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ISOLATION AND CHARACTERIZATION OF PLASMIDS IN *XANTHOMONAS MANIHOTIS*[1,2]

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Abstract

Three extrachromosomal deoxyribouncleic acids (DNAs) were isolated from *Xanthomonas manihotis* by the technique of ethidium bromide cesium chloride density gradient centrifugation, while attempts to isolate plasmid DNA from *X. citri, X. oryzae or X. pruni* by the same technique were unsuccessful. The plasmid DNAs extracted from *X. manihotis* have three different sizes of supercoiled DNA. The GC molar ratio of both plasmid DNAs and chromosomal DNA are the same based on the melting temperature (96.2°C) and the density (1.724 g/cm^3).

Introduction

Plasmid is an extrachromosomal genetic element which is capable of autonomous replication (Lederberg, 1952). It has been recently one of the rapidly growing research topics. Many bacteria were found to contain one or more than one kind of plasmids in the cytoplasm. They also play different functional roles in their own existence, namely: as a resistance factor exhibiting resistance to antibiotics or heavy metals; as a fertility factor that conjugates male and female bacteria; as a virulence factor that induces plant tumor and as an enzymatic factor that produces restriction endonuclease (Reanney, 1976). The most important and attractive function of plasmid is probably its ability to serve as a genetic cloning vehicle. Itakura *et al.* (1977) demonstrated that a piece of DNA, the man made somatostatin gene, joined with plasmid pBR322 DNA was introduced into *E. coli* cell by transformation. Somatostatin was not only produced in this bacterium, also exhibited its biological function. This has been another milestone since the first recombinant DNA was made (Morrow *et al.*, 1974) where an eukaryotic gene was introduced and expressed in the transcriptional level in a prokaryotic cell. In regard to the virulence factor, plasmid was found to be associated with all the pathogenic strains

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in *Agrobacterium tumefaciens*, a causal agent of crown gall disease, but not with the avirulent strain. It was then hypothesized that plasmid was responsible for the tumorigenesis (Zaenen, *et al.*, 1974). Later plasmid DNA was successfully isolated from an avirulent strain *A. radiobacter* and another avirulaent strain *A. tumefaciens* (Lin, 1976). This finding challenged previous statement about the hypothesis that avirulent strains have no plasmid (Zaenen *et al.*, 1974). Merlo and Nester (1977) were able to confirm this finding. Therefore, this method for isolation of a plasmid may be useful for other bacteria.

A survey for the presence of plasmids among plant pathogenic *Xanthomonas* species was conducted on strains of *X. citri*, *X. oryzae* 604, *X. pruni* and *X. manihotis*, causal agents for various diseases on citrus, rice, stone fruits and cassava, respectively. We found only *X. manihotis* contained plasmids. After a series of pruification, pure DNA samples were examined under electron microscope and analyzed with gel electrophoresis. The results indicate that this bacterium contained three different kinds of plasmids. This paper presents a fast and efficient procedure to isolate and characterize the cryptic plasmids from *X. manihotis* without using radioisotope.

Materials and Methods

Bacteria and media

Bacteria employed in this study were shown in Table 1. Media used in this study depended on bacterial species. The enriched minimal 9 medium (EM9) containing casein hydrolysate 2 g, yeast extract 1 g in addition to M9 medium (Miller, 1972) was employed for most species except *X. oryzae*, which could not grow well in EM9 medium. The potato sucrose medium (PS) (Kuo *et al.*, 1974) was used for the culture of *X. oryzae*.

Table 1. *Bacterial strians*

Bacteria	Virulence	Host	Original location	Source
X. citri	+	Orange	Taiwan	T.T. Kuo, Inst. of Botany, Academia Sinica.
X. manihotis	+	Cassava	Taiwan	B.C. Lin, Inst. of Botany, Academia Sinica.
X. oryzae 604	+	Rice	Taiwan	T.T. Kuo, Inst. of Botany, Academia Sinica.
X. oryzae 507	−	derive from 604	Taiwan	T.Y. Chow, Inst. of Botany, Academia Sinica.
X. purni 13D52	+	Plum	California	C.I. Kado, Univ. of California, Davis.

+: virulence; −: avirulence.

Chemicals

Tris (hydroxymethyl)-aminomethan (Tris), analytical CsCl and casein hydrolysate were purchased from Merck, West Germany, while EDTA (disodium ethylenediamine tetraacetate), lysozyme (eggwhite grade I), RNase, DNase and triton-X100 were from Sigma Chemical Co., USA., and agarose was from Bio.Rad Laboratories, USA.

