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EXTRACTION OF HIGH QUALITY DNA FROM POLYSACCHARIDES-SECRETING XANTHOMONADS

by

Y. JAUFEERALLY-FAKIM[1] and A. DOOKUN

Department of Biotechnology, Mauritius Sugar Industry Research Institute,

Reduit, Mauritius;

[1] Faculty of Agriculture

University of Mauritius, Réduit, Mauritius

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ABSTRACT

A DNA extraction method using CTAB was used for the isolation of genomic DNA from ten *Xanthomonas campestris* pathovars, ten isolates of *Xanthomonas albilineans* and one isolate of *Pseudomonas rubrisubalbicans.* High quality DNA was obtained that was ideal for molecular analyses. Extracellular polysaccharides were effectively removed thus resulting in DNA that dissolved easily and was well digested by restriction enzymes. All of the other methods tested resulted in the coprecipitation of the polysaccharides together with the nucleic acids upon addition of alcohol so that even high yields could not compensate for this contamination. DNA obtained by the CTAB method was used for cloning, Southern hybridizations and PCR for up to three years after the extraction.

Keywords: Polysaccharides, CTAB, polymerase chain reaction, DNA.

INTRODUCTION

Good quality DNA is a prerequisite for the application of molecular techniques when studying any organism. Standard methods for bacterial DNA extractions rely on the lysis of the cells with sodium dodecyl sulphate (SDS) or N- lauryl sarcosine (Sarcosyl) in a buffer containing proteinase. This is followed by phenol/chloroform extractions to remove proteins and precipitation of the nucleic acids with ethanol. This work very well for most bacteria. However, with organisms that secrete large amounts of extracellular polysaccharides (EPS), the DNA thus obtained is highly contaminated with the EPS. The latter prevents the dissolution and restriction digestion of the DNA making it very difficult to work with.

Xanthomonads are gram-negative, phytopathogenic bacteria affecting many commercially important plants. They produce typical yellow pigments known as xanthomonadins which have been used as chemotaxonomic markers (Starr *et al.*, 1977). Two major diseases of sugarcane are caused by members of the Xanthomonas genus, and they are *Xanthomonas campestris* pv. *vasculorum* and *Xanthomonas albilineans* (Ricaud *et al.* 1989). In order to develop molecular tools for diagnostic purposes and for epidemiological studies, it was essential to have good quality DNA that was not contaminated with inhibitors of DNA modifying enzymes and polymerases. Both pathogens secrete excessive amounts of polysaccharides even in liquid cultures.

X. campestris pv. *vasulorum* produces large quantities of xanthan gum responsible for the gumming aspect of the disease. Xanthan gum has high commercial value and is industrially produced for use as gelling and stabilising agent in foods and other commodities. These molecules are important determinants of the pathogenicity of the organisms producing them and reduced production of EPS has been associated with reduced aggressiveness (Tang *et al.* 1990).

Members of the *Xanthomonas campestris* species and *Xanthomonas albilineans* produce EPS that are difficult to remove by washing. It has previously been reported that washing Xanthomonas cells grown in TY-MOPS medium (Gabriel *et al.* 1989), in NE (50 mM EDTA, 0.15 M NaCl, pH 8.0) buffer makes the EPS float above the pellet of cells (Gurr *et al.* 1992). This did not work in our hands with the above-mentioned bacteria. Extraction of DNA was first attempted by lysing with either SDS or Sarcosyl then performing deproteination with the phenol/chloroform steps and precipitating the nucleic acids by addition of ethanol. These methods have been described by Cook *et al.* (1989) and Gurr *et al.* (1992). The DNA solution obtained was very viscous and difficult to pipette, particularly after thawing. Restriction digestions were often partial even with high enzyme concentrations.

Procedures for extraction of plant genomic DNA often calls for an incubation step with CTAB (cetyl-trimethylammonium bromide) which is a cationic detergent for the removal of contaminants. Depending on the concentration of NaCl in the solution, CTAB either precipitates the DNA or the contaminants. At 0.7-0.8 M NaCl, polysaccharides precipitate out, while the nucleic acids remain in solution.
The method used here is based on that of Ausubel *et al.* (1994). It was scaled up to accommodate 50ml cultures. The modifications were a thorough washing of the cells with a sodium chloride/ EDTA buffer and longer centrifugation and incubation times. This protocol is quick, inexpensive and above all yields high quality DNA for PCR and hybridisation purposes.

MATERIALS AND METHODS

DNA extraction

CTAB/ NaCl solution (4.1 g NaCl added to 80 ml of water, 10 g of CTAB is slowly added while heating. The volume is made up to 100 ml).

