Molec. gen. Genet. 166, 259–267 (1978)

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The Relationship Between Molecular Structure and Transformation Efficiency of Some *S. aureus* Plasmids Isolated from *B. subtilis*

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Summary. DNA preparations of the chloramphenicol resistance determining *S. aureus* plasmids pC194, pC223, and PUB112 can be fractionated by gel electrophoresis into various bands. Electronmicroscopic investigations of these various molecular species obtained with pC194 indicated that, depending on the preparations, 70 to 80% of the molecules were monomers, while the rest consisted of various classes of concatemeric and/or interlocked multimers. Measurements of the specific transforming activity of the various molecular classes indicated that the monomers had less than one thousandth the activity of the multimeric plasmid DNA. pC194 DNA of high specific transforming activity could also be obtained by ligation of *Hin*dIII generated monomers into concatemeric DNA.

In recent years it has been shown that the 168 derivatives of *B. subtilis* can be transformed with plasmids (Ehrlich, 1977; Gryczan and Dubnau, 1978). Such plasmids, either derived from plasmid carrying strains of *B. subtilis* (Bernhard et al., 1978) or from *S. aureus* (Gryczan et al., 1978; Löfdahl et al., 1978), persist in the transformed cells and replicate. Some staphylococcal plasmids, which carry genes for antibiotic resistance convey antibiotic resistance also to the transformed *B. subtilis* recipients (Ehrlich, 1977) and thereby allow the selection of plasmid transformed recipients. Beside the interest in developing *B. subtilis* borne plasmids as cloning vehicles, such plasmids represent an ideal material for the study of the mechanics of DNA uptake in transformation. Plasmids, carrying selectable markers share with transfecting phage DNA the properties of being small replicons of defined molecular structure and of not interacting with the recipient genome. In common with transforming DNA they introduce a permanent and readily detectable genetic change of the transformed cell without destroying its viability. It is this interest in the mechanism of uptake of DNA in transformation which has motivated us to study plasmid transformation. In this communication we analyze the molecular requirements for plasmid transformation.

Materials and Methods[1]

Bacteria. The strains used in this study and the plasmids they contain are described in Table 1. The plasmid carrying *B. subtilis* strains were kindly provided by S.D. Ehrlich.

Media. HS and LS media (Rottländer and Trautner, 1970) were used for the growth of cells to competence. To obtain plasmid DNA, plasmid carrying cells were grown in TY medium (Biswal et al., 1967) supplemented with the antibiotic to which such cells

Table 1. List of *B. subtilis* strains and plasmids

Strain	Chromosomal markers	Plasmid	Phenotype	Reference
222	*trp arg*	–	Cm^S	Schlaeger and Spatz (1974)
SB 634	*thy aroB tyr*	pC194	Cm^R	Ehrlich (1977)
SB 634	*thy aroB tyr*	pC223	Cm^R	Ehrlich (1977)
SB 634	*thy aroB tyr*	pUB112	Cm^R	Ehrlich (1977)

Symbols: *trp, arg, thy, aroB, tyr* indicate respectively requirement for tryptophan, arginine, thymine shikimic acid and tyrosine. Cm^R, resistance, Cm^S sensitivity to chloramphenicol

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[1] *Abbreviations used:* CCC = supercoiled covalently closed circle; CM = chloramphenicol; d = dimer; DTT = dithiothreitol; EtBr = ethidium bromide; m = monomer; OC = relaxed open circle; tet = tetramer; tr = trimer

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were resistant. Plates with nutrient agar (Difco) supplemented with 5 μg/ml of CM were used to select for Cm^R transformants.

Transformation. Plasmid DNA was prepared as described below. Competent cells were exposed to DNA for 30 min at 37° in the absence of aeration. DNA uptake was terminated by the addition of pancreatic DNAse. After exposure to DNA, cells were plated on selective plates without prior incubation.

Preparation of Plasmid DNA. Cells of plasmid carrying strains were grown at 37° to late log phase in 1 l of TY supplemented with 50 μg/ml CM. The cells were sedimented by centrifugation, washed several times with TES buffer (Tris-HCl, 0.05 M, NaCl 0.05 M, EDTA 0.005 M, pH 7.5) and resuspended in 50 ml of TES. Lysozyme (Serva) 0.5 mg/ml and pancreatic RNAse A (Sigma), DNAse free, 0.1 mg/ml were added. The cells were lysed after 30 min at 37°. 50 ml TES, 10 ml of 8% N-Laurylsarkosin-Na-salt (Serva) and 0.3 mg/ml of pronase P (Serva) predigested at 37° for 1 h were added. Incubation at 37° continued for 1 h. The lysate was centrifuged for 20 min at 17.000 g. EtBr (Sigma) was added to the supernatant at a final concentration of 300 μg/ml. 6.9 g of CsCl were added to 8 ml of the EtBr containing lysate. This solution was centrifuged for 16–20 h at 45.000 rpm at 15° in a Spinco V50 Ti rotor. Two bands of DNA were detectable. The band which predominantly contained plasmid DNA was collected and subjected to a second isopycnic centrifugation (40 h, 40.000 rpm, 15°) in a 50 Ti rotor. Plasmid bands were collected and freed from EtBr by extraction with CsCl saturated isopropanol. This DNA/CsCl solution was then diluted 1/5 with 5 mM Tris-HCl pH 7.5 and brought to 0.3 M sodium acetate. Two volumes of cold isopropanol were added to precipitate the DNA. The tubes were then quickly frozen in a ice/methanol mixture and thawed in ice water. The DNA was sedimented by centrifugation for 1 h at −5° at 27.000 g and dissolved in 5 mM Tris-HCl, pH 7.5. [^{3}H]-labelled plasmid DNA was prepared by the same method except that the TY medium used for growth contained 3 μCi/ml of [5,6-^{3}H]-Uridine (Amersham). Such DNA had a specific activity of 10.000 cpm/μg.

