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Short Communication

Rapid and Efficient Protocols for Throughput Extraction of High Quality Plasmid DNA from Strains of *Xanthomonas axonopodis* pv *malvacearum* and *Escherichia coli*

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Efficient protocols developed to isolate low copy plasmid DNA from *Xanthomonas axonopodis* pv *malvacearum (Xam)* and high copy recombinant plasmid DNA from *Escherichia coli* are described. The protocol for extraction of low copy plasmid DNA from strains of *Xam* yielded high concentrations of plasmid DNA and used easily available and inexpensive chemicals in simple steps. The protocol for plasmid extraction from *E. coli* was rapid, cost-effective and yet yielded high concentrations of plasmid DNA. The procedures are simple and can be used to process several samples at one time. The plasmid DNA extracted by two methods was sufficiently pure, free from protein and other cellular contaminants and amenable to various molecular manipulations.

Key words: Plasmid DNA, Extraction protocol, *Xanthomonas axonopodis* pv. *malvacearum*, *Escherichia coli.*

Plasmids are extrachromosomal genetic material found in bacteria where, they govern variety of phenotypes right from pathogenicity to various biological processes (1). Genes governing pathogenicity and EPS production in a strain of *Xanthomonas axonopodis* pv *malvacearum* (*Xam*) was found to be plasmid-borne (2). Besides, plasmids are the most indispensable component of recombinant DNA technique, as vector for gene cloning.

Isolation of plasmid from strains of *Xam* is usually tricky due to a thick layer of exoploysaccharide, forming the outer envelope of this bacterium. Moreover, the plasmids in Xanthomonads, a group of bacterium that infect several economically important crop plants (3), are low copy plasmid. These limitations make it difficult to harvest plasmid in high concentration in single operation, when needed in larