ISOLATION AND CHARACTERIZATION OF COVALENTLY CLOSED CIRCULAR DNA ASSOCIATED WITH CHROMOSOMAL AND MEMBRANE FRACTION FROM *STREPTOMYCES AMBOFACIENS*

HARUO IKEDA, HARUO TANAKA and SATOSHI ŌMURA*

School of Pharmaceutical Sciences, Kitasato University, and The Kitasato Institute, Minato-ku, Tokyo 108, Japan

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Covalently closed circular (ccc) DNAs were isolated by a technique involving alkaline denaturation from the spiramycin producer *Streptomyces ambofaciens* KA-1028 and also from spiramycin non-producing strains AF-11 and QN-25; plasmids could not be detected in these strains by a cleared lysate method. It was found that most of the ccc DNA in these strains was present in the chromosomal and membrane fractions.

These ccc DNAs had identical mobilities in agarose gel electrophoresis. The size was calculated to be 53.1×10^6 daltons from the contour length measurements. The ccc DNA gave one fragment on digestion with *Hin*d III, three fragments with *Eco* R1, and twenty-eight fragments with *Bam* H1.

Various ccc DNAs have been isolated from streptomycetes[1~13]. Their involvement in various biological functions, such as antibiotic production[14] and resistance[4,14], fertility[2,15], differentiation[16~18], and release of extracellular enzyme[19,20], has been studied; the involvement of plasmids in antibiotic production in streptomycetes has been suggested by "curing experiments"[16]. Isolation of ccc DNAs from streptomycetes has generally been performed by cleared lysate methods although YAGISAWA *et al.*[4] and ŌMURA *et al.*[12] employed alkaline denaturation.

In bacteria other than streptomycetes, for example, in *Escherichia coli*[21~23], it was recently reported that more than 80% of F, R6K and NR1 plasmid DNAs were associated with the folded chromosome though relaxed multi-copy plasmids free from the folded chromosome are well known[22]. Plasmid DNAs associated with the folded chromosome can be isolated from the *E. coli* lysate by an alkaline denaturation method[24]. Recently, the alkaline denaturation technique was applied to the isolation of bound plasmid DNAs in *Agrobacterium*[25], *Rhizobium*[26] and *Pseudomonas*[27,28]. To our knowledge, there has been no report of a bound form of plasmid DNA from streptomycetes.

We have reported the presence of plasmid DNA (pSA1) in the spiramycin-producing *Streptomyces ambofaciens* KA-1028 (ISP-5053) by a "cleared lysate" method and discussed the possible involvement of the plasmid in the biosynthesis of spiramycin[6]. However, only a small amount of pSA1 DNA, could be recovered from the cleared lysate of the organism. Recently, we detected ccc DNA in the spiramycin-producing strain and also in the spiramycin non-producing strains AF-11 and QN-25 using an alkaline denaturation method[12], the latter strains were obtained from the spiramycin-producing strain by treatment with acriflavine and quinacrine, respectively, and in which no plasmid DNA had been detected previously. Most of the ccc DNA in the spiramycin-producing strain and of the non-producing strains was present in the chromosomal and membrane fractions.

* To whom inquiries should be directed.

This paper deals with the isolation and distribution of plasmid DNA from spiramycin-producing and non-producing strains of *S. ambofaciens* and with the characterization of pSA1 DNA.

Materials and Methods

Organisms

Streptomyces ambofaciens KA-1028 (ISP-5053), a spiramycin-producer, and strains AF-11 and QN-25, spiramycin non-producing strains, were used. Strains AF-11 and QN-25 were non-producing strains obtained by treatment of *S. ambofaciens* KA-1028 with acriflavine and quinacrine, respectively.