Restriction Endonuclease Digestion and Ligation. Digestion of plasmid DNA with *Hin*dIII (Boehringer) followed the procedure described by the manufacturer. Ligation of DNA was performed with T4 ligase (Miles). 30 μg of *Hin*dIII digested DNA were diluted in 120 μl 50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 0.2 mM ATP, pH 7.5 and 2 U of enzyme were added and the mixture was incubated for 4 h at 30° or 15 h at 4°. The reaction was stopped by 5 min heating at 68°.

Agarose Gel Electrophoresis. Plasmid DNA molecules of different tertiary structure and size were resolved on vertical 0.8% agarose (Bio-Rad) gels. Analytical gels were run on slabs 13 × 15 × 0.4 cm, preparative gels were 22 × 14 × 0.5 cm. Tris-acetate buffer (0.04 M Tris-HCl, 0.001 M EDTA, 0.005 M Na-acetate, pH 7.9) was used throughout. gels were run with 0.2% bromphenol blue as tracking dye at 3–4 V/cm at room temperature for 4 to 5 h and stained with EtBr (1 μg/ml final concentration). Fluorescence of DNA bands was visualized with a mineralight short-wave UV lamp and photographed with Polaroid type 665 positive/negative land films. For molecular weight determination, an *Eco*RI digest of SPP1 DNA was added to provide markers.

Extraction of DNA from Agarose Gels. DNA was reisolated from agarose gels by the freeze-squeeze method described by Thuring et al. (1975). The extracted DNA was brought to a final concentration of 0.3 M sodium acetate. DNA was precipitated by the addition of two volumes of cold ethanol. After at least 5 h at −20°,

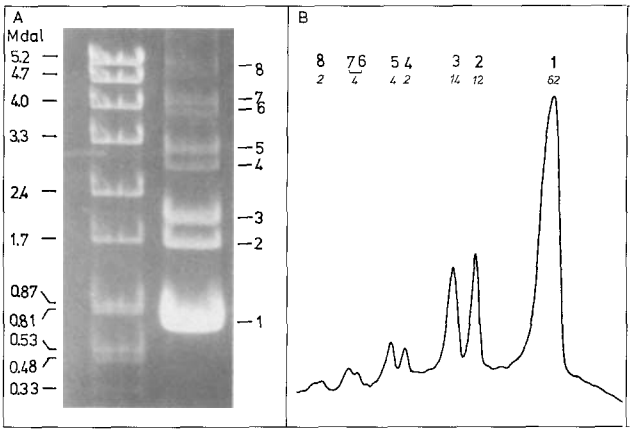
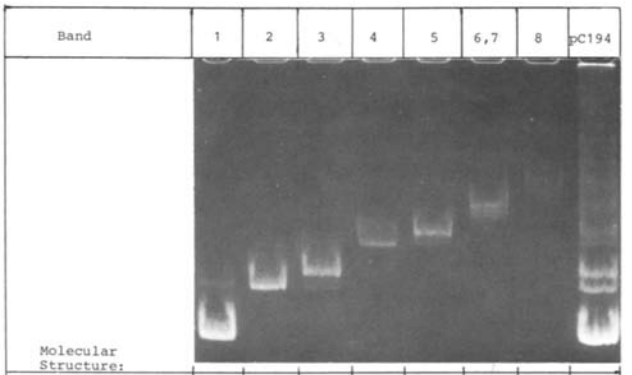


Fig. 1A and B. Gel electrophoretic analysis of pC194 DNA. **A** Agarose gel pattern of the plasmid (right track) compared to that of an *Eco*RI digest of SPP1 DNA (left track); Molecular weights of *Eco*RI SPP1 fragments were taken from Ganesan et al. (1976). **B** Scan of the pC194 gel. Number in italics indicate the percentage of DNA found in each band

Table 2. Fractionation and composition of pC194 DNA bands. The left column describes the species of molecules which could be detected in the various purified DNA bands shown in the agarose gel on top. "+" indicates interlocking of components. The number of such molecules within each band is given in the columns below the corresponding tracks



Molecular Structure:	1	2	3	4	5	6,7	8
linear m	1	2					
OC m	103	89	14	8	3	6	11
linear d						3	
OC d			27	17	1	34	
m + m			77	89	6	8	1
linear tr						2	2
OC tr					3		
d + m					30	19	7
m + m + m					21	16	4
linear tet						1	1
d + d						3	1
d + m + m						8	14
tr + m						6	
m + m + m + m							6
d + d + m							4
d + m + m + m							1
tr + m + m							2

