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ISOLATION AND PROPERTIES OF PLASMIDS FROM DEINOCOCCUS RADIODURANS SARK

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Radioresistant bacterium, Deinococcus radiodurans, can repair completely almost all of DNA damages including double strand breaks induced by gamma-rays up to about 5 kGy. In order to reveal the repair mechanism, it is necessary to develop a cloning vector available for the genetic analysis. We tried to isolate plasmids from D.radiodurans Sark strain. In the present paper the isolation and properties of plasmids were described.

Keywords: Radioresistant Bacterium, Deinococcus radiodurans, Plasmids, Vector, Repair, Restriction Map, Molecular Size

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Deinococcus radiodurans サーク株からのプラスミドの分離と性質

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（1990年4月5日受理）

放射線抵抗性細菌であるDeinococcus radioduransは，5 kGyまでの線量で生じるところの2本鎖切断を含めた種々のDNA損傷を修復することができる。この修復機構を明らかにするためには，遺伝子解析に使用するクローニングベクターを開発する必要があり，著者らはそのためにD. radioduransサーク株からプラスミドの分離を試みた。本報告では，ベクター用プラスミドの分離方法と分離されたプラスミドの分子サイズ及びその制限酵素地図について得られた結果を述べる。

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1. Introduction

Micrococcus radiodurans was originally isolated in 1956 from canned meat which was irradiated with a sterilizing dose of gamma-rays [1]. This bacterium has been classified into a new genus, Deinococcus, by Brooks and Murray [2] in 1980, that can grow everywhere in sewage sludge, animal feeds, and sawdust medium of mushrooms [3,4]. A radioresistance in bacteria is associated in general with its spore-forming capability. However, the radioresistance is also occured in Deinococcus radiodurans eventhough it has no spore-forming ability. D. radiodurans has been known to be radioresistant to both of ionizing and ultra-violet radiation. The resistance of D. radiodurans to the lethal and mutagenic action of UV, ionizing radiation and some chemical compounds such as N-methyl-N'-nitro-N-nitrosoguanidine(MNNG), nitrous acid(NA), ethylmethanesulphonate(EMS) and β-propiolactone(BPL) has been compared with that of Escherichia coli B/r. It was evidenced that D.radiodurans was more resistant than E.coli B/r to the lethal effects of UV, ionizing radiation and MNNG and NA, but sensitive to EMS and BPL [5].

D.radiodurans was isolated not only from irradiated but also from unirradiated sources. This indicates that the radioresistance is an inherent characteristic which is not acquired as a result of one or a few exposures to radiation. It is known that the radioresistance of this organism is due to high ability of repair on DNA damages [6]. In fact, D.radiodurans can repair not only single- but also double-strand breaks of DNA induced by radiation [7]. However, the repair mechanism in the bacterium has not been revealed. On the purpose to reveal the repair mechanism, the transformation using homologous chromosomal DNA was only the method so far available for genetic analysis in the bacterium. According to Moseley and Copland [8], however, there is a limitation in direct use of chromosomal DNA. In spite of a long search, no phages have been found out from Deinococcus spp. Further, an attempt to mobilize the chromosomal genes by introducing plasmids from other species of bacteria as a vector have been unsuccessful [9]. To overcome this problem, a search of plasmids in Deinococcus spp. has been carried out. It was found that several species of radio-resistant bacteria such as D.radiodurans Sark, D.radiophyrus,

D.proteolyticus and others contained plasmids except for D.radiodurans R_1 strain [10]. The plasmid of Sark strain may be useful as a cloning vector for the type strain of D.radiodurans R_1. Therefore, the isolation of plasmids from Sark strain and their properties were investigated.

2. Materials and Methods

2.1 Radiation sensitivity

Two strains of D.radiodurans Sark and R_1 were grown in TGY medium consisted of Bacto-tryptone 5 g, Bacto-yeast extract 3 g and glucose 1 g per liter. These bacteria were cultivated in shaking incubator at 30 °C for two days which were in a stationary phase. The cells were collected by centrifugation, washed with 0.01 M phosphate buffer(pH 7), then resuspended in the same buffer to adjust to 1.0 of optical density at 660 nm. 20 ml of the suspension was irradiated with gamma-rays of Co-60 at a dose rate of 3 kGy/hr. The suspensions were irradiated with the following doses; 3.3, 4.8, 7.0, 8.4 and 10.4 kGy. Nitrogen gas was bubbled through the sample during irradiation. The first dose given, 3.3 kGy, was a single dose, while the following doses were fractional doses. Aliquot of suspension was taken out from irradiated sample at intervals of certain minutes. The dilution of suspension was maken in 0.01 M phosphate buffer containing 0.1% Tween 80, and plating for viable cell counts were done on TGY agar medium. The plates were incubated at 30°C for 2 days until colonies were shown off.

2.2 Plasmid isolation

D.radiodurans Sark strain was used in this experiment. The Sark was cultivated in TGY-glycine medium containing 0.2% glycine. The separation of plasmids was conducted on agarose gel electrophoresis by considering some conditions such as growth phase of bacterium, kinds of agarose gel, concentration of agarose, pH of buffer and voltage of electrophoresis. Buffers used in the experiments were TAE and TBE. TAE buffer was maken up of 40 mM Tris-HCl, 1 mM acetate and 1 mM EDTA,

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while TBE buffer consists of 89 mM Tris-borate, 89 mM boric acid and 2 mM EDTA. Electrophoresis was held under two different conditions. One was carried out in circulation of buffer cooled in ice, and another in cold chamber(4 °C). The buffer circulation was mostly used for electrophoresis which was run in room temperature under the voltage up to 100 V.

