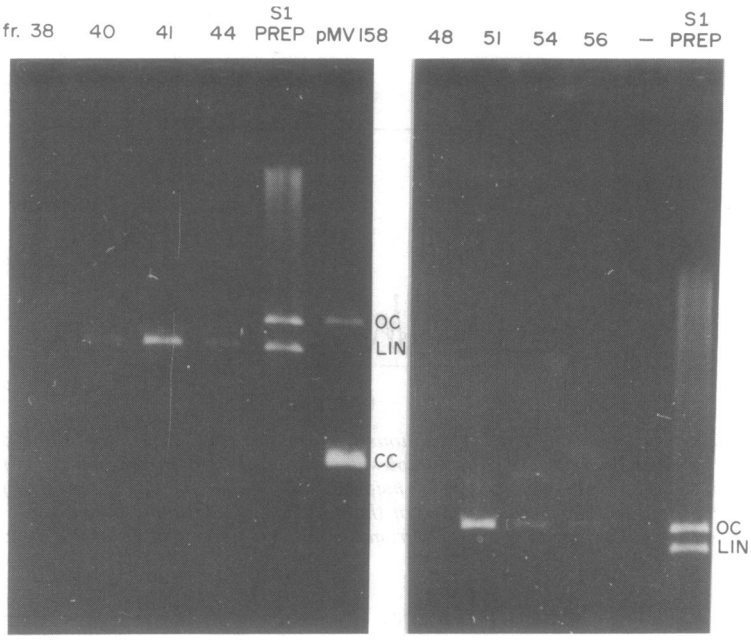

FIG. 6. *Detection of linear and OC monomers in fractions from the preparative gel separation shown in Fig. 4. Analytical gels were run at 1.0 V/cm in 0.5% agarose. fr., Fraction; LIN, linear; S1 PREP, S1 nuclease-treated preparation.*

DISCUSSION

In pneumococcus (20, 21; this paper) and *B. subtilis* (3, 10, 17), plasmid transformants arise from a number of DNA forms, of which some are much more active than others and can be detected readily only by their transforming activity. To identify which forms have activity, we used gel electrophoresis to separate DNA species that comigrated in sucrose velocity gradients, and vice versa. Coupled with dye-buoyancy analyses, these procedures gave strong evidence as to which plasmid species contributed a particular transformation event, even if it comigrated with another species in any single procedure.

The results showed that non-CC forms of pMV158 could transform pneumococcus and that the proportions of plasmid transforming activity found in the CC and non-CC regions of dye-buoyancy gradients depended on the history of the preparation. For this species, therefore, finding transforming activity only in non-CC forms cannot be taken as proof that a phenotype is not plasmid-associated; other criteria, as have been used to characterize resistance elements in the chromosomes of clinical isolates (23), are needed.

Monomer OC, dimer linear, and dimer OC forms of pMV158 were shown to be active by the criterion that the activity eluting in a given fraction sedimented with the velocity expected for the form expected to elute at that R_f value. The possibility that any of these activities were due to the trace of surviving CC DNA in the

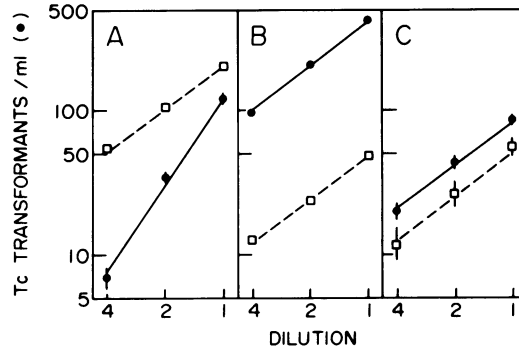

FIG. 7. *Dose-response curves for selected fractions from Fig. 4. (A) Fraction 51, monomer OC; (B) fraction 68, dimer linear; (C) fraction 152, dimer OC. Each point represents 5 ml of cells transformed with 0.5 ml of the indicated DNA and assayed for pMV158 and chromosomal ery-2 transformants by plating appropriate volumes. Statistical errors were small except where indicated by the error bars (± 1 standard deviation of the colony counts). The results are expressed as pMV158 transformants per ml (●) in each panel. For ery-2 (□), the results are expressed as transformants per 0.1 ml (A), 0.01 ml (B), or 1.0 ml (C) of culture.*

TABLE 2. *Effect of S1 nuclease on separated forms of pMV158*

DNA form[a]	No. of transformants/ml after the following incubation conditions:[b]			Survival (%)
	NaCl	Buffer	Buffer + S1	
CC monomer	4,500	5,020	132	2.6
CC dimer	17,200	16,200	460	2.8
OC dimer	705	930	670	72
Chromosomal	575	540	400	74

[a] The indicated forms were identified in fractions 29, 54, and 158 of a preparative gel separation as described in the text. Chromosomal *ery-2* was assayed in fraction 158.