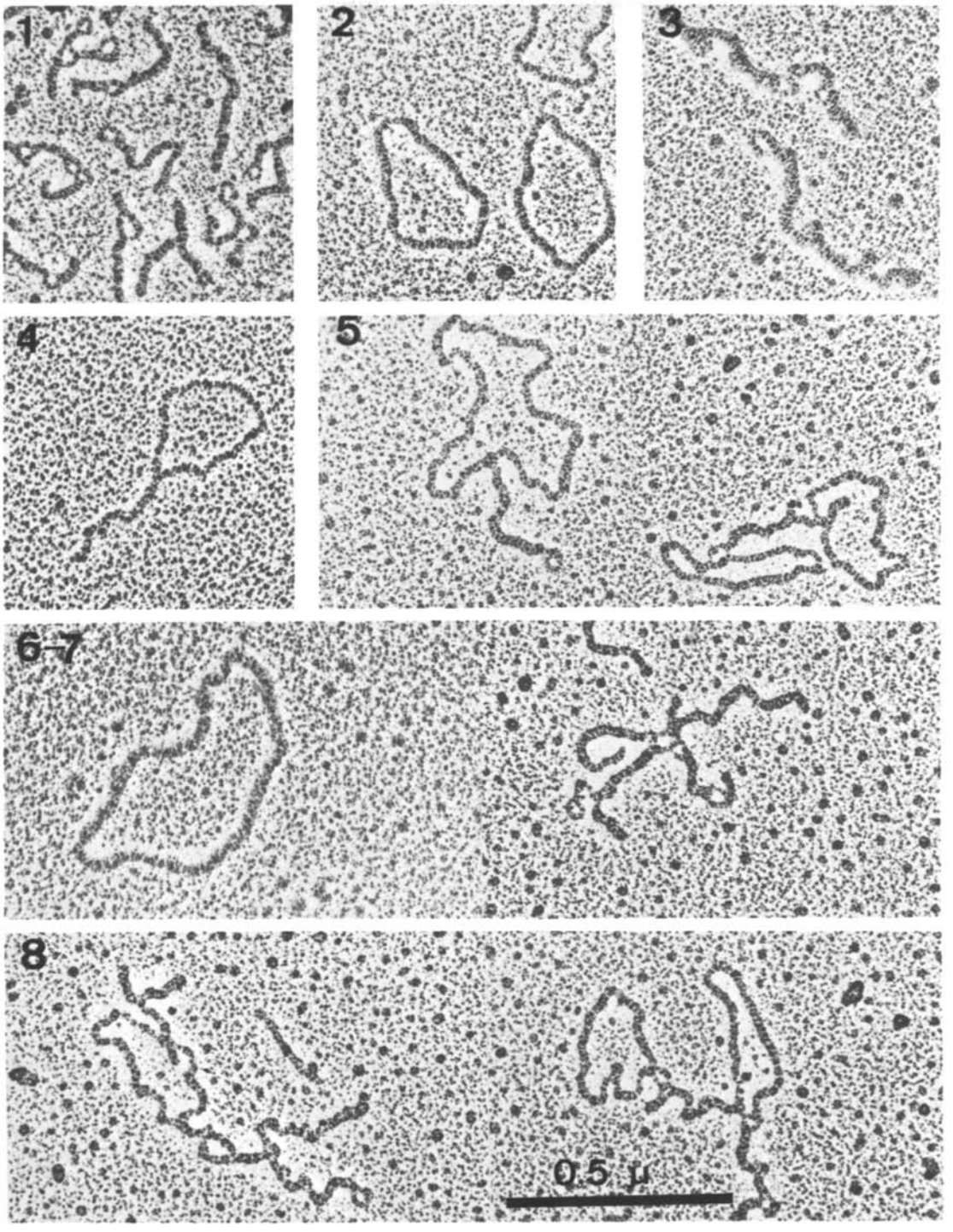


Fig. 2. Electronmicrographs of representative molecules from pC194 bands 1 through 8

the DNA was collected by centrifugation (1 h, 27.000 g, −5°) and dissolved in 1/20 volume of 5 mM Tris-HCl, pH 7.5.

Sucrose Gradients. Neutral linear sucrose gradients from 20% to 5% in TES buffer (total volume: 5 ml) were prepared in Spinco SW50 tubes. About 0.1 ml of DNA solution were layered on top of such gradients. The tubes were run for the times indicated in a Spinco SW50-1 rotor at 44.000 rpm at 20°. Fractions were collected from the bottom.

Electron Microscopy. DNA was spread according to two procedures: 1) in 0.5 M ammonium acetate at neutral pH following

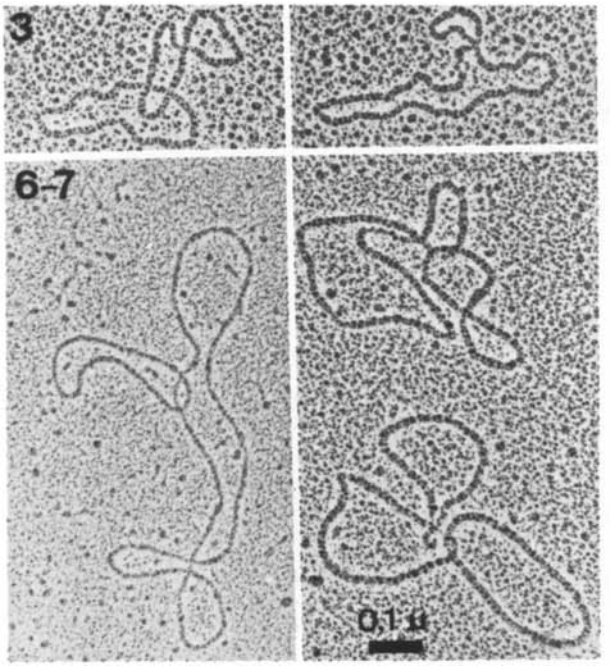


Fig. 3. Electronmicrographs of molecules from bands 3 and 6,7 after alkaline/formamide spreading