For recovery of plasmids, "EpiGene"(EpiGene Inc.), "GENE CLEAN"(BIO 101 Inc.) and low-melting-temperature agarose were used in relation to the concentration of agarose and kinds of buffer.

(1)EpiGene: In this method, bands of plasmid DNA detected on agarose gel by staining with 0.5 μg/ml of ethidium bromide(EtBr) were cut into as small pieces as possible and then transfered to a specific electro transfer chamber which contains DEAE matrix. Buffer was poured into the chamber which was connected to a power electric source to allow DNA to come out of the gel and attach to the DEAE matrix. DNA was eluted from DEAE matrix using 0.5 to 1.0 ml of TE buffer(10 mM Tris, 1 mM EDTA, pH 8.0) containing 1.0 M NaCl, then recovered by ethanol precipitation.

(2)GENE CLEAN: In this method, DNA band excised from agarose gel was dissolved in NaI stock solution, and then glass milk suspension was added. After the supernatant was discarded, the remained glass milk pellet was washed in NEW (NaCl/Ethanol/Water) solution (BIO101 Inc.). DNA was eluted in water or low salt buffer. No ethanol precipitation was required in this method.

(3)LMTA: In low-melting-temperature agarose(LMTA) method, DNA band was cut into as small pieces as possible, and put into two volumes of TE buffer(20 mM Tris-HCl, 1 mM EDTA, pH8.0), then melted completely in 70°C water bath. This solution was then extracted by phenol saturated with 0.1 M Tris buffer-0.2 % 2-mercaptoethanol, followed by phenol--chloroform(1:1) and chloroform-isoamylalcohol (24:1) before DNA was collected by ethanol precipitation.

2.3 Digestion of DNA by restriction enzymes

Several restriction endonucleases were used for a single or double digestion of DNA in this experiment. Reaction mixture was made up into final 15 μl with 1 μl of each endonuclease, 3 μl of concentrated

buffer, 4 μl of DNA solution and 7 μl of distilled water. The digestion was carried out at 37°C for 3 hr. Two μl of loading buffer containing 50 % glycerol and 0.1 % bromophenolblue was added to the mixture, then incubated at 60°C for 10 min. Electrophoresis was taken part in TAE buffer(pH 8) with 0.9 % agarose gel. The voltage used for the first 30 minutes was constant at 50 V, then changed to 100 V for 3 hr. During 100 V electrophoresis, the buffer was circulated through ice to keep it cool. The molecular size of DNA fragments produced on electrophoresis was calculated from migration of standard DNA. In this experiments Sma I was used for identification of plasmids, and EcoR I, Hind III, Pst I, Dra I, Sca I, EcoT22 I and Cla I were also used for making the restriction map of plasmid.

On the purpose to confirm the possible use of plasmid from rifampicin resistant Sark strain, the plasmid of antibiotic resistant Sark strain was compared with a plasmid of non-resistant one. The restriction endonucleases used on rifampicin-resistant Sark plasmid were Apa I, Bgl II, Dra I, EcoR I, Hind III, Pvu II, Sal I, BamH I, Pst I, Sca I, Sma I and Xho I. These enzymes were also used to the plasmid of rifampicin non-resistant Sark strain as a control.

3. Results and Discussion

3.1 Radiation resistance

Sensitivies of D.radiodurans Sark strain and R_1 strain are shown in Fig.1. Although Sark strain is more sensitive than R_1 strain up to 10 kGy, the sensitivity more than 10 kGy seems not to be so different. Many chromosomal genes may be responsible for the radioresistance of the cells as stated above. In order to carry out genetic analysis of the resistance, it is desirable to construct a cloning vector. It is reported that R_1 strain has no plasmid [11], what we confirmed in this experiment. So Sark strain was used for isolation of plasmids.

3.2 Conditions available for separation of plasmid

In the plasmid separation, the concentration of agarose and

buffer, 4 μl of DNA solution and 7 μl of distilled water. The digestion was carried out at 37°C for 3 hr. Two μl of loading buffer containing 50 % glycerol and 0.1 % bromophenolblue was added to the mixture, then incubated at 60°C for 10 min. Electrophoresis was taken part in TAE buffer(pH 8) with 0.9 % agarose gel. The voltage used for the first 30 minutes was constant at 50 V, then changed to 100 V for 3 hr. During 100 V electrophoresis, the buffer was circulated through ice to keep it cool. The molecular size of DNA fragments produced on electrophoresis was calculated from migration of standard DNA. In this experiments Sma I was used for identification of plasmids, and EcoR I, Hind III, Pst I, Dra I, Sca I, EcoT22 I and Cla I were also used for making the restriction map of plasmid.

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3.2 Conditions available for separation of plasmid

In the plasmid separation, the concentration of agarose and

duration of electrophoresis tend to take an important part in this process. The relative mobilities of plasmid DNA are dependent primarily on the agarose concentration and are also influenced by the strength of applied current or voltage and the ionic strength of the buffer [12]. Therefore, for the plasmid separation from bacterial cells, various conditions were examined such as different growth phase, kind or concentration of agarose, and kind or pH of buffer. The DNA in the gel was stained with low concentration of ethidium bromide, then detected by direct illumination of the gel under ultraviolet light.