[b] The DNAs were incubated as indicated in 120 mM NaCl (NaCL), in S1 buffer (Buffer), or in S1 buffer plus S1 nuclease (Buffer + S1) and then assayed for transforming activity.

preparation could be eliminated because (i) no activity eluted from the gel where monomer CC was expected (Fig. 4) and (ii) dimer or higher CC forms would have sedimented much more rapidly than observed (Fig. 3). In the preparative fractionation used for the results of Table 2, the activity eluting near fraction 158 was shown to band as non-CC DNA in a dye-buoyancy gradient and to have the velocity expected for dimer OC (data not shown), in agreement with the results in Fig. 3E.

Too little activity eluted at fraction 41 to use either sedimentation velocity or kinetics to confirm that it was due to the monomer linear species. However, it was the first to elute and showed a well-resolved peak that coincided with the position of observed monomers to ±0.025 in R_f value; no other pMV158 species was expected to elute at this position, and the relative activity in this fraction was comparable to that seen in a mixture of linear DNAs created by two restriction enzymes (Table 3).

A small part of the total activity in cleared lysates sedimented more rapidly than expected for dimers (Fig. 3A) (20). This might correspond to the small peaks of activity at fractions 80, 85, and 95 whose R_f values were in the range expected for trimers, tetramers, etc. (compare with Fig. 5). In *B. subtilis*, such multimers are present in readily detected amounts, have specific activities much greater than that of dimers, and appear to contribute the great majority of transforming activity in that system (3, 17). If such multimers also have high specific activity in pneumococcus, their small contribution to the total profile suggests that their occurrence is rare.

Relative contributions of various plasmid forms. Comparison of the activities of dimers and monomers is complicated by the difference in kinetics and will be discussed below. Within a size class, CC DNA was much more active than OC and linear forms: (i) only a trace of activity sedimented with monomer OC in Fig. 3A, although it represented about half of the DNA in the gradient (Fig. 1); (ii) S1 nuclease digestion reduced transformation by purified monomer or dimer CC to 2.6 to 2.8% (Table 2); and (iii) this result was consistent with a direct comparison of separated CC, OC, and linear forms in which the latter two showed about 2 and 1% activity, respectively, relative to CC.

The observation that S1 nuclease treatment reduced total transformation only seven- to eightfold was not in conflict with these results, because 10% of the transformants were due to non-CC DNA before treatment (Table 1). Preparative gel fractionation of a comparable preparation (used in Table 2) showed 10% of total transformants associated with the dimer OC

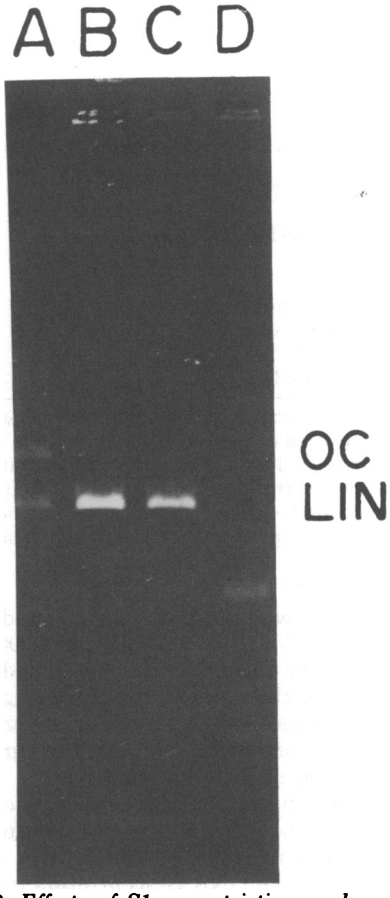

FIG. 8. *Effects of S1 or restriction nucleases on cleared lysates of pMV158 with S1 (A), HindIII (B), BglI (C), and HindIII and BglI (D).*