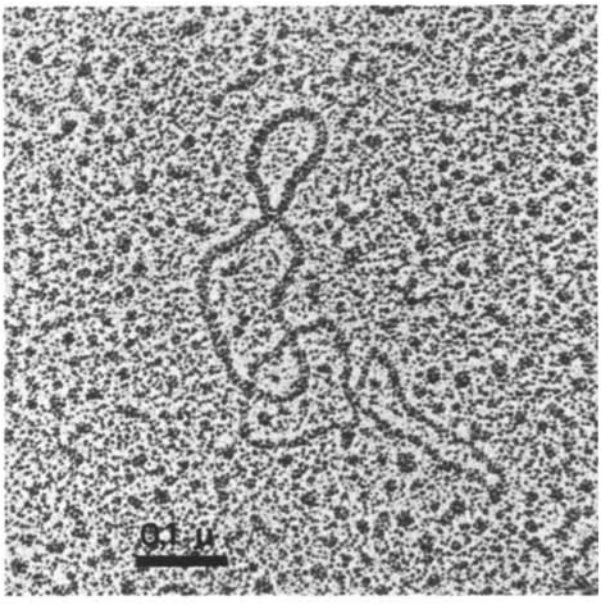


Fig. 4. Electronmicrographs of a mixed double/single stranded interlocked dimer (from band 4, alkaline/formamide spreading)

the method of Kleinschmidt and Zahn (1959); 2) in 10 mM Na_2CO_3, 2 mM EDTA, 50% (v/v) repurified formamide at pH 10 according to Morris et al. (1975). DNA was collected on parlodion coated grids, which were subsequently rotary shadowed with platinum-palladium. Electron micrographs were obtained using a Philips EM 301 electron microscope.

Results

Characterization of Plasmid DNA from B. subtilis

The DNA of several *S. aureus* plasmids, reisolated from *B. subtilis*, separates into several bands when analyzed in agarose gel electrophoresis (Gryczan and Dubnau, 1978). This is shown here again in Figure 1 A for the plasmid pC194. Similar banding patterns (data not shown) were obtained when pC194 was isolated from various rec$^-$-mutants of *B. subtilis* (*rec*H342, *rec*30, *rec*F, *rec*G, *rec*D, *rec*K [Mazza et al., 1975]). Several species of DNA were also observed after agarose gel electrophoresis of plasmid pC223 and pUB112 isolated from *B. subtilis* (data not shown). An evaluation of a scan of the gel obtained with pC194 (Fig. 1 B) indicated that under the conditions of preparation used 62% of the total plasmid DNA was located in the fastest moving band No. 1. Decreasing amounts of DNA are found in the resolvable slower moving bands 2 to 8.

In order to characterize the various kinds of DNA, we have extracted the DNA from bands 1 through