Chemicals

CsCl was purchased from Nakarai Chemicals Ltd., Kyoto, Japan, lysozyme and ethidium bromide from Sigma Chemicals Co., RNase A from Boehringer Mannheim GmBH, restriction endonucleases, *Eco* R1, *Hin*d III, and *Bam* H1, from Takara Shuzo Co., Ltd., Kyoto, Japan, and [methyl-^{3}H]thymidine (41 Ci/mmole) and [2-^{14}C]thymidine (42 mCi/mmole) from New England Nuclear.

Media and Growth

Strains were incubated in Tryptic Soy Broth (BBL) at 27°C for 48 hours, and 0.2 ml of the culture was transferred into 10 ml of a medium containing 0.25% glucose, 0.5% low phosphate content Casamino Acids (when low phosphate content Casamino Acids was not used, mycelial pellets were often formed that interfered with lysozyme digestion), 0.3% L-asparagine·H_2O, 0.3% NaCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% $NaNO_3$, 0.01% $CaCl_2 \cdot 2H_2O$, 0.009% KH_2PO_4, 0.4% trace elements solution, 0.02% 2′-deoxyadenosine, 0.1 M tris-(hydroxymethyl)aminomethane hydrochloride (tris-HCl), pH 7.2, and 1~10 μCi of [methyl-^{3}H]thymidine (41 Ci/mmole) or 1 μCi of [2-^{14}C]thymidine (42 mCi/mmole) per ml and then cultivated at 27°C for 18 hours. Mycelia were harvested by centrifugation at 12,000×g for 10 minutes. Low phosphate content Casamino Acids was prepared as follows. Twenty grams of Casamino Acids (Difco) was dissolved in 100 ml of distilled water and then 2.54 g of $MgCl_2 \cdot 6H_2O$ and 11 ml of concentrated NH_4OH were added. The stirred mixture was chilled at 0°C for at least 2 hours, and centrifuged at 2,000×g for 5 minutes at 0°C to remove the precipitate. The excess ammonia was evaporated from the supernatant fluid under reduced pressure. After the pH was adjusted to 7.0 with 1 N HCl, the solution was lyophilized to give a powder of low phosphate content Casamino Acids.

Preparation of Cleared Lysates

Mycelia obtained from a 10-ml culture were washed twice with 2 ml of a buffer containing 30 mM tris-HCl, 50 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0. Washed mycelia were suspended in 1 ml of a buffer containing 25% (w/v) sucrose, 50 mM tris-HCl, 25 mM EDTA, pH 8.0, and 1 mg of egg-white lysozyme was added to the suspension which was incubated at 37°C for 10 minutes. The protoplasts formed were lysed by the addition of one-ninth volume of 10% (w/v) sodium dodecyl sulfate (SDS) solution. The lysate was centrifuged at 20,000×g for 30 minutes at 18°C and the supernatant was decanted. The pellet was used for the preparation of chromosomal and membrane fractions. To the supernatant a quarter volume of 5 M NaCl was added and the mixture allowed to stand for 2 hours at 0°C. After SDS was removed by centrifugation at 4,000×g for 10 minutes, polyethyleneglycol #6,000 was added to make a final concentration of 10% (w/v) and the solution was kept at 0°C for 3 hours to complete DNA precipitation. The precipitated DNA was harvested by centrifugation at 1,200×g for 5 minutes and dissolved in TE buffer (10 mM tris-HCl, 1 mM EDTA, pH 8.0).

Preparation of Partially Purified Plasmid DNA by Alkaline Denaturation

Partially purified plasmid DNA fraction was prepared as described previously[12].

Preparation of DNA from Chromosomal and Membrane Fraction

The pellet obtained from the clearing step in preparation of a cleared lysate was suspended in 2 ml of TE buffer and 200 μg of RNase A was added to the suspension. After the mixture was incubated at 37°C for 30 minutes, one quarter volume of 5 M NaCl was added to the mixture. The DNA was isolated by two extraction with an equal volume of phenol saturated with TE buffer and the phases separated by centrifugation. To the aqueous phase an equal volume of chloroform was added and residual phenol was extracted. Two volumes of ethanol were added to the aqueous phase and the mixture was kept at

−20°C for 4 hours to precipitate DNA. The precipitated DNA was collected by centrifugation at 12,000×*g* for 10 minutes, and dissolved in TE buffer.