Preparation of plasmid

Forty milliliters of overnight culture in gyratory water bath shaker (New Brunswick model G76) at 30°C were harvested by centrifugation (Sorvall rotor SS-34) at 8,000 rpm, 4°C for 5 min. The precipitated cells were washed once with TE buffer (0.01 M Tris-HCl, 0.001 M EDTA, pH 8.0), and resuspended in 4 ml of solution A (0.1 M NaCl, 0.02 M EDTA and 20% sucrose pH 8.0). Four mg of fresh lysozyme powder were added while the suspension was held in an ice bath. After 10 min the solution B (2% triton X-100 or other detergents described in Table 2) was slowly added and mixed gently with a vortexgenie mixer (Scientific Product Co.), and finally the mixture was allowed to stand at room temperature at 28°C for 10 to 30 min. As soon as a clear lysate was obtained, the lysate in a 35 ml Sorvall centrifuge tube was sheared at highest speed in the vortex-genie mixer for 30 sec., and then ehtidium bromide was added to the sheared lysate and adjusted to 100 μg/ml as the final concentration (Lin and Kado, 1977).

Table 2. *Conditions for isolating plasmid from various Xanthomonas spp.*

Detergents	Treatments	*X. citri*	*X. manihotis*	*X. oryzae* 604	*X. oryzae* 507	X. pruni
Brij 1.0% (w/v)	No	–	+	–	–	–
SDS 0.1% (w/v)	No	–	–	–	–	–
SDS 0.5% (w/v)	No	–	+	–	–	–
SDS 1.0% (w/v)	No	–	–	–	–	–
Sarkosyl NL-30 1.0% (v/v)	No	–	+++	–	–	–
Triton X-100 1.0% (v/v)	No	–	+++	–	–	–
Triton X-100 1.0% (v/v)	Proteinase	–	+++	–	–	–
Triton X-100 1.0% (v/v)	Heat 70°C	–	+++	–	–	–
Triton X-100 1.0% (v/v)	RNase	–	+++	–	–	–

– No plasmid was detected.
\+ Small amount of plasmid was obtained.
+++ Good yield of plasmid was obtained. At least this was two time as much as singal "+".

Ethidium bromide-CsCl buoyant density gradient centrifugation

Clewell and Helinski's modified method (1969) from Radloff *et al.*, (1967) was mainly followed. The sheared lysate with ethidium bromide was adjusted with CsCl powder to a refractive index (η) of 1.3900 by a refractive meter (Bausch and Lomb Co.). The final volume of this CsCl solution was about 13 ml. They were then divided into two centrifuge tubes (Beckman rotor type 65, polyallomer tubes) and filled up the remaining space with paraffin oil. The buoyant density gradient centrifugation was running at 44,000 rpm, 20°C for 44 hr. The plasmid band was observed under long wave length UV light (UV-56, UltraViolet Production Inc., California). The plasmid DNA was extracted with isopropanol or n-butanol and dialyzed overnight against 3 liters of TE buffer with two exchangers. The ethidium bromide free plasmid DNA was stored at 4°C for further studies.

CsCl equilibrium density gradient

The ethidium bromide free plasmid and chromosomal DNA were separately recentrifuged in ethidium bromide-CsCl buoyant density gradient. DNAs from these two gradients were collected and the ethidium bromide was also extracted by the same procedures described above. Plasmid DNAs, chromosomal DNA and *E. coli* chromosomal DNA solutions were added with CsCl powder and adjusted to a refractive index (η) of 1.400 because these DNAs had no ethidium bromide and their density had not been reduced. The centrifugation was performed at 44,000 rpm, 20°C for 68 hr and DNA bands were formed in the centrifuge tubes. These DNA bands were scanned by Gilford UV-spectrophotometer (model 24000), and these DNAs were collected by fraction collector.

The determination of DNA melting temperature

The technique of using UV absorbance-temperature profile for determining the guanine-cytosine content of a double strand DNA was described by Mandel and Marmur (1968). The purified DNA from either chromosomal DNA, linear plasmid DNAs, which was previously treated with DNase, or both were adjusted to a concentration of about 0.8 (A_{260}) in 1x SSC solution (0.15 M NaCl, 0.015 M Na_3citrate, pH 7.0), the DNA sample in the cuvette was degassed in vacuum chamber, and the stoppers of the cuvette were then tightened. The temperature of DNA solution must be allowed to be in equilibrium for each temperature increment. Gilford UV-spectrophotometer with recorder and temperature sensor was employed in this investigation.