TABLE 3. *Effect of restriction nucleases on plasmid transformation*[a]

Treatment	No. of transformants/ml	
	pMV158	Chromosomal
None	19,700	3,350
*Hin*dIII buffer	22,900	3,100
*Bgl*I buffer	18,600	5,450
*Hin*dIII, *Bgl*I buffer	18,600	4,590
*Hin*dIII	5	2,700
*Bgl*I	20	2,300
*Hin*dIII + *Bgl*I	5	860
*Hin*dIII + heat	5	1,600
*Bgl*I + heat	20	2,660
Mixed heated digests	500	3,420

[a] DNA was incubated in the buffers with or without the enzymes, as indicated. Where indicated, the samples were heated after digestion for 50 min at 65°C to inactivate the enzymes before mixing the digests. A 10-μl portion of each sample was used as donor with 1 ml of cells, except that in the mixture, 10 μl of each digest was used.

peak, which was only slightly affected by S1 nuclease. In Table 1, loss of over 4,000 transformants from the CC region led to addition of 132 (3.2%) in the non-CC peak. Although these data are subject to the problems of concentration dependence discussed next, the agreement with expectation is satisfactory.

The difference in kinetics between monomers and dimers means that the proportion of the total transformants due to each in a mixture depended on the DNA concentration in the assay tube (for an example, see Fig. 5 of reference 20). Therefore, the shape of the profiles in Fig. 3A and 3B, the relative peak heights in Fig. 4, and quantitative interpretations such as those discussed above for Table 1 were all dependent on the concentrations used for assay. Further, effects of competing DNA were much greater on monomers than on dimers (data not shown), and the contribution due to monomers was not detected in crude lysates (20). Thus, in many situations the dimer forms contributed essentially all of the transformants even though they represented only a few percent of the plasmid DNA.

Absolute efficiencies. The following considerations, although only approximate, show that transformation was rare even when the cell had taken up the minimum number of donors indicated by the kinetics. The results suggest, first, that in a typical small-scale cleared lysate, almost all of the transformants, N, came from CC forms and that $N_{cc} \simeq a_{12}n_1^2 + a_{21}n_2$, in which the a values are coefficients and n_1 and n_2 are numbers of monomer and dimer CC molecules per milliliter. Second, at 1.0 ng of total pMV158 DNA per ml, the monomer CC ($\sim 1.5 \times 10^8$/ml) gave $\sim 10^3$ transformants per ml; when the contribution of monomers could be subtracted from the total, the dimers present ($\sim 4 \times 10^6$ per ng of total DNA) gave 10^3 to 2×10^3 transformants per ng of total DNA (20; data not shown). These figures lead to an a_{12} value of $\sim 4 \times 10^{-14}$ as the coefficient for the two-hit monomer CC process and an a_{21} value of $\sim 3 \times 10^{-4}$ transformants per dimer CC in solution, for the conditions used with these batches of competent cells. For these conditions (DNA concentration limiting, 3×10^7–4×10^7 cells per ml, and 100% competent), our experience, consistent with that of Lacks (12), is that roughly 10% of the DNA in solution reacts with the cells. Therefore, the probability of success appeared to be less than 10^{-2} per dimer taken up. Similarly, the limitation on monomers was not simply that very few cells took up parts of two or more donor molecules. Again, for 10% uptake from a population of 1.5×10^8 monomers per ml, the mean was about 0.5 per cell, from which the Poisson distribution predicts that $\sim 3 \times 10^6$ cells received two plasmid strands. Since only 10^3 cells were transformed, the intracellular efficiency was less than 10^{-3}. Comparable figures result at higher DNA concentrations in which most cells received at least two strands. Finally, when DNA is in excess, it may be shown that the average cell takes in at least 10 donor strands, yet transformation by a cleared lysate of pMV158 approaches saturation at a level at which less than than 1% of the cells are transformed (20). Although not precise, these numbers show clearly that the intracellular processes are very inefficient for establishing new replicons in the recipient cell, whereas they are efficient for adding DNA to the chromosome or to homologous phage (8) or plasmid (10) replicons already present.

Model. Figure 9 summarizes our interpretation of the pathway of plasmid transformation in pneumococcus, extending a previous model (21) to include linear and OC forms and modifying it slightly to take into account the low efficiency. Results on phage transfection have been interpreted similarly (19). The essential feature is that replicons are assembled by pairing of single-strand fragments that have entered the cell separately, either from two monomer particles or from parts of opposite strands of a single dimer particle. Repair synthesis and, if necessary, an intramolecular circularization event, are needed to create an intact replicon that is of monomer size.