Preparation of Total DNA Fraction

Mycelia were lysed as described in "Preparation of Cleared Lysates" except that sodium lauryl sarcosinate (final concentration 0.5%) was substituted for SDS. The lysate was sheared by trituration in a 5-ml pipette and DNA was extracted from the lysate and purified as described above.

Molecular Weight

The molecular weight of pSA1 plasmid was determined from contour length measurements in the electron microscope[29] and sucrose density gradient centrifugation using λ *c*I 857 *Sam* 7 DNA as internal standard.

Results

Isolation of ccc DNA from *S. ambofaciens* KA-1028 and its Mutant Strains AF-11 and QN-25

In order to examine the relationship of spiramycin production and plasmid presence we attempted the isolation of plasmid DNA from the spiramycin producing organism *S. ambofaciens* KA-1028 and its spiramycin non-producing mutant strains AF-11 and QN-25. A small amount of plasmid DNA was detected in the cleared lysate of the parent strain, KA-1028 (Fig. 1-A), while no plasmid DNAs were detected in cleared lysates from the spiramycin non-producing mutant strains (Fig. 1-B and C). On the other hand, using a different method[12] for plasmid isolation, plasmids could be detected from both the spiramycin-producing parent strain KA-1028 and the non-producing strains (Fig. 2).

The relative mobilities on agarose gel electrophoresis of ccc DNAs of the two mutant strains coincided with that of the parent strain (Fig. 2-B). The recovery of ccc DNA from the parent strain calculated from the result of Fig. 2-A (alkaline denaturation method) was higher than that calculated from Fig. 1-A (cleared lysate). To examine the distribution of the ccc DNA, total DNA and chromosomal and membrane fractions were submitted to dye-buoyant centrifugation after the removal of proteins and RNAs, when satellite peaks of ccc DNA were detected in all strains as shown in Fig. 3 (A, B and C). These data suggest that most of the ccc DNA in the parent strain KA-1028 and all that in mutant strains AF-11 and QN-25 is found in the chromosomal and membrane fractions. When the chromosomal and membrane fractions were subjected to dye-buoyant centrifugation. Satellite bands of ccc DNA were detected in the (chromosomal and membrane) fractions of three strains as well as in the total DNA fractions (Fig. 3-D, E and F). The amounts of ccc DNAs to DNAs in cleared lysates, mycelial lysates (total DNA), and chromosomal and membrane fractions from the spiramycin-producing and non-producing strains of *S. ambofaciens* are summarized in Table 1. The data indicate that most (83%) of ccc DNA is bound to the chromosomal and membrane fraction and only a small portion (17%) occurs intracellularly as a free form in the spiramycin-producing parent strain KA-1028, and that in the spiramycin non-producing mutant strains AF-11 and QN-25 all ccc DNA is associated with the chromosomal and membrane fraction.

It has been reported[25,30] that large ccc DNAs frequently precipitate with chromosomal and membrane fraction during a clearing spin in the presence of SDS, we asked therefore whether an SDS-ccc DNA complex is formed in the preparation of the cleared lysate from strains AF-11 and QN-25 or not. ^{14}C-Labeled ccc DNA prepared from cultures of strain AF-11 or QN-25 grown in the presence of ^{14}C-thymidine was added to a mycelial lysate which was prepared from the corresponding culture grown in

Fig. 1. CsCl-ethidium bromide equilibrium centrifugation of cleared lysates from cultures of *S. ambofaciens* strains KA-1028 (parent strain) (A), AF-11 (B) and QN-25 (C) grown in the presence of ^{3}H-thymidine (10 μCi/ml).