Agarose gel electrophoresis

The preparation of an agarose gel was modified from the method described by Sharp *et al.*, (1973). Agarose solution (0.7%, w/v) was boiled in TBEE buffer (Tris, 89 mM, borate 8.9 mM, EDTA, 1 mM and 0.5 μg/ml of ethidium bromide, pH 8.0) for 30 min. After cooling the homogenous gel solution to 60°C, the agarose gel solution was poured into a disc gel apparatus (5 mm in diameter, 100 mm in length). Gelatination was

completed within 20 min at room temperature. Five microliters of a mixture of 33% (v/v) glycerol, 0.7% bromophenol blue were added to the 45 μl of DNA sample before loading on the gel. The remaining volume of the well was filled with TBEE buffer. Electrophoresis was carried out at one mA and fifteen volts per tube for 16 hr.

Electron microscopy

The procedures for preparation and observation of DNA under the electron microscope were essentially the same as that described by Davis *et al.*, (1971) and later modified by Lin and Kado (1977). Plasmid DNA was adjusted to 2 μg/ml in the TE buffer. The DNA molecules would be laid separately on a two dimensional protein film, which was made by spreading the hydrophilic and hydrophobic cytochrome C protein on the hypophase of a glass-double-distilled water. A 300 mesh copper electron microscope grid (Electron Microscopy Sciences, Fort Washington, Pennsylvania, U S A.) coated with 0.35% formvar was picked up by a fine forceps and the coated side of the grid was touched to the surface of the basic protein film to stick DNA molecules. The grid was dipt into a fresh solution of uranyl acetate in acidic ethanol for 30 seconds, and then submerged in 95% ethanol for 5 seconds. Excess ethanol on the grid was drained on filter paper and the grid was mounted on double coated tape attached to a stainless steel circular plate. The DNA was shadowed by the rotary method (Lin and Kado, 1977) using platinium or uranium. These metals were prewashed with petroleum ether. The preparations of DNA on the grid were shadowed at a distance of 8 cm from the wire and 1 cm below the wire where the platinium or uranium were coiled, and the rotation rate of the circular plate made by stainless steel was set at 90 rpm under high vacuum condition (10^{-5} torr). The Hitach model HU-11 A electron microscope was employed for this study.

Results

The proper condition of plasmid isolation

One of the convenient methods for determining the efficiency of cell lysis (if any) was to see whether the lysate would be clear within 20 or 30 min after detergent was added (Lin, 1976). In this study all bacteria tested were easily shown to have a clear lysate within 15 min. This indicates that the process of lysis was in a good condition, however, we obtained the plasmid DNA only from *X. manihotis*. One percent of sodium dodesyl sulfate (SDS) was always employed as an efficient detergent for the plasmid isolation (Gurry *et al.*, 1973; Clewell and Helinski, 1969), but we could not obtain any plasmid from *X. manihotis* in 1% SDS. The efficiency of recovering the plasmid DNA from bacterial cells was shown to be different in various detergents, therefore, we tried the lower concentration of 0.5% and 0.1% SDS, respectively and found that 0.5% SDS was a suitable concentration for isolating plasmid from *X. manihotis*. However, the yield of plasmid was not so good as compared to other detergents listed in the Table 2, in which triton X-100 was the best for the plasmid DNA isolation, However, protease, heat

and RNase treatments could not produce a significant increase in plasmid yield.

Isopycnic dye CsCl Buoyant density gradient centrifugation

The ethidium bromide-CsCl density gradient was the most efficient method for isolating plasmid DNA. Since most plasmid DNAs have the same or similar density as their host chromosomal DNA, it is difficult to separate them by equilibrium density gradient centrifugation. Vinograd's group (Radloff *et al.*, 1967) introduced this plasmid DNA isolation technique which is based principally on the different topology of the DNA structures between linear and supercoiled form. The ethidium bromide was a dye and also a DNA interchelating agent, which could make non-covalently binding to DNA (Waring, 1965) and made the natively helix DNA turn to positively helix DNA (Vinograd and Lebowitz, 1966). Therefore, the supercoiled DNA was restricted in its binding with ethidium bromide by the tightness of positive helix DNA, while the linear DNA was free to uptake this dye. As a consequence of this difference, the density of linear DNA with dye would be lower than that density of supercoil DNA with the same dye. Based on this concept, the plasmid DNA of *X. manihotis*, as shown in Figure 1, formed a band which was separated from chromosomal DNA.