Entry appears to use the normal pathway for donor DNA in pneumococcus (20). For linear duplexes, this involves nonspecific cutting on the cell surface at mean spacings that are greater than 7 kilobases when the donor is large, fol-

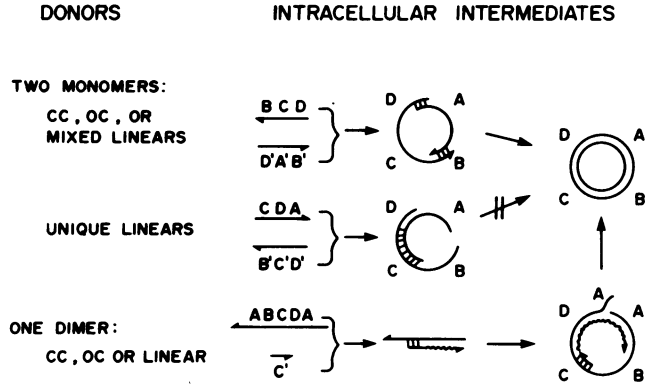

FIG. 9. *The pathway of plasmid transformation in pneumococcus.*

lowed by entry of one of the strands (11, 16). Closed circular duplexes are also cut on the cell surface (12). Preexisting cuts in OC and linear forms should force the average entering strand to be shorter than from a CC donor, leading to the expectation that activity would be reduced substantially but not to zero, as was observed. However, since half of the strands in singly nicked OC forms are intact, one might expect the average entering strands from OC donors to be at least one half as efficient as those from CC donors. The fact that they are not raises the possibility that a nick on one strand inhibits the entry of its complement. Alternatively, the great majority of the OC forms may have had nicks on both strands, either because of the S1 nuclease treatment itself or the buffer. However, the buffer alone did not reduce transformation (Table 2), and the mechanism of action of S1 nuclease on CC DNA (7, 13, 24) makes the occurrence of second nicks at sites removed from the first ones unlikely.

The model readily explains the lack of activity in a unique linear species produced by a restriction enzyme. No circularization is likely, although rare pairings of the 3 or 4 bases at the restriction site cannot be excluded at this time. Mixing two different restriction digests restored activity, a result comparable to that reported for phage DNA transfection (6).

The modification of the prior model is that we now suggest that the extent of overlap for pairing of donor strands is minimal rather than extensive. Although we would expect that entering strands from monomer CC donors (5.2 kilobases) would often be nearly full length, the low efficiency of transformation suggests either that they are cut more often on the cell surface than are linear donors, or that they are degraded extensively after entry while awaiting the entry of a suitable complement with which to react. Intracellular inactivation has been described for chromosomal DNA when conditions for integration into the chromosome are not optimal (5, 22) and for rescue of markers on phage DNA when entry of the helper phage is delayed (8). Such inactivation is greater when the donor strands are shorter (5), suggesting that the degradation may be exonucleolytic. If this is a major cause of the low efficiency of plasmid transformation, one expects that the rare successes involve minimal overlaps. Quantitative differences in the degree of such degradation might account for the failure of monomer plasmids to transform naturally competent *B. subtilis* (3, 17), particularly since the minimum size of chromosomal DNA that transforms *B. subtilis* (14) is larger than in pneumococcus (15).

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LITERATURE CITED

1. **Bauer, W., and J. Vinograd.** 1968. The interaction of closed circular DNA with intercalative dyes. I. The superhelical density of SV40 DNA in the presence and absence of dye. J. Mol. Biol. **33**:141-171.
2. **Burdett, V.** 1980. Identification of tetracycline resistant R-plasmids in *Streptococcus agalactiae* (group B). Antimicrob. Agents Chemother. **18**:753-760.
3. **Canosi, U., G. Morelli, and T. A. Trautner.** 1978. The relationship between molecular structure and transformation efficiency of some *S. aureus* plasmids isolated from *B. subtilis*. Mol. Gen. Genet. **166**:259-267.
4. **Clowes, R. C.** 1972. Molecular structure of bacterial plasmids. Bacteriol. Rev. **36**:361-405.
5. **Collins, C. J., and W. R. Guild.** 1972. Events occurring near the time of synapsis during transformation in *Diplococcus pneumoniae*. J. Bacteriol. **109**:266-275.
6. **Duncan, C. H., G. A. Wilson, and F. E. Young.** 1978. Biochemical and genetic properties of site-specific re-