To the DNA solution (4.80 ml) in TE buffer obtained as described in the text, 5.5 g of solid CsCl and 0.5 ml of an ethidium bromide solution (4.75mg/ml) were added. The mixture was centrifuged in a polyallomer tube on an RP55T fixed angle rotor at 36,000 rpm for 48 hours using a Hitachi 55P-2 ultracentrifuge.

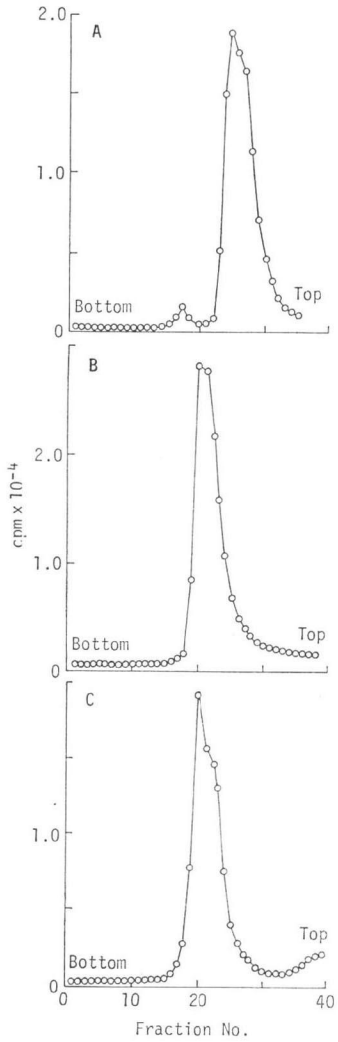
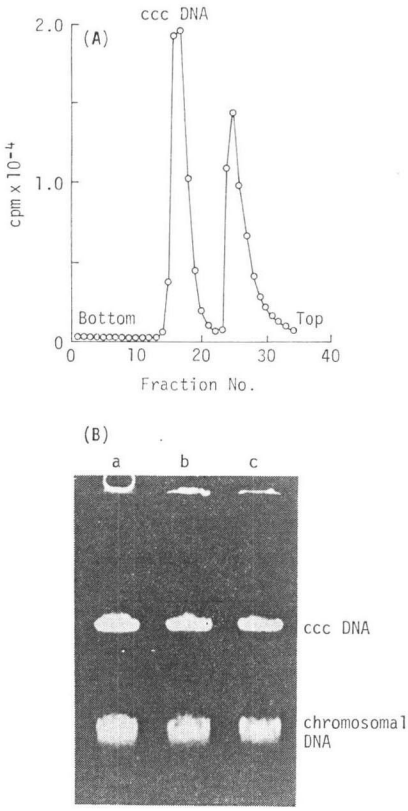


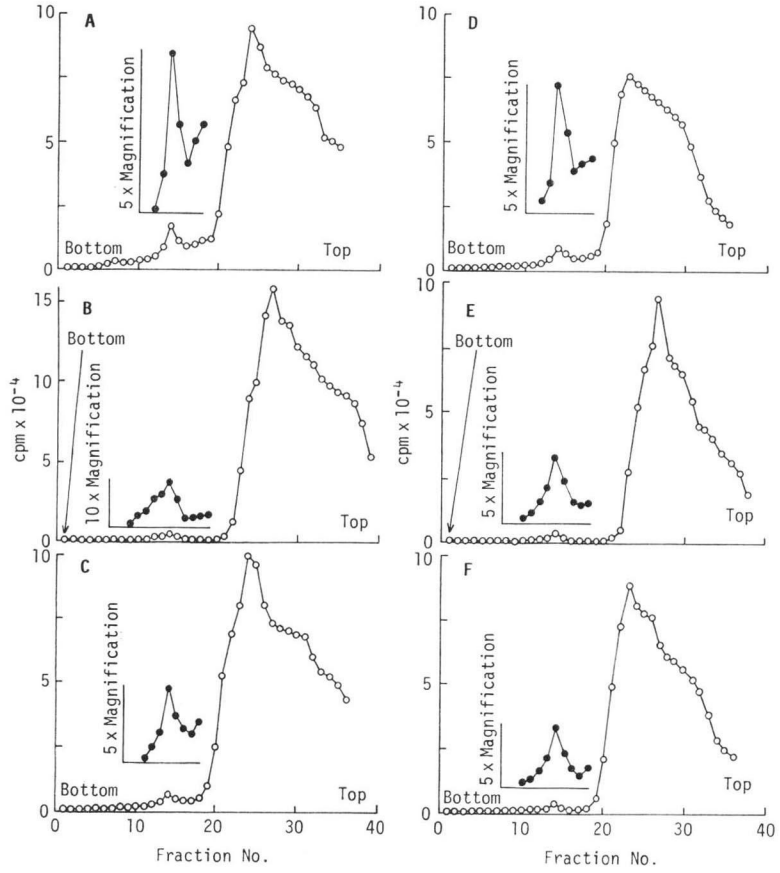
Fig. 2. Analysis of partially purified plasmid DNA fractions obtained from *S. ambofaciens* strains.