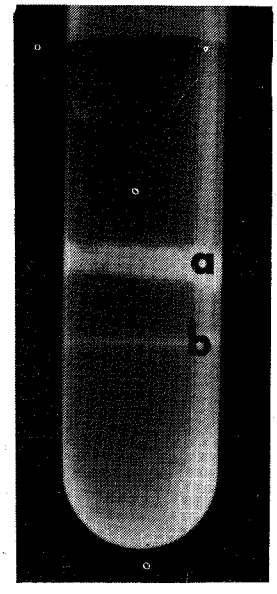

Fig. 1. Ethidium bromide-CsCl density gradient showing the plasmid DNA band (b) under the chromosomal DNA band (a) of *X. manihotis*.

Base composition of plasmid DNA

The base composition of plasmid DNA and chromosomal DNA were the same.

1). *Density of plasmid DNA*: Plasmid DNAs purified twice from a dye-CsCl

density gradient was analyzed by a CsCl equilibrium density gradient centrifugation. DNA from *E. coli* was employed as a marker of a known denisty, 1.710 g/cm^3, and Figure 2b shows the density of plasmid DNAs was the same as that of chromosomal DNA. This density of 1.724 g/cm3 corresponded to a guanine-cytosine content of 65.3 mole percent GC as calculated by the equation of

$$GC = \frac{\rho - 1.660}{0.098}$$

(Schildkraut *et al.*, 1962; Mandel 1968).

Fig. 2. CsCl equilibrium density gradient of plasmid DNAs and chromosomal DNA from *X. manihotis*. A. showing the standard *E. coli* DNA, 1.710 g/cm^3 and plasmid DNAs, 1.724 g/cm^3. B. showing the mixture of both plasmid DNAs and chromosomal DNA are the same as 1.724 g/cm^3.

2). Hyperchromicity: Melting temperature of DNA was proportional to the GC molar ratio of DNA. When the temperature of DNA solution was elevated, the UV absorbance temperature profile would be significantly increased. Because the arrangement of the DNA nucleotide sequences in a double helix causes a reduction in their molar extinction coefficient, hypochromicity will occur. On the other hand, when the double helix DNA was disassociated and hyperchromicity will occur again. Figure 3 shows the linear plasmid DNAs having a melting temperature of 96.5°C closed to 96.2°C, another melting temperature of their host chromosomal DNA. According to the equation

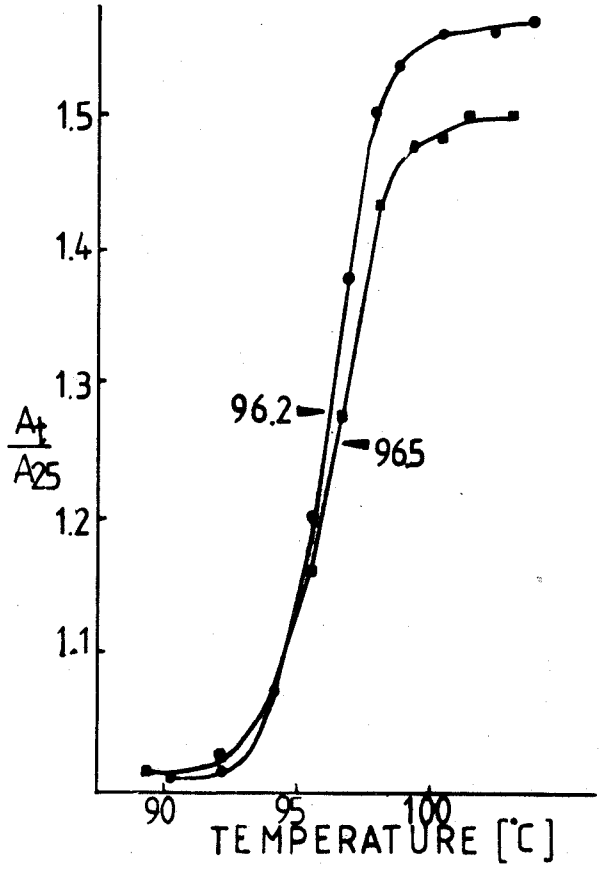

Fig. 3. The melting temperature of chromosomal DNA (96.2°C •————•) and plasmid DNAs (96.5°C. ■————■) from *X. manihotis.* A_t/A_{25} is the corrected absorbance at each temperature divided by the initial absorbance at 25°C.

of GC = (Tm- 69.3) x 2.44 (Mondel *et al.,* 1968), we calculate the GC content of plasmid DNAs to be 66.4%, while their host chromosomal DNA was 65.6%.