Fig.2 shows plasmid DNA recovered in two growth phases of early stationary phase(24 hr cultivation) and late stationary phase(45 hr cultivation). There is no qualitative and quantitative difference in plasmids isolated from the bacteria. The effect of running time on electrophoresis is shown in Fig.3. Using high-melting-temperature agarose (m.p.>85°C), the concentration of 0.6% either in TAE or TBE buffer gave a good separation. The clean separation of plasmid bands was appeared after 9 to 12.5 hr of electrophoresis. The four bands of plasmid DNA were detected by staining, and named as P1, P2, P3 and P4. The electrophoresis was carried out at 50 V for the first 30 minutes and then at 100 V for the remaining time.

On the other hand, in low-melting-temperature agarose(m.p. 65°C), two concentrations of 0.6% and 0.75% were examined. As shown in Fig.4, define plasmid bands can be obtained by using 0.6 % agarose gel in TAE buffer(pH 8.0). The times required for the plasmid separation in low-melting-temperature agarose was 5.5 hr, where the voltage was kept at 150 V throughout the run. When the electrophoresis was carried out either in circulation of ice-cold buffer or in cold chamber at 4°C without buffer circulation, the former condition gave better separation than the later (Fig.5). As far as the kinds of buffer is concerned, TAE and TBE were favourable to be used for the separation.

3.3 Recovery of plasmid from agarose gel

Four bands of plasmids separated by electrophoresis were tried to recover using EpiGene, GENE CLEAN or LMTA methods. Amongst the three methods, GENE CLEAN and LMTA methods gave promising results in purity

and yield of plasmid. GENE CLEAN method can be used either for high- and low-melting-temperature agarose in TAE or TBE buffer. But more linearization had occored by GENE CLEAN compared with LMTA method. It may occur during handling the suspension of glass milk pellet on which plasmids attached. EpiGene method did not give any good result. From the comparison of three methods, it was clear that LMTA method was most favourable for the separation and recovery of the plasmids.

3.4 Identification of plasmid

To confirm the purity and identification of plasmids, a restriction endonuclease Sma I was used. Fig.6 shows an illustration of fragments produced from each plasmid of P1, P2, P3 and P4. By comparing the density and the composition of fragments separated by electrophoresis, the purity of plasmids was determined. The digested DNA fragments of P2, P3 and P4 show different electrophoretic patterns one another(Fig. 6, lanes 2, 3 and 4). On the other hand, P1 seems to be a mixture of P2, P3 and P4, because P1 identical bands of No.1,7,9,12,13,14,15,16 and 17 can be seen in lane 2. Whereas, bands, No.2,4,5,6,10,11 and 19 are also seen in lane 4, and No.3 and 8 bands may be come from P4 plasmid. Herein, it can be seen that P1 plasmid is dominated by P2, then followed by P3, while P4 is contaminated slightly in P1 plasmid. Thus it is clear that P2, P3 and P4, which molecular size is 36, 45 and 87 kbp, respectively, are the inherent plasmids in D.radiodurans Sark. Two of those plasmids have been found by Mackay et al.[10] as pUE10 and pUE11 which correspond to P2 and P3, respectively. However, P4 plasmid was first found out in this experiment.

3.5 Antibiotic resistant gene as a marker

A vector suitable for gene cloning must have at least the following properties: (1)copy number in a cell is high, (2)there are many recognition sites for restriction enzymes, (3)it posseses distinguishable genetic markers, such as antibiotic resistance, to allow selection on agar plates of cells containing the recombinant plasmid. The plasmid of small molecular size carring a marker such as antibiotic resistance is favorable as a vector. Therefore, P2 plasmid

of the smallest molecular size was examined to construct a vector. It is hoped that antibiotic resistant strain would carry the resistant gene on its plasmid. So Sark strain was cultivated in TGY medium containing rifampicin to select mutant strain resistant to the drug, and then plasmids were isolated. From the digestion of whole plasmid by several restriction enzymes, it was found that no different patterns of fragments were given on plasmid from rifampicin resistant strain compared with normal one from sensitive strain (Fig.7). Furthermore, the plasmids from rifampicin resistant strain did not transform to the sensitive strain. These results suggest that the rifampicin resistant gene might be located on chromosomal DNA. So a search would be required for transforming the antibiotic resistant gene from chromosomal DNA to plasmid DNA as a marker before it is used as a vector.

3.6 Restriction map of plasmid

In order to construct cloning vector, the recognition sites of restriction enzymes have to be known. Therefore, P2 plasmid was digested by 9 kinds of enzymes, i.e., BamH I, EcoR I, Hind III, Pst I, EcoT22 I, Sal I, Not I, Cla I and Xba I. A part of results is shown in Fig.8. The fragment size after digestion with BamH I was calculated to be 0.9, 9.8 and 26.6 kbp. As shown in restriction map of P2 plasmid (Fig.9), three cleavage sites were also found by Cla I (4.0, 6.4 and 24.2 kbp), and two sites by Hind III (15.3 and 20.5 kbp) and Pst I (8.5 and 27.3 kbp). Whereas EcoT22 I, Sal I, Not I and Xba I had introduced single cut. From the analysis of these data obtained by electrophoresis, 18 cleavage sites were determined.