The partially purified plasmid DNA fractions prepared by alkaline denaturation technique were analyzed by (A) CsCl-ethidium bromide equilibrium centrifugation (parent strain KA-1028) and (B) agarose gel electrophoresis: a, KA-1028; b, AF-11; c, QN-25. Centrifugation was performed as described in the legend to Fig. 1.



the presence of ^{3}H-thymidine, a cleared lysate was prepared and subjected to dye-buoyant centrifugation. As shown in Fig. 4, 80 to 90% of the ^{14}C-labeled ccc DNA added was recovered from the cleared lysate. These results indicate that the ccc DNAs in the strains AF-11 and QN-25 do not form complexes with SDS in the preparation of cleared lysate. Since the size of pSA1 is estimated to be 53×10^{6} daltons as described below, the content of the plasmid in the spiramycin-producing parent strain KA-1028 was calculated from the results shown in Table 1 to correspond to two copies per chromosome, assuming that the content of streptomycete chromosomal DNA is about 7×10^{9} daltons[31]. The plasmid contents of strains AF-11 and QN-25 were calculated to be one copy per chromosome.

Fig. 3. CsCl-ethidium bromide equilibrium centrifugation of total DNA fractions (A, B and C) and chromosomal and membrane fractions (D, E and F) from cultures of *S. ambofaciens* strains KA-1028 (A and D), AF-11 (B and E), and QN-25 (C and F). The conditions of centrifugation are as described in the legend to Fig. 1.



Characterization of pSA1 DNA from *S. ambofaciens*

The physical properties of pSA1 DNA from *S. ambofaciens* KA-1028 were investigated; 100 to 150 μg of the ccc DNA could be obtained from 1 liter of the culture of strain KA-1028, and the DNA was analysed by sucrose density gradient centrifugation. As shown in Fig. 5, strain KA-1028 contained one species of ccc DNA sedimenting at 77 S and 155 S under neutral and alkaline conditions, respectively. Using the equation of CLAYTON and VINOGRAD[32], the molecular weight of the ccc DNA (pSA1) was calculated from these sedimentation values to be about 53×10^6.

The contour length of pSA1 DNA molecule was measured and found to be 25.6 ± 0.9 μm. The molecular size was estimated using λ *c*I 857 *Sam* 7 DNA molecule as internal standard (32.3×10^6 daltons) to be $53.1 \pm 1.2 \times 10^6$ daltons.

Three endonucleases were used to digest pSA1 DNA and the digested samples were subjected to agarose gel electrophoresis (Fig. 6-A). *Hin*d III cleaved the ccc DNA into single linear molecule which could be distinguished from ccc-form by mobility on gels (data not shown). *Eco* R1 cleavage gave three fragments: A1 ($>20 \times 10^6$ daltons), A2 (*ca.* 17×10^6 daltons) and A3 (8.3×10^6 daltons). *Bam* H1

Table 1. Distribution of ccc DNA in spiramycin-producing (spp$^+$) and non-producing (spp$^-$) strains of *S. ambofaciens*.