Three different molecular sizes of plasmids

Figure 4 shows three plssmid DNAs in 0.7% agarose gel. To use TBEE buffer system for the observation of DNA bands under UV light during electrophoresis is a very powerful parameter to measure the structures and molecular weights, therefore, these three DNA bands in Figure 4 might be interpreted as three different forms of plasmid DNA of the same molecular wieght; namely linear, open circle and supercoil forms could be separated well in the agarose gel electrophoresis.

In order to make sure whether these three bands represent three different sizes

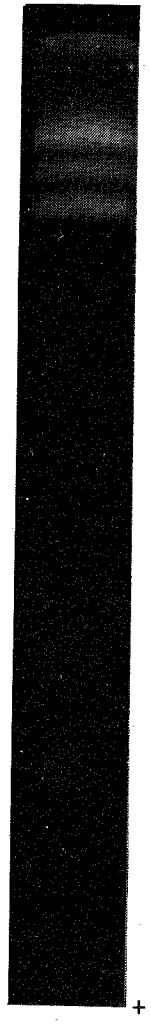

Fig. 4. Three distinct plasmid DNA bands in 0.7% agarose gel. The electrophoresis was running from cathode to anode.

of molecules rather than three different forms, we carried out the following procedures: First we employed the plasmid purified twice by dye-CsCl densitsy gradient to prevent the contamination of linear form plasmid DNA. If there was any open circle form DNA contamination, it would limited to a minute amount. Second we treated these plasmid DNAs with alkaline solution up to pH 12.2 for 15 min and then neutralized by the same normal of HCl (0.1 N) The neutralized plasmid DNAs were run in the 0.7% disc agarose gel and the same result was obtained (Figure 4). These results confirm that the three bands of DNA were of different molecular weights instead of different forms.