4. Conclusions

The several conditions for isolation of plasmids and the properties of plasmids isolated from radiation resistant bacterium, Deinococcus radiodurans Sark strain, were investigated.

In the isolation of plasmids, it was found that the growth phase of Sark strain did not influence the constitution of plasmids. Low-melting-temperature agarose at 0.6 % concentration in TAE

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In the isolation of plasmids, it was found that the growth phase of Sark strain did not influence the constitution of plasmids. Low-melting-temperature agarose at 0.6 % concentration in TAE

buffer(pH 7-8) gave the most promising results in separating the plasmids. Voltage used for electrophoresis was 150 V with circulation of cooling buffer. For recovery of plasmids from the gel, low-melting-temperature agarose gave the best results among the several methods tested.

The agarose gel electrophoresis showed 4 bands of plasmid DNA named as P1, P2, P3 and P4. The digestion of each plasmid by restriction enzymes provided clear evidence that P2, P3 and P4 were different plasmid one another and their molecular size was 36, 45 and 87 kbp, respectively. Besides P1 was a mixture of P2, P3 and P4. The two of them have been found by Mackay et al.[10] as pUE10 and pUE11 which correspond to P2 and P3, respectively. P4 was newly found in this study.

To obtain a better plasmid for cloning vector, plasmids from rifampicin-resistant Sark strain were compared with those from non-resistant strain. From comparative digestion with several restriction enzymes, it was found that no rifampicin-resistant gene was located in the plasmids. The digestion of P2 plasmid by 9 kinds of restriction enzymes gave 18 specific cleavage sites for the enzymes, what allowed the construction of restriction map.

Acknowledgements

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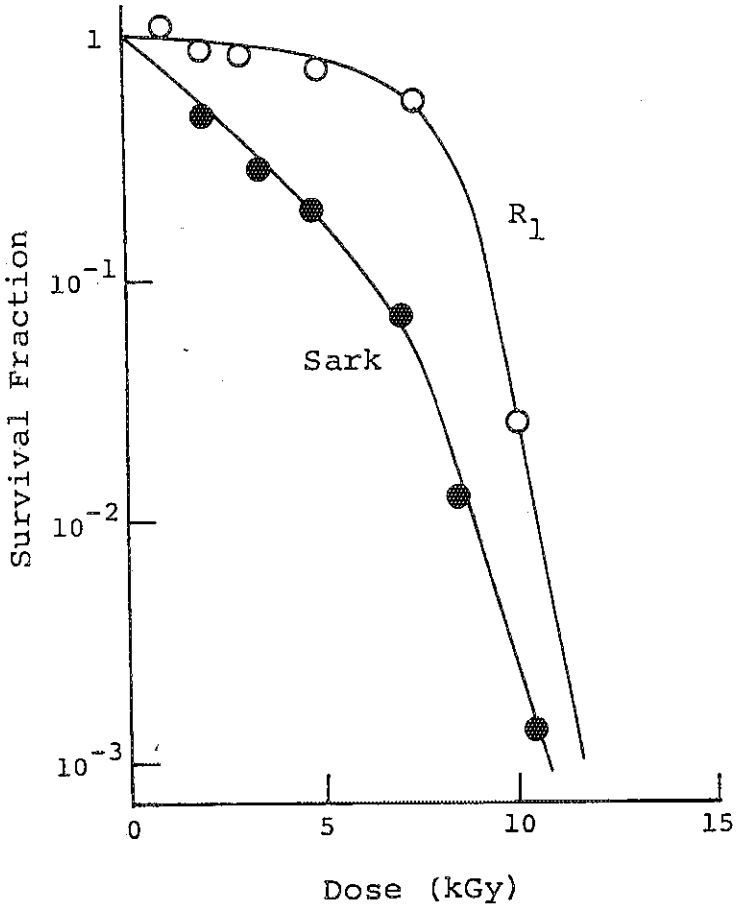


Fig. 1 Survival curves of Deinococcus radiodurans Sark strain and R_1 strain.

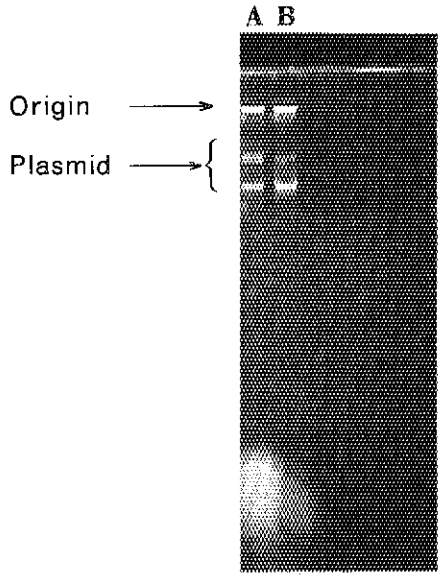


Fig. 2 Electrophoresis of plasmids isolated from Sark strain cultivated for 45 hr(A) and 24 hr (B).