Data obtained from the results of Figs. 1 and 3 were summarized.

Strain	DNA fraction	ccc DNA (cpm)	Chromosomal DNA (cpm)	*Content of ccc DNA in total DNA(%)	**Rate of associated ccc DNA(%)
KA-1028 (spp$^+$)	Total DNA	20,281	1,247,297	1.6	83
	Chromosomal & membrane	15,625	1,033,213		
	Cleared lysate	3,160	84,804		
AF-11 (spp$^-$)	Total DNA	14,094	1,747,601	0.8	100
	Chromosomal & membrane	8,180	1,014,300		
	Cleared lysate	no peak	138,295		
QN-25 (spp$^-$)	Total DNA	9,184	1,138,759	0.8	100
	Chromosomal & membrane	8,305	1,029,875		
	Cleared lysate	no peak	93,764		

* $\frac{\text{ccc DNA}}{\text{ccc DNA}+\text{chromosomal DNA}} \times 100$, each datum is from total DNA fraction.

** $\frac{\text{ccc DNA (chromosomal \& membrane fraction)}}{\text{ccc DNA (chromosomal \& membrane fraction)}+\text{ccc DNA (cleared lysate fraction)}} \times 100$

digestion produced at least 22 fragments and a densitometry trace of the gel showed that *Bam* H1 digestion of pSA1 DNA gave about 28 fragments (Fig. 6-B). A *Hin*d III and *Eco* R1 double digestion of pSA1 DNA produced four fragments B1 ($>20\times10^6$ daltons), B2 (*ca.* 17×10^6 daltons), B3 (8.3×10^6 daltons), and B4 (5.5×10^6 daltons) as shown in Fig. 6-A. Partial digestion with *Eco* R1 of the linear duplex pSA1 DNA generated by *Hin*d III produced at least seven fragments as shown in Fig. 7-A.

From these results, a cleavage map with the relative positions of the restriction sites of *Hin*d III and *Eco* R1 in the pSA1 DNA was deduced as shown in Fig. 7-B.

Discussion

In the previous paper[6] dealing with the occurrence and functions of a plasmid (pSA1) in the spiramycin producer *S. ambofaciens* KA-1028, we reported that treatment of the strain with 10 μg/ml of acriflavine provided spiramycin non-producing strains at a frequency of 10%, and that the plasmid DNA was detected in the parent strain but not in a spiramycin non-producing strain AF-30 using a cleared lysate method.

The results described in the present paper demonstrate that most (83%) of the ccc DNA in the spiramycin-producing strain KA-1028 is associated with the chromosomal and membrane fraction and only a small amount (17%) of ccc DNA, detected by cleared lysate method, occurs in the cytoplasm. On the other hand, in the spiramycin non-producing strains AF-11 and QN-25 no free ccc DNA can be detected in cleared lysates since it is associated with chromosomal and membrane fraction. All of spiramycin non-producing strains other than AF-11 and QN-25 (each 20 strains obtained by the treatment with acriflavine or quinacrine were tested) were found to harbor ccc DNA bound to chromosomal and membrane fraction (data not shown). The size of ccc DNA isolated from the spiramycin non-producing strain AF-11 or QN-25 was shown to agree with that of pSA1 DNA in agarose gel electrophoresis.

Since no ccc DNAs other than pSA1 could be detected *S. ambofaciens* is considered to harbor only

Fig. 4. CsCl-ethidium bromide equilibrium centrifugation of a cleared lysate prepared from a mixture of a mycelial lysate and ^{14}C-labeled ccc DNA.