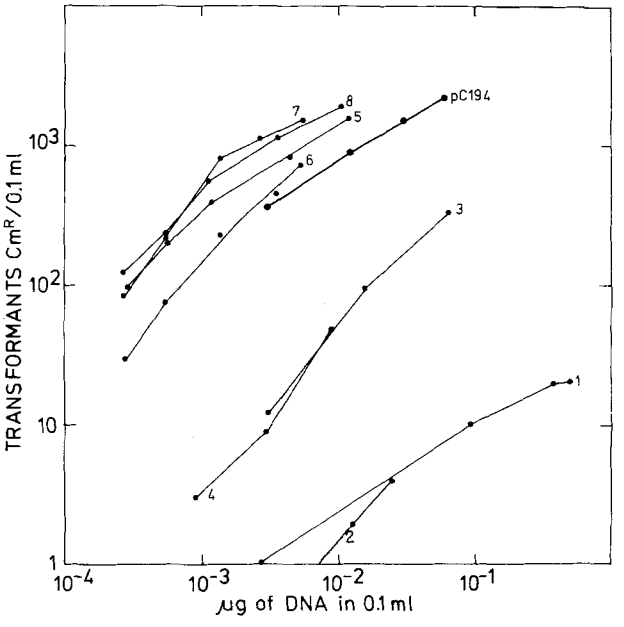
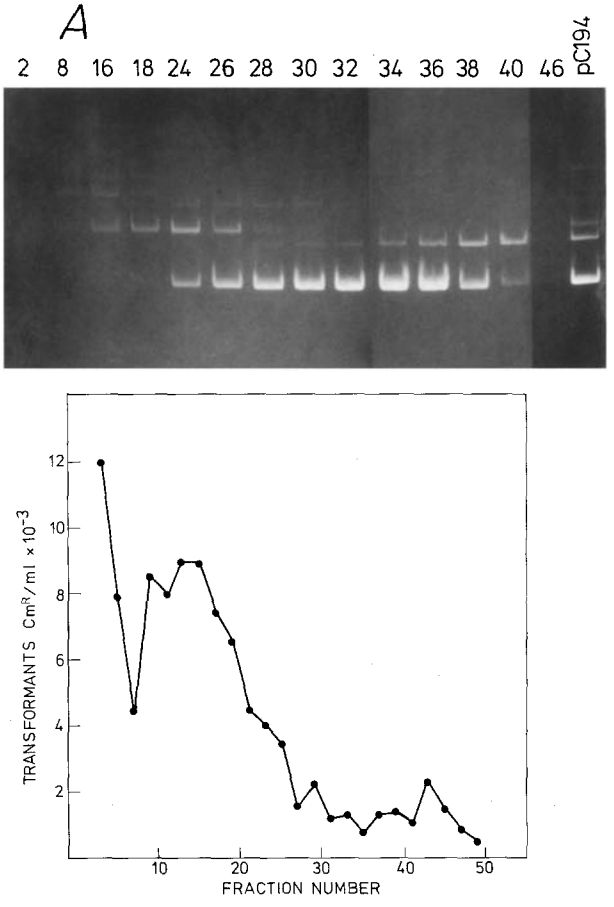
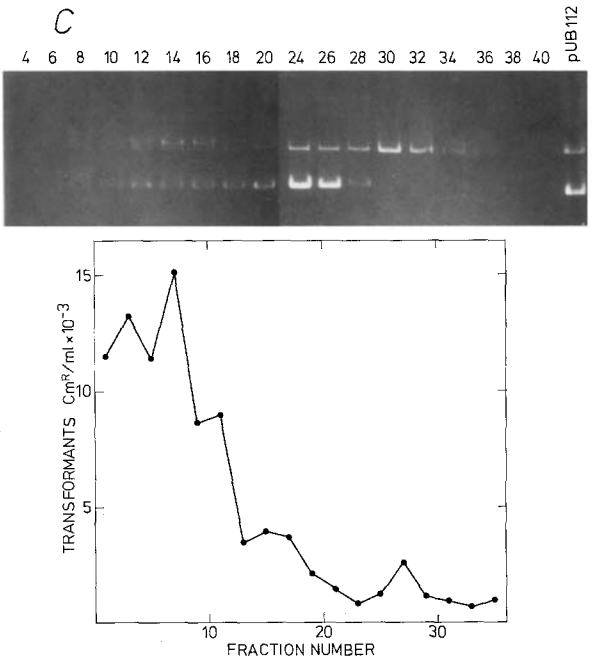


Fig. 5. Dose response in transformation with purified pC194 bands and with unfractionated plasmid DNA

8. The electrophoretic mobility of these bands is shown in Table 2. DNA isolated from purified bands was analyzed by electron microscopy. Representative electron micrographs of DNA from individual bands are shown in Figure 2. Band 1 corresponds to monomeric CCC DNA, band 2 to OC monomers, band 3 to dimeric CCC DNAs. Material in bands 4 and above





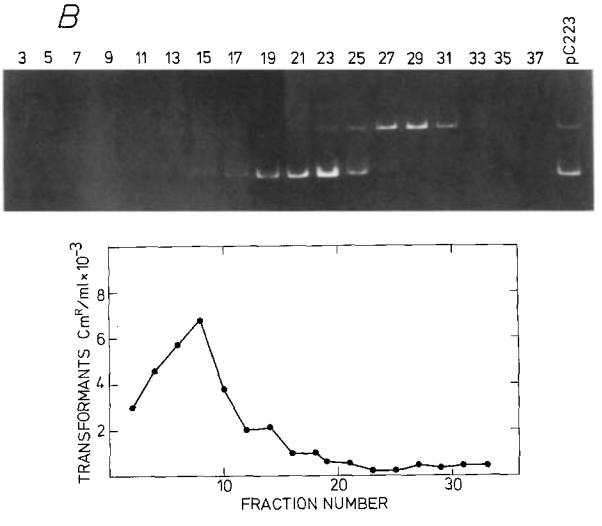


Fig. 6A–C. Sucrose gradient analysis of plasmid DNA. Alternating fractions were analyzed by gel electrophoresis to identify the DNA (top panel) or by transformation to determine biological activity (bottom panel). Agarose gel electrophoresis was run on 30 µl of each fraction. For transformation 20 µl of the fractions from A and C and 10 µl from B were added to 0.1 ml of competent 222 cells. Sedimentation was from right to left. Conditions in centrifugation: **A** 25 µg of DNA, pC194, 3 h; **B** 20 µg of pC223 DNA, 2.5 h; **C** 16 µg of pUB112 DNA, 2.5 h

represents composite molecules consisting of various combinations of monomers, dimers, and rarely higher polymers of DNA in relaxed and supercoiled forms. Essentially no linear monomers of DNA were observed.

A better resolution of composite DNA molecules was obtained when the DNA was analyzed under alkaline spreading conditions in the presence of formamide which would resolve supercoiled DNA. Table 2 shows the distribution seen in random electron microscopic fields of various species of combined double stranded molecules in the bands analyzed. As an example of molecular diversity, Figure 3 shows representative electron micrographs of various molecules

observed in bands 3 and 6,7. Catenated molecules as we observe here have been described for various plasmids and virus DNAs (Kupersztoch and Helinski, 1973; Novick et al., 1973; Meinke and Goldstein, 1971; Jaenisch et al., 1969) and have been interpreted as replication intermediates. Catenated *dimers* of staphylococcus plasmids have first been discovered by Novick et al. (1973) but the larger combinations, as described here after pC194 isolation from *B. subtilis* were not described.