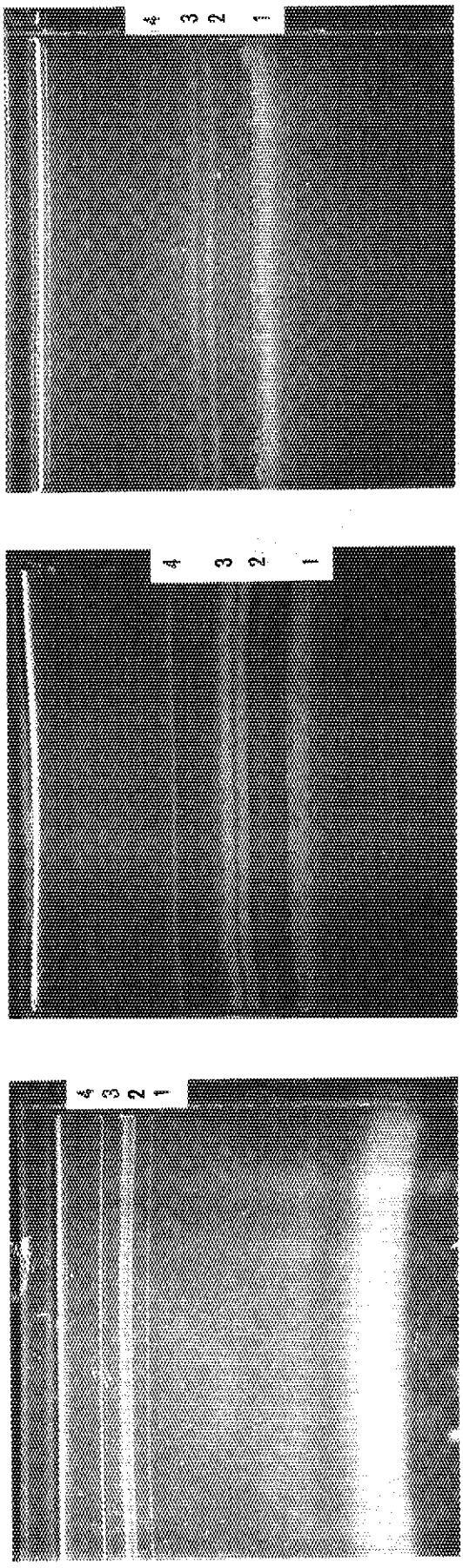


Fig. 3 Effect of running time on plasmid isolation.
The electrophoresis was run using high-melting-temperature agarose with a concentration of 0.6 % in TAE buffer(a & b) and TBE buffer(c) for 4 hr(a), 9 hr(b) and 12.5 hr(c), where the voltage were set up at 50 V for the first 30 minutes and then changed to 100 V for the rest of time.

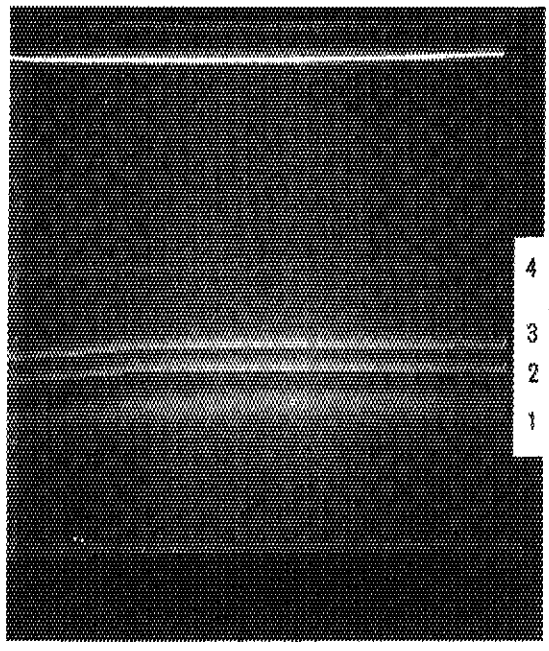


Fig. 4 Plasmid isolation on electrophoresis using low-melting-temperature agarose with a concentration of 0.6 % in TAE buffer(pH 8.0).

The electrophoresis was run for 5.5 hr. The voltage was set up at a constant of 150 V.