Mycelial lysate was prepared by lysozyme digestion and SDS lysis from a culture of *S. ambofaciens* mutant strain AF-11 (A) or QN-25 (B) grown in the presence of ^{3}H-thymidine (1 μCi/ml) as described in the text. ^{14}C-Labeled ccc DNA was prepared from the culture of strain AF-11 (A) or QN-25 (B) grown in the presence of ^{14}C-thymidine. The mycelial lysate and corresponding labeled ccc DNA were mixed and then centrifuged at 18°C and 20,000×*g* for 30 minutes. The supernatant fluid (cleared lysate) was subjected to dye-buoyant centrifugation as described in the legend to Fig. 1.

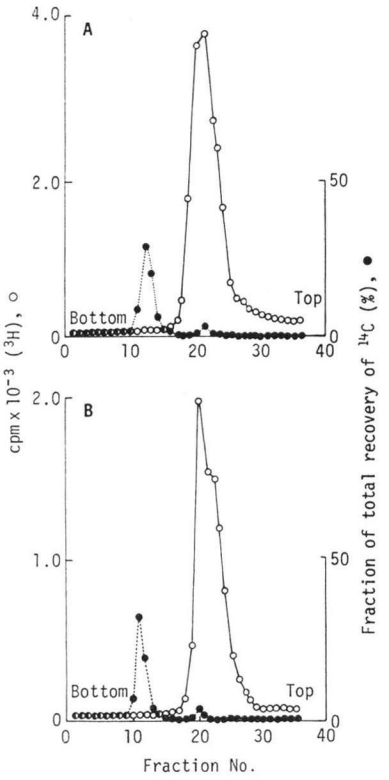
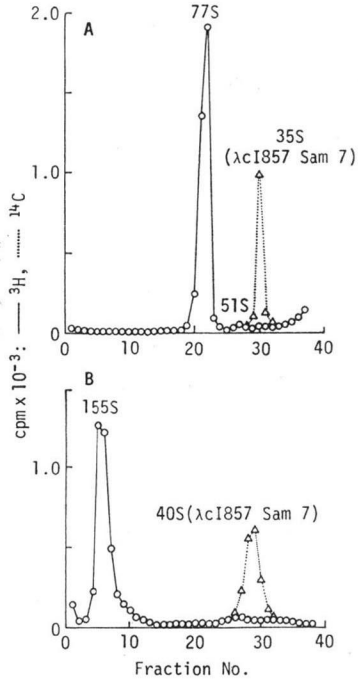


Fig. 5. Sedimentation in neutral (A) and alkaline (B) sucrose gradients of the dialyzed denser DNA satellite band obtained by CsCl-ethidium bromide equilibrium centrifugation of *S. ambofaciens* KA-1028.

A DNA sample (0.05 ml) was layered on a neutral or alkaline 5~20% sucrose gradient (5 ml) and centrifuged at 30,000 rpm for 60 minutes at 20°C in an RPS 40T-2 swing rotor using a Hitachi 55P-2 ultracentrifuge. Sedimentation was from right to left. Sedimentation velocities given were related to linear duplex ^{14}C-labeled λ *c*I 857 *Sam* 7 DNA, which was used as internal marker and which sedimented at 35S under neutral and 40S under alkaline conditions.



one plasmid DNA. The size of pSA1 was calculated to be 53.1×10^6 daltons from the contour length measurements.

The cleared lysate technique is useful for the isolation of multiple copy plasmids free from chromosome and membrane. However, for the isolation of plasmid DNA associated with chromosomal and membrane fractions, an alkaline denaturation method is more effective. To our knowledge, the isolation of plasmid DNA associated with the chromosomal and membrane fractions of streptomycetes has not been reported previously.

Fig. 6. (A) Analysis of pSAl DNA from parent strain KA-1028 (pSAl) cleaved with restriction endonucleases.