In addition to the double stranded forms of DNA, we have observed under alkaline spreading conditions mixed multimers of double and single stranded circles (Fig. 4). Such molecules would arise from those composite molecules in which a circular molecule carried a single strand nick. Under alkaline spreading conditions the strand carrying such a nick would diffuse away from the original composite molecule, leaving the complementary closed circular strand behind. In agreement with this, we have also found in the DNA preparations monomeric and seldomly dimeric linear single stranded DNA. The preparative conditions, however, did not permit quantitative assessment of the frequency of these molecules.

Biological Activity of the Various Species of DNA

[^{3}H] labelled pC194 DNA was separated into its various bands by gel electrophoresis. The separated bands 1 through 8 were isolated, the DNA extracted and their transforming activity tested at several DNA concentrations (Fig. 5). Also included in Figure 5 is the dose response with unfractionated pC194 DNA. It is obvious that material isolated from bands 1 and 2, representing monomeric CCC or OC DNA molecules, have the lowest biological specific activity. The specific activity of DNA isolated from bands > 5 is about 1000 times that of monomeric DNA. The slope of the dose response curves of Figure 5 never exceeds the value of 1. This would indicate that transformation with plasmid DNA does not require cooperation between more than one transforming entity in contrast to what is found in transfection (Trautner and Spatz, 1973).

The significantly higher biological specific activity of multimeric DNA was also observed in a different type of experiment: Total DNA of plasmids pC194, pC223, and pUB112 was sedimented in sucrose gradients. Fractions were collected to determine their DNA composition by agarose gel electrophoresis and their transforming activity (Fig. 6). With all plasmids the peaks of biological activity sediment much more rapidly than the monomeric DNA. Very little DNA is detectable in those fractions of the gradient which show high transforming activity, indicating high specific activity of faster sedimenting plasmid DNA. To quantitate this observation, we have performed the same experiment described in Figure 6 with [^{3}H] labelled pC194 DNA of known specific activity. From the distribution of radioactivity and transforming activity we obtain a curve of specific biological activity (Fig. 7), which indicates a greater than 400 fold difference between the monomeric and multimeric molecules species.

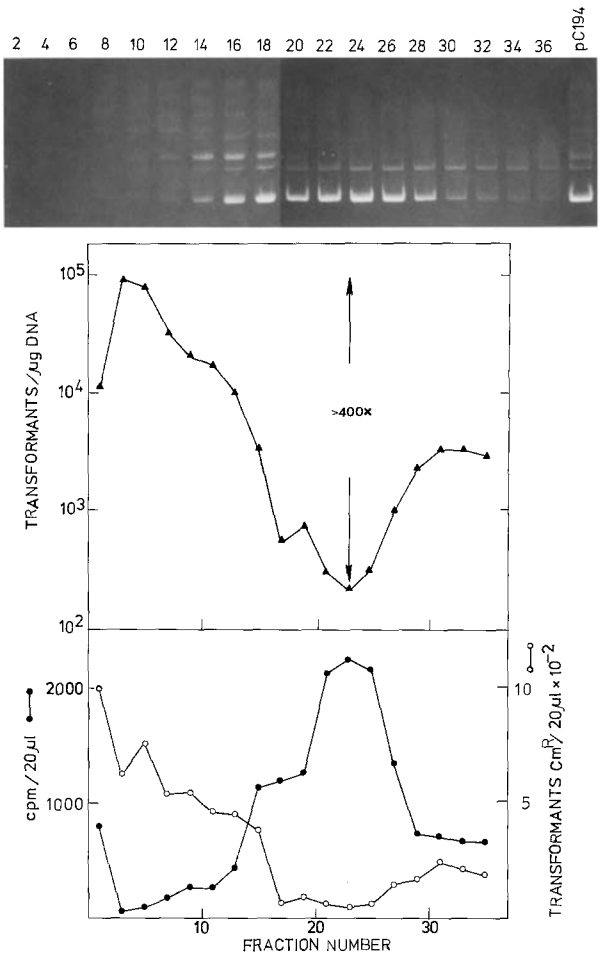


Fig. 7. Sucrose gradient analysis of ^{3}H-pC194 DNA. 18 µg of DNA (specific activity 10^4 cpm/ µg) were sedimented (3 h). Alternating fractions were used to either characterize DNA (30 µl) or to measure radioactivity (20 µl) and transforming activity (20 µl). The middle panel indicates the specific activity of DNA. The increase of specific activity at fractions greater than 23 was caused by an artifact in the collection of this gradient. No such increase is seen in the gradients of Figure 6

Biological Effects of Ligation of Monomeric Plasmid DNA

The species of natural molecules which show a high specific biological activity contain both concatenated and interlocked monomers in CCC and OC forms.