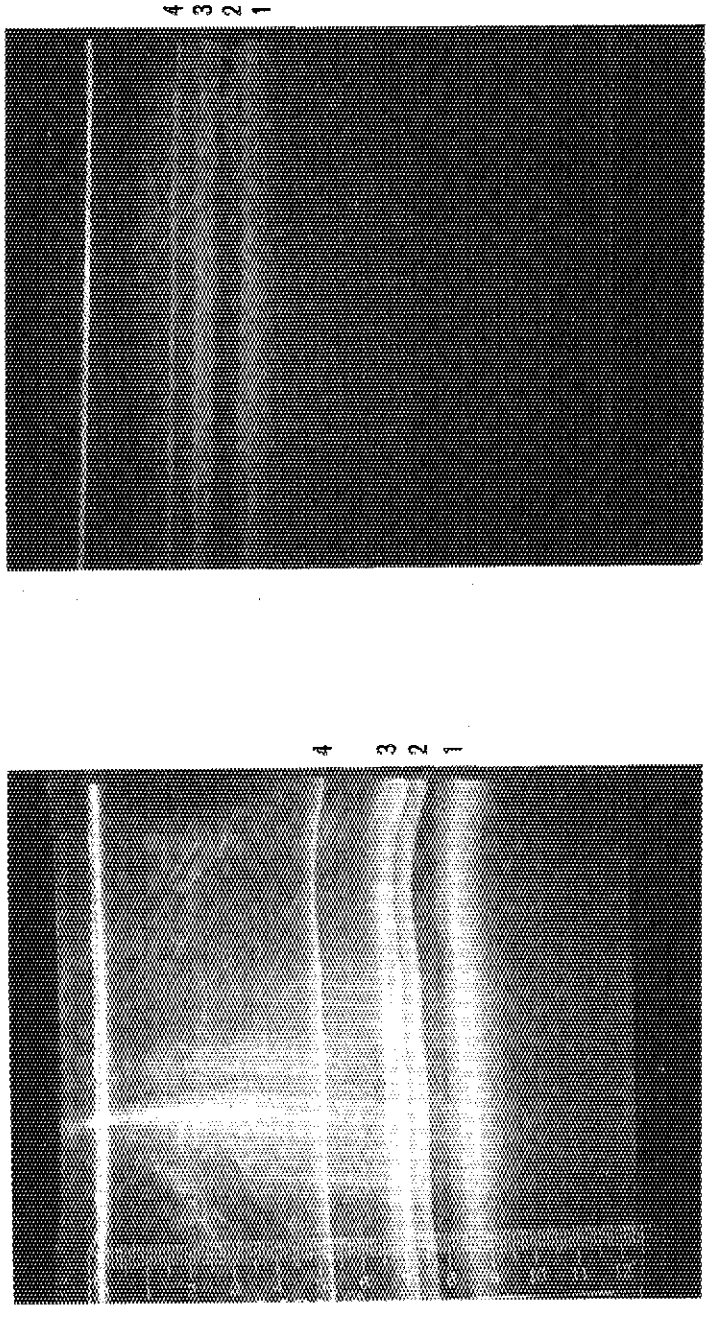


Fig. 5 Effect of cooling buffer on electrophoresis.
The electrophoresis was carried out using 0.6 % low-melting-temperature agarose and TBE buffer for 6 hr in buffer circulation(a) and in cold chamber(b). The voltage was 150 V.

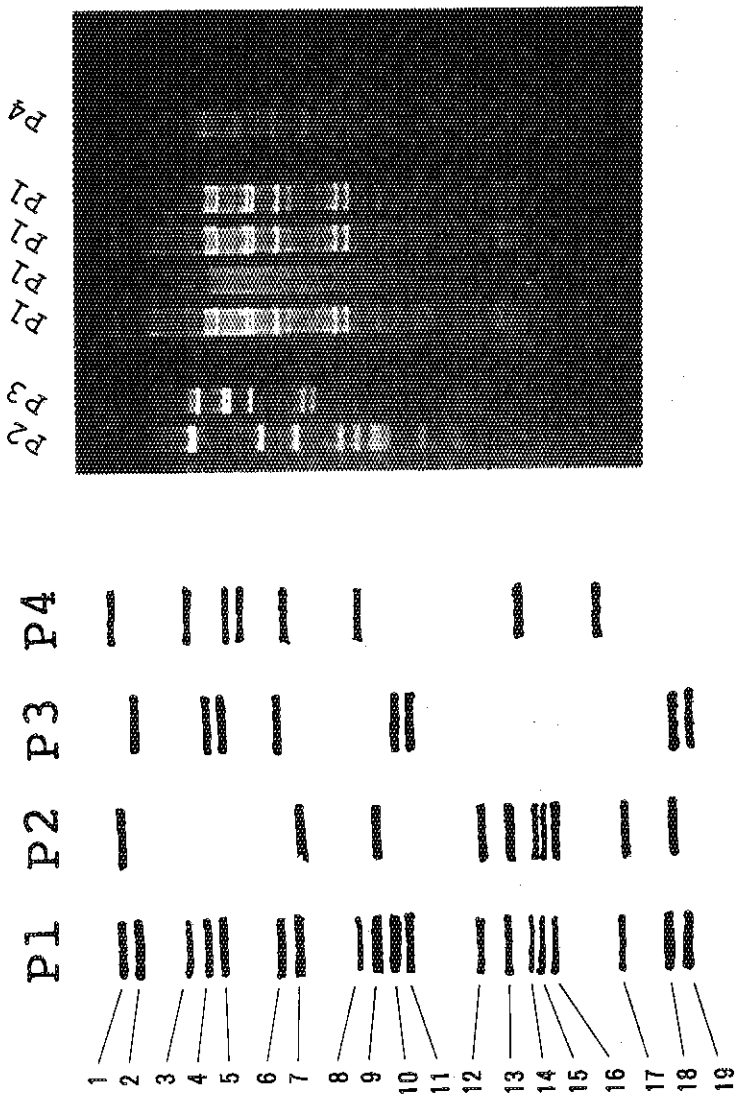


Fig. 6 Digestion of P1, P2, P3 and P4 plasmid by restriction enzyme, Sma I.