Contour length

Figure 5 and Figure 6 show the different sizes of plasmid DNAs. According to the

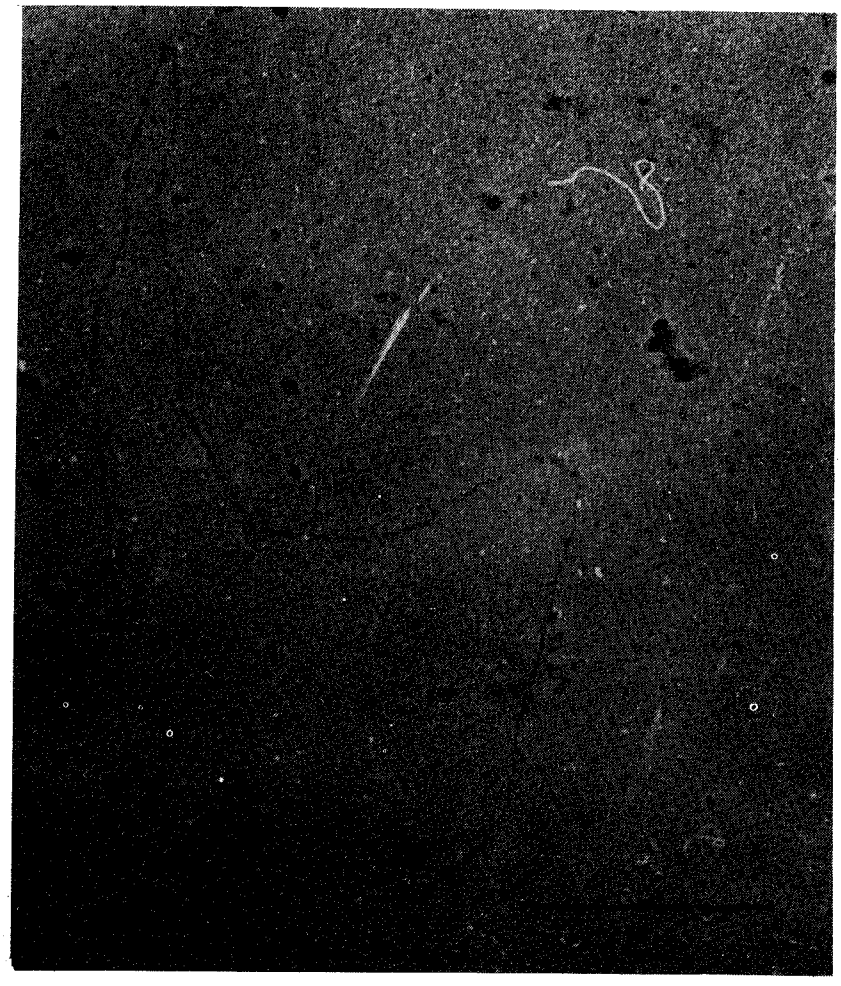

Fig. 5. A tyical open circular plasmid DNA molecule of *X. manihotis*. The length of bar is equal to one micrometer.

contour length, these three plasmids have moleuclar wieght of 18x, 40x and 72x 10^6 daltons, respectively (Lang *et al.*, 1967). The evidence that the plasmid DNA was autonomous in replication is shown at Figure 6A, which indicates that the DNA isolated from *X. manihotis* was a really plasmid DNA.

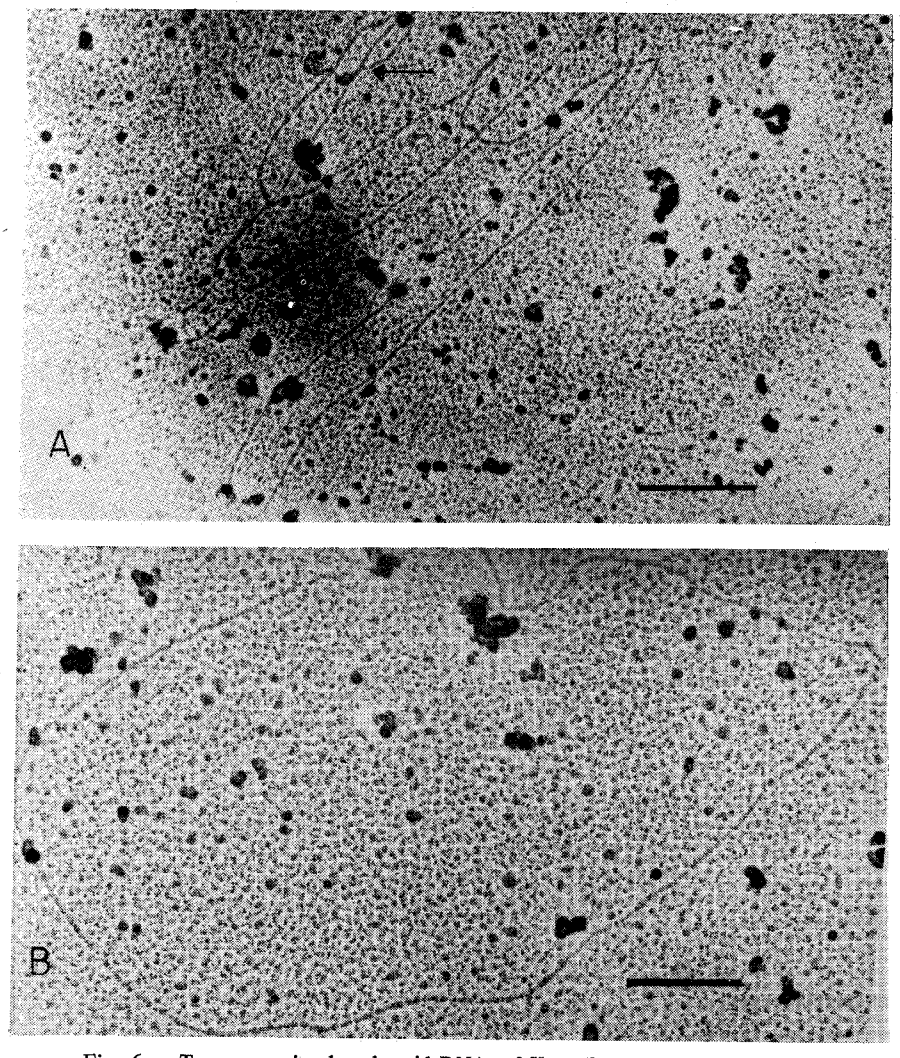

Fig. 6. Two open circular plasmid DNAs of *X. maihotis.* The eye structure of plasmid DNA is showing at the up of picture A. The contour length of these molecules are derived from the bar of one micrometer.