Digested DNA samples were subjected to electrophoresis through a 0.7% agarose gel on a horizontal slab apparatus. Samples were digested with *Hin*d III (2), *Eco* R1 (3), *Bam* H1 (4), *Eco* R1-*Hin*d III (5). Reference DNA fragments are λ *c*I 857 *Sam* 7 digested by *Hin*d III (1) and *Eco* R1 (6).

(B) Densitometry trace of the gel of *Bam* H1 endonuclease-digested fragments of pSA1 DNA.

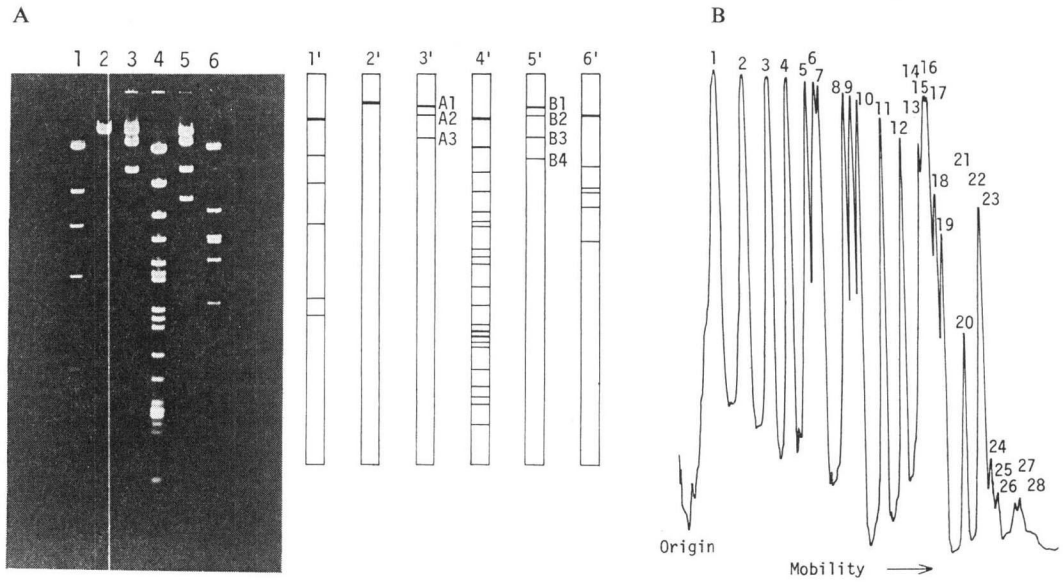
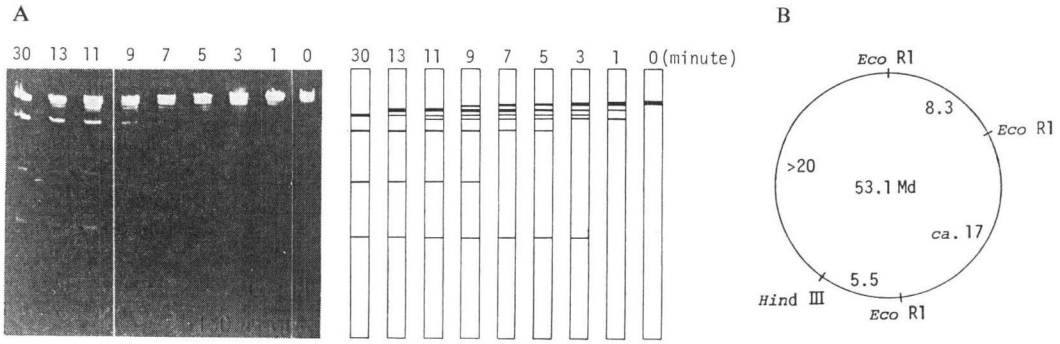


Fig. 7. (A) The partial digestion with *Eco* R1 of the linear duplex pSA1 DNA generated by *Hin*d III digestion. (B) Cleavage map of pSA1 DNA for the *Eco* R1 and *Hin*d III endonucleases.



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