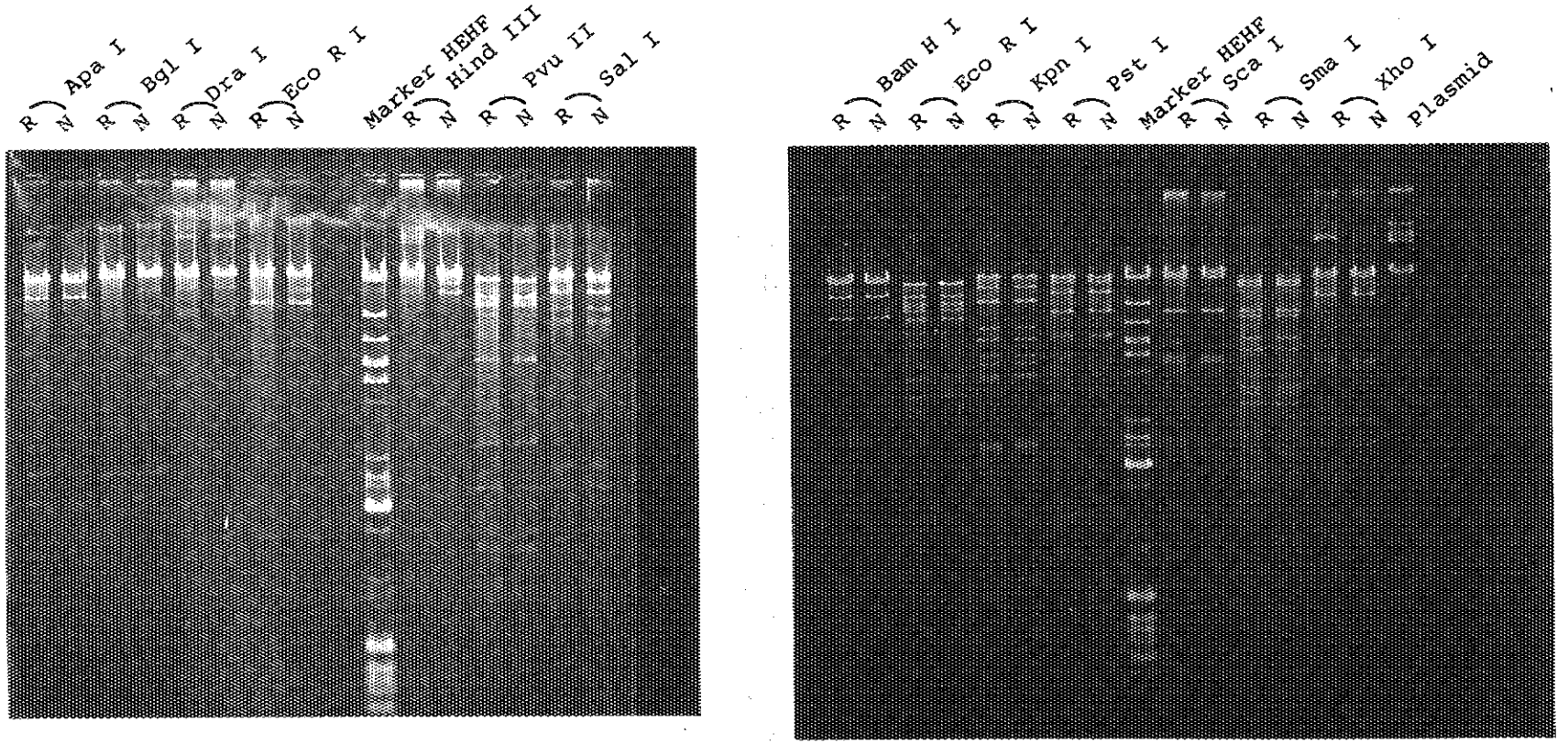


Fig. 7 Digestion of whole plasmid including P1 to P4 by various kinds of restriction enzymes.

Apa I, Bgl I, Dra I, EcoR I, Hind III, Pvu II, Sal I, BamH I, Kpn I, Pst I, Sca I, Sma I and Xho I were used as enzymes.