1. Cultures of bacterial cells were made in Wilbrink (1920) medium at 22°C. 50 ml of liquid culture was centrifuged for 10 minutes. The supernatant was removed and the pellet washed twice in NE buffer (0.15 M NaCl/ 50 mM EDTA).
2. Pellet was resuspended in 3860 ml of TE (Tris 10 mM; EDTA 1mM) buffer; 40 ml of proteinase K (10 mg/ml) and 100 ml of 20% SDS are added. Mixture was incubated for at least 2 hr to allow complete lysis.
3. 667 ml of 5 M NaCl was added to give a final concentration of 0.7 M.
4. 533 ml of CTAB/NaCl (10% CTAB; 0.7 M NaCl) solution was added and the solution was mixed thoroughly and incubated at 65°C for 30 min.
5. An equal volume of chlorofrom/isoamyl alcohol (24:1) was added and the tube was centrifuged for 15 minutes.
6. The aqueous phase was removed into a clean 15-ml tube and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) added. After mixing and centrifuging the supernatant was removed.
7. 0.6 volume of isopropanol was added to the supernatant to precipitate the nucleic acids. At this point a white precipitate was clearly visible and could be removed with a sealed pasteur pipette (when the polysaccharides coprecipitate with the nucleic acids a clear, gelatinous mass is obtained).
8. The precipitate was washed by dipping the pasteur pipette in 70 % ethanol. It was then allowed to dissolve in 500 ml of sterile water and the solution was stored at -20 °C.

The volume of TE added in step 2 should be carefully measured by taking into consideration the volume of cells present. This is important in order to add the right volume of 5 M NaCl to give a final concentration of 0.7 M. When the aqueous phase is viscous and is difficult to pipette after addition of chloroform/isoamyl

alcohol, this is an indication that all of the polysaccharides have not been removed and the above volumes should be scaled up.

The bacteria used for DNA extraction are the following:
1. *Xanthomonas campestris* pv. *vasculorum* (sugar cane).
2. *Xanthomonas campestris* pv. *phaseoli* .
3. *Xanthomonas campestris* pv. *malvacearum* .
4. *Xanthomonas campestris* pv. *holcicola.*
5. *Xanthomonas campestris* pv. *translucens.*
6. *Xanthomonas campestris* pv. *vesicatoria.*
7. *Xanthomonas campestris* pv. *graminis.*
8. *Xanthomonas campestris* pv. *vasculorum* (Broom bamboo).
9. *Xanthomonas campestris* pv. *citri.*
10. *Xanthomonas campestris* pv. *campestris.*
11. *Pseudomonas rubrisubalbicans.*
12. *Xanthomonas albilineans* (ten isolates).

Restriction enzyme digestions.

Digestions were carried out in 70 ml volumes containing approximately 20 mg of DNA and 10 units of enzyme in the appropriate buffer. The tubes were incubated at 37° C overnight and the products electrophoresed on a 0.8 % agarose gel.

Polymerase chain reaction

The PCR was carried out in a 30 ml reaction volume containing 1.5 mM MgCl, 200 mM dNTPs, 0.5 unit *Taq* polymerase (Boehringer Mannheim) and 14 ng DNA. The primers used were derived from the sequence of a probe used to study *Xanthomonas albilineans* and had the following sequence.

F20 A (19 mer): 5’ -TTC GCC CTT AAC ACC GCC C- 3’
F20 D (19 mer): 5’ -ACC GCT GTA CAA CAA TAG C- 3’

The cycling program was as follows:
1. 94°C 5 min.
2. 35 cycles of:
   94°C 1 min
   50°C 1 min
   72°C 1 min
3. 72°C 10 min

The PCR products were visualised in a 1.5 % agarose gel.

RESULTS AND DISCUSSION

When the above protocol was adopted, pure DNA was obtained from all of the bacteria used. It went into solution easily and the latter was not viscous. They have all been used for restriction digestions, PCR and Southern hybridisations. Fragments of the genomic DNA of two *X. albilineans* isolates have been cloned and sequenced.

Fig. 1 shows the DNA obtained by this method. A single high molecular weight band is observed in all cases. Their digestibility by restriction enzymes, after repeated freezing and thawing, is revealed in Fig. 2. *Eco* R1 was used for all the samples in this figure. DNA contaminated with polysaccharides is not well digested with restriction enzymes and gives smears in hybridisation studies.