Discussion

This paper is the first report about plasmids detected in *X. manihotis.* Although Lai *et al.* (1977) reported that *X. campestris, X. corylina, X. diffenbachia, X. hederae, X. incane, X. juglandis, X. malvacearum, X. pelargoni* and *X. vesicatoria* had RP4, RK2 or R18-1 plasmids inside the bacterial cell, those plasmids were actually transconjugated either from *Pseudomonas aeruginosa* (R18-1), *E. coli* (RP4) or *E. coli* (RK2) into each receptor *Xanthomonas* species and they did not have physical evidence of the plasmid contained. However, we have directly isolated three kinds of plasminds from *X. manihotis* and characterized some features of the plasmid DNAs.

Protease, heat and RNase treatments followed the clear lysate step were set for increasing the plasmid yield. Protease was self digested at 37°C for 90 min before applying to the clear lysate and this treatment was assumed to increase the amount of plasmid released from cell debris (Womble *et al.*, 1977), the heat treatment was reported as an efficient method for obtaining plasmid DNA from small amount of bacterial lysate (Barnes, 1977), and the DNase free RNase was reported to help the plasmid releases from chromosomal DNA (Pettijohn *et al.*, 1973). However, we failed to get the good yield of plasmid by these three treatments as shown in Table 2, it may require radioisotope labelling for a more accuracy measurement.

The same GC value of plasmid DNAs as their host chromosomal DNA (Figure 2b) indicates that the plasmid DNAs are indigenous in *Xanthomonas manihotis*. This is quiet different from Lai's report (Lai *et al.*, 1977) which showed plasmid in *Xanthomonas* species was from other genus' bacteria. The isolated plasmid DNAs in our studies demonstrate a symmetric density profile of absorbance at wave length 260 nm (Figure 2). The GC content of chromosomal DNA derived from the UV absorbance temperature profile gave the same value as that derived from CsCl equilibrium density gradient analysis. From these data we consider that the DNAs we isolated are quiet pure DNA. However, the melting temperature of these three plasmid DNAs gave a slightly higher UV absorbance temperature profile than that of a linear DNA. This difference might be resulted from partial contamination with DNase undigested supercoil DNA. The shape of the melting temperature curve shown in Figure 3 clearly suggests that these plasmid DNAs have no unusual GC distribution among their nucleotide sequences arrangements.

The contour length of plasmid DNAs are measured by the calculation of 1 μ = 2.07 x 10^6 dalton, assuming a proper ionic strength in the DNA spreading solution (Lang *et al.*, 1967). Gel electrophoresis is another common method to calculate the molecular weight. In this case, when the molecular weight is over 20 x 10^6 daltons, it is out of the accuracy range of the gel system (Sharp *et al.*, 1973). The precise measurement of molecular weight of these plasmid DNAs are undertaking.

The eye structure shown in Figure 6A seems to suggest that some plasmid DNA is in the early replication stage (Cairns, 1963). This evidence also proves that these plasmid DNAs isolated from *X. manihotis* fit the original Lederberg's definition of plasmid (Lederberg, 1952).

Since *X. manihotis* is sensitive to antibiotics such as chloramphenical, kanamycin, mitomycin, neomycin, novobiocin and tetracycline, the plasmids under studies are possible to have other biological function(s). Furthermore, there is no record about the genetic marker or any recombination system found in this bacterium, therefore, more information must be obtained before analyzing plasmid's function, particularly the recombination system must be worked out.

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樹薯細菌性萎凋病原菌中質體DNA的分離與其理化特性

林伯仟　戴鴻璋　陳淑珍　簡名正

中央研究院　植物研究所

樹薯細菌性萎凋病原菌 (*Xanthomonas manihotis*) 中有三種不同大小的質體。但以同樣染料一氯化銫的浮力密度階梯法，不能在柑桔潰瘍病菌 (*X. citri*) ，水稻白葉枯病菌 (*X. oryzae*) 或梅樹萎凋病菌 (*X. pruni*) 中分離出質體DNA。

依據雙股DNA加熱變成單股DNA的融點 96.2°C 和密度每立方公分有 1.724 公克。推測出這三種質體 DNA 的理化性質和細菌內的染色體的烏苷酸和胞苷酸都是相似的。