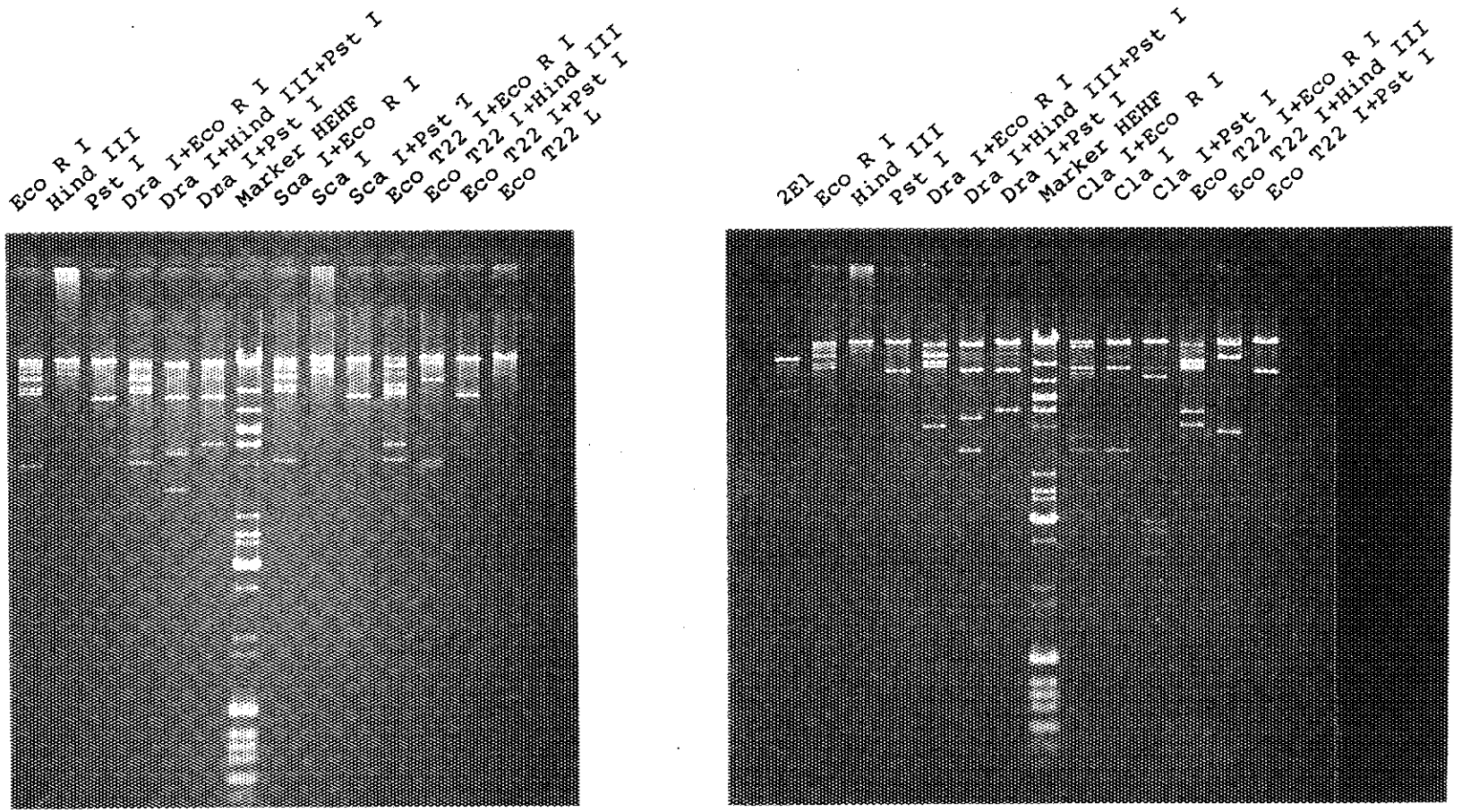
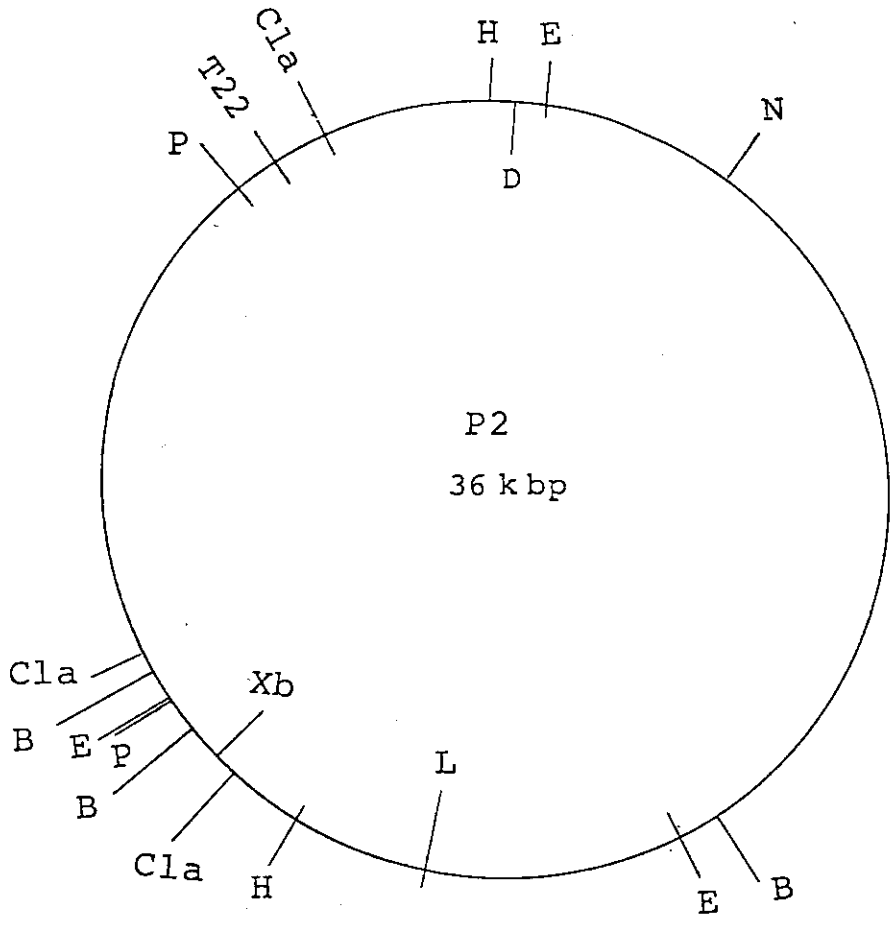


Fig. 8 Digestion of P2 plasmid by various kinds of restriction enzymes.

The enzymes used were EcoR I, Hind III, Pst I, Dra I, Cla I, Sca I and EcoT22 I.



The number of fragments produced by enzyme:

B	(BamH I)	: 3	L	(Sal I)	: 1
E	(EcoR I)	: 4	N	(Not I)	: 1
H	(Hind III)	: 2	Cla	(Cla I)	: 3
P	(Pst I)	: 2	Xb	(Xba I)	: 1
T22	(EcoT22 I)	: 1			

Fig. 9 Restriction map of P2 plasmid.