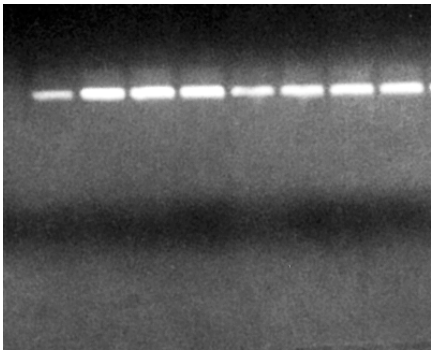

Fig. 1. Genomic DNA extracted from various pathovars of *X.campestris* and *X. albilineans* on a 1 % agarose gel. Lanes 1- 6: *Xanthomonas campestris* pv. *vasculorum* (sugar cane), *Xanthomonas campestris* pv. *phaseoli, Xanthomonas campestris* pv. *malvacearum, Xanthomonas campestris* pv. *holcicola, Xanthomonas campestris* pv. *graminis, Xanthomonas campestris* pv. *campestris;* lanes 7,8: *Xanthomonas albilineans.*

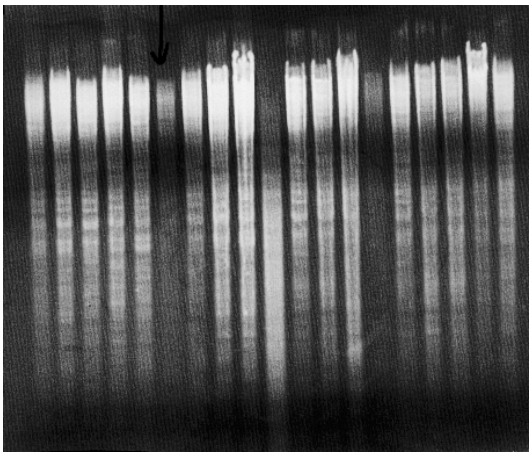

Fig. 2. *Eco* R1/ *Hind* III digests of genomic DNA. Lane 1: sugarcane genomic DNA; lane 2: *Xanthomonas campestris* pv. *vasculorum* (sugar cane); lane 3: *Xanthomonas campestris* pv. *phaseoli* ; lane 4: *Xanthomonas campestris* pv. *malvacearum* ; lane 5: *Pseudomonas rubrisubalbicans*; lane 6: DNA molecular weight marker III (B.Mannheim); lanes 7-13: *Xanthomonas albilineans.*

Fig. 3 shows a PCR performed on the bacterial DNA obtained using primers F20A and F20D. This set of primers gives amplification polymorphism among the strains of *Xanthomonas albilineans*.

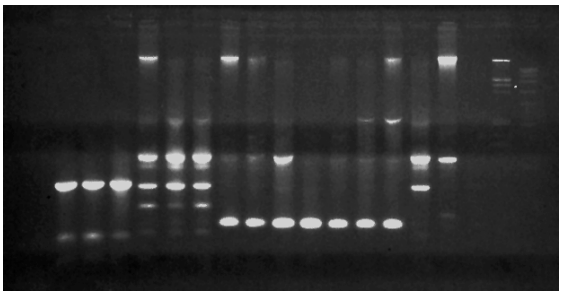

Fig. 3. Amplification with primers F20 A/D. Lanes 1-3: *X. campestris* pv. *vasculorum* races 1, 2 and 3; (lanes 4-17: *X. albilineans*); lanes 4-6: 3508, 3514, 3516 (Mascarene); lanes 7-10: 3509, 3510, 3511, 3518 (African serotype from Mauritius); lane 11: 3193 (African serotype from Burkina Faso); lane 12: 3194 (I. Coast); lane 13: Xa 23 (Florida); lane 14: Xa 97 (Dominican Republic) ; lane 15: Xa 6 (Kenya); lane 16 water control; lanes 17 and 18: DNA molecular weight markers III and VII respectively.

Yields varied between 50 and 70 mg of DNA per 50 ml of bacterial culture. No signs of degradation were observed. Proteinase K and the phenol/chloroform steps were adequate for removing proteins. There was no need to include the additional ethidium bromide/phenol/chloroform step as recommended by Chan and Goodwin (1995) for *Xanthomonas campestris* pv. *phaseoli*. The DNA obtained from *Xanthomonas campestris* pv. *vasculorum* by the above method was of as good quality as with the other pathovars. Satisfactory PCR has been performed on the samples even though, xanthan gum, which is secreted by this organism is an acidic extracellular polysaccharide. Acidic polysaccharides have been shown to inhibit PCR (Demeke and Adams, 1992) and no such inhibition was observed, thus confirming their effective removal. This DNA extraction method does not require that the bacteria be cultured in any special medium and there is no need for additional and lengthy purifications such as those using cesium chloride or ethidium bromide /phenol/ chloroform. It can be easily carried out in laboratories with limited resources and is particularly attractive for the clean DNA that is obtained. The DNA can be stored for several years and still retain its integrity after multiple freezing and thawing, as evidenced by PCR amplification and Southern hybridisations.

DNA solutions which were already contaminated with polysaccharides could not be cleaned by the method described. Addition of NaCl and CTAB to such a solution

did not result in separating the DNA from the polysacharrides. Two attempts were made towards this end and in both cases the DNA was lost together with the polysaccharides at the interphase, following the addition of chloroform. The amounts of DNA obtained from 50 ml cultures are ample for several Southern transfers and thousands of PCR reactions.

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