striction endonucleases in *Bacillus globigii*. J. Bacteriol. **134**:338–344.
7. **Germond, J., V. Vogt, and B. Hirt.** 1974. Characterization of the single-strand-specific nuclease S1 activity on double-stranded supercoiled polyoma DNA. Eur. J. Biochem. **43**:591–600.
8. **Green, D. M.** 1966. Intracellular inactivation of infective SP82 bacteriophage DNA. J. Mol. Biol. **22**:1–13.
9. **Guild, W. R. and N. B. Shoemaker.** 1976. Mismatch correction in pneumococcal transformation: donor length and *hex*-dependent marker efficiency. J. Bacteriol. **125**:125–135.
10. **Gryczan, T., S. Contente, and D. Dubnau.** 1980. Molecular cloning of heterologous chromosomal DNA by recombination between plasmid vector and a homologous resident plasmid in *Bacillus subtilis*. Mol. Gen. Genet. **177**:459–467.
11. **Lacks, S.** 1977. Binding and entry of DNA in bacterial transformation, p. 179. *In* J. Reissig (ed.), Microbial interactions. Chapman and Hall, London.
12. **Lacks, S.** 1979. Uptake of circular deoxyribonucleic acid and mechanism of deoxyribonucleic acid transport in genetic transformation of *Streptococcus pneumoniae*. J. Bacteriol. **138**:404–409.
13. **Lilley, D.** 1980. The inverted repeat as a recognizable structural feature in supercoiled DNA molecules. Proc. Natl. Acad. Sci. U.S.A. **77**:6568–6472.
14. **Morrison, D. A., and W. R. Guild.** 1972. Activity of deoxyribonucleic acid fragments of defined size in *Bacillus subtilis* transformation. J. Bacteriol. **112**:220–223.
15. **Morrison, D. A., and W. R. Guild.** 1972. Tranformation and deoxyribonucleic acid size: extent of degradation on entry varies with size of donor. J. Bacteriol. **112**:1157–1168.
16. **Morrison, D. A., and W. R. Guild.** 1973. Breakage prior to entry of donor DNA in pneumococcus transformation. Biochim. Biophys. Acta **299**:545–556.
17. **Mottes, M., G. Grandi, V. Sgaramella, U. Canosi, G. Morelli, and T. A. Trautner.** 1979. Different specific activities of the monomeric and oligomeric forms of plasmid DNA in transformation of *B. subtilis* and *E. coli*. Mol. Gen. Genet. **174**:281–286.
18. **Polsky, F., M. H. Edgell, J. G. Seidman, and P. Leder.** 1978. High capacity gel preparative electrophoresis for purification of fragments of genomic DNA. Anal. Biochem. **87**:397–410.
19. **Porter, R. D., and W. R. Guild.** 1978. Transfection in pneumococcus: single-stranded intermediates in the formation of infective centers. J. Virol. **25**:60–72.
20. **Saunders C. W., and W. R. Guild.** 1980. Properties and transforming activities of two plasmids in *Streptococcus pneumoniae*. Mol. Gen. Genet. **180**:573–578.
21. **Saunders, C. W., and W. R. Guild.** 1980. Monomer plasmid DNA transforms *Streptococcus pneumoniae*. Mol. Gen. Genet **181**:57–62.
22. **Shoemaker, N. B., and W. R. Guild.** 1972. Kinetics of integration of transforming DNA in pneumococcus. Proc. Natl. Acad. Sci. U.S.A. **69**:3331–3335.
23. **Shoemaker, N. B., M. D. Smith, and W. R. Guild.** 1979. Organization and transfer of heterologous chromosomal and tetracycline resistance genes in pneumococcus. J. Bacteriol. **139**:432–441.
24. **Shishido, K.** 1980. Relationship between S1 endonuclease-sensitivity and number of superhelical turns in a negatively-twisted DNA. FEBS Lett. **111**:333–336.
25. **Smith, M. D., N. B. Shoemaker, V. Burdett, and W. R. Guild.** 1980. Transfer of plasmids by conjugation in *Streptococcus pneumoniae*. Plasmid **3**:70–79.