Table 3. Effect of *Hin*dIII Degradation and Ligation on the Transforming Activity of pC194 DNA

Treatment	Cm^R transformants/μg DNA
None	1.26×10^4
*Hin*dIII digestion	4.4
Ligation of *Hin*dIII digest	1.1×10^5

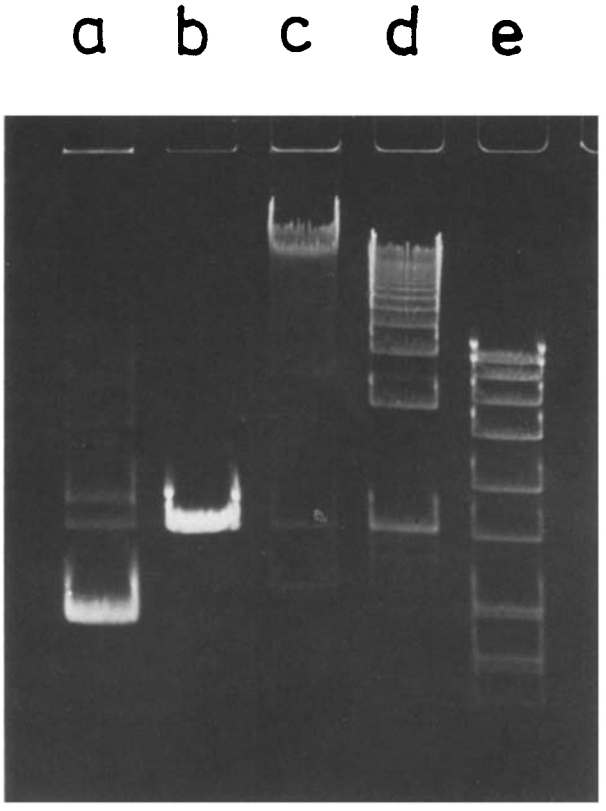


Fig. 8a–e. Agarose gel electrophoresis of pC194 DNA after *Hin*dIII digestion and ligation. **a** Untreated, **b** *Hin*dIII digested, **c** digested and ligated with T4 ligase for 16 h, 4°, **d** the same but ligated for 4 h at 30°, and **e** *Eco*RI degraded SPP1 DNA (see Fig. 1 A)

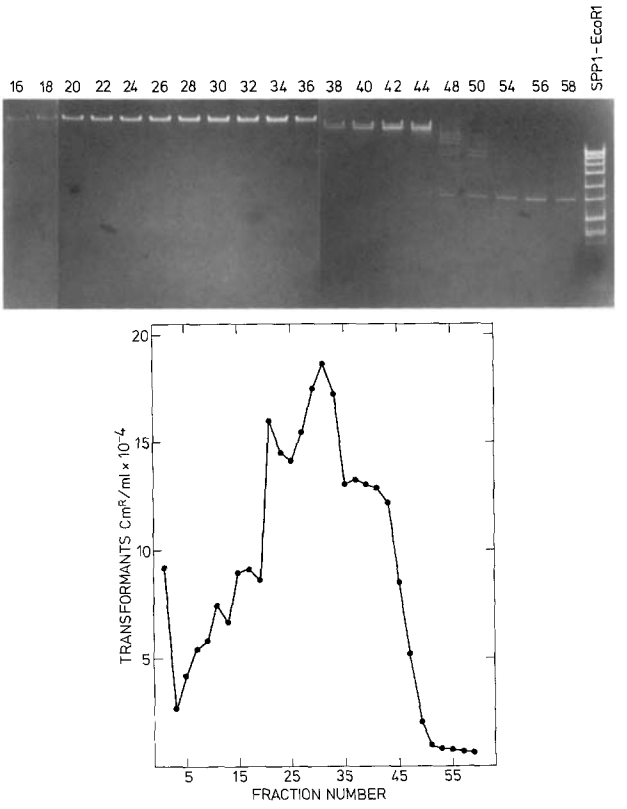


Fig. 9. Sucrose gradient analysis of pC194 DNA after *Hin*dIII digestion and ligation (4 h, 30°). Conditions of gradient: 25 μg of DNA, 1.5 h. 20 μl of alternating fractions were used to identify DNA by gel electrophoresis and to determine biological activity

It was of interest to determine whether concatenation of molecules alone would suffice to construct molecules of high specific activity. We have therefore digested pC194 DNA with *Hin*dIII restriction endonuclease to linear monomers (pC194 carries one *Hin*dIII site/monomer [Ehrlich, 1977]). As first reported by Ehrlich (1977) *Hin*dIII digestion destroys the transforming activity of plasmid DNA (Table 3). We have then ligated the digested DNA to produce concatemeric pC194 DNA. Figure 8 shows the electrophoretic mobility of pC194 after *Hin*dIII digestion and after the subsequent ligation under two experimental conditions. During ligation most of the DNA has been converted to high molecular weight DNA. Electron microscopic investigations have shown that all molecules greater than monomers were in a linear form. This is parallelled by a significant recovery of specific transforming activity which is at least eight times higher than the level which was observed prior to *Hin*dIII digestion (Table 3). Sucrose gradient centrifugation performed with the ligated material (track d of Fig. 8) showed a peak of biological activity sedimenting much faster than monomeric DNA (Fig. 9). Since no interlocked molecules are produced in the ligase reaction, this result indicates that concatemerization renders such molecules highly active in transformation. It remains to be established whether interlocking of molecules has a similar effect.

Discussion

The major part of staphylococcal plasmid DNA which can be reisolated from *B. subtilis* exists in a monomeric form. The minority material represents concatenated oligomers or discrete classes of interlocked combinations of monomers and oligomers in supercoiled and relaxed forms. The latter types of molecules have been observed with various types of DNA, such as plasmids, circular animal virus DNAs and mitochondrial DNA (Novick et al., 1973; Meinke et al., 1971; Hudson and Vinograd, 1967). Novick

et al. (1973) and Kupersztoch et al. (1973) have shown with some plasmid DNAs that interlocked dimers are replication intermediates and most likely the precursors of the monomeric CCC forms of the plasmid. Higher oligomers than dimers were not reported in their studies. Our observation that the recombination deficient plasmid carrying cells contain the same multimeric plasmid forms as the corresponding rec^+ strains also support the replicative rather than recombinational origin of multimeric molecules. The occurrence of oligomers *greater* than dimers which we report here could be interpreted to mean that replication of *S. aureus* plasmids in *B. subtilis* proceeds normally but that in *B. subtilis* the part of termination, which leads to the separation into two monomeric circles does not operate as efficiently. Since degradations of total pC194 DNA with restriction enzymes like *Hin*dIII, *Bgl*I, or *Bsu* (data not reported) which cut the monomer at only one site, produce only unit length DNA it must be concluded that monomers are arranged in tandem, rather than in head-head combinations in concatemeric DNA.

Ehrlich (1977) and Gryczan et al. (1978) have reported that transformation of *B. subtilis* with plasmids originally isolated from *S. aureus* is less efficient than transformation with the same concentration of plasmid DNA reisolated from a plasmid carrying transformant of *B. subtilis*. They attributed this higher efficiency of the *B. subtilis* grown plasmid to its modification mediated resistance against some *B. subtilis* specific restriction, which had developed during the growth of the plasmid in *B. subtilis*. An alternative to this interpretation is suggested by the results presented here: since the transforming activity of a plasmid preparation is almost exclusively determined by the minor subclass of oligomeric molecules in the preparation, one might assume that the frequency of oligomeric DNA is higher in plasmids derived from *B. subtilis* than in preparations of the same plasmid directly isolated from *S. aureus*.

The reason for the high specific activity of oligomeric DNA as opposed to the monomeric form remain to be elucidated. This difference does not appear to reflect differences in the expression of the transformed genes from the two molecular species. Transformation with monomeric DNA remained at the low level observed even when the transformants were incubated for up to two hours under nonselective conditions before plating on antibiotic containing medium (data not reported). Therefore we attribute the dependence of transforming activity on the molecular configuration to the mechanics of DNA processing during the uptake into competend cells. Based on the experiment described (Fig. 9) in which we have shown that ligation of *Hin*dIII produced monomeric DNA into oligomers is parallelled with a manifold increase in the transforming activity of such DNA, we conclude that it is the concatemeric form which is responsible for the high specific biological activity in the higher molecular weight bands of Figure 5. This is also consistent with a positive correlation between the specific transforming activity of DNA (Fig. 7) and the incidence of multimeric DNA which is summarized in Table 2. We have no information at this time as to whether interlocking of monomeric DNA molecules to produce multimeric combinations would also increase the specific activity of such DNA. Neither do we have information on how the *size* of a concatenated molecule affects its transforming activity, i.e. whether the specific activity of a dimeric molecule is different from that of a trimer.

The dependence of biological activity on the existence of polymeric plasmid DNA resembles the situation described by Rutberg and his associates for transfection with ϕ105 DNA (Rutberg et al., 1969; Rutberg and Rutberg, 1970). These authors have observed that the monomeric form of ϕ105 DNA, isolated from mature phage has an extremely low specific activity, whereas prophage DNA, concatemeric replicating DNA, or mature ϕ105 DNA to which DNA had been added in vitro to the molecular ends (Flock, personal communication) is highly active in transfection. Their evidence indicates that during transfection some DNA is obligatorily removed from either DNA terminus. Such DNA degradation would consequently severely affect transfection by mature DNA (and thus explain its low transfecting activity) but would not inactivate a ϕ105 molecule which was sandwiched between additional DNA at both molecular ends.

A similar situation has been described for ϕ29 transfection in which the major contribution to the transfecting activity of phage DNA comes from multimeric molecules. In this case, however, the ϕ29 DNA associated protein (Hirokawa, 1972) is responsible for joining unit-length ϕ29 DNA into multimeric DNA containing complexes (Hirokawa et al., 1977). A significant difference between these transfection processes and transformation with plasmid DNA as described here is that monomeric DNA both in transfection with mature ϕ105 DNA or purified unit length ϕ29 DNA does give a *multiple* hit dose response indicating a cooperation of several molecules in a transfection event. The dose response with monomeric pC194 DNA is always observed to be less than one suggesting that monomeric plasmid DNA can, at a low frequency, successfully transform a *B. subtilis* cell.

The processing of pC194 DNA during transformation is presently being studies. Of particular interest are the questions of whether plasmid DNA in trans-

formation is converted to single stranded molecules and whether it is subject to the same kind of degradation which occurs during ϕ105 transfection.

Acknowledgement. We are grateful to Dr. S.D. Ehrlich for sending us the plasmid carrying strains of *B. subtilis*, to W. Messer and J. Reeve for helpful discussions, and to C. Fisseau and G. Lüder for technical assistance.

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Communicated by E. Bautz

Received August 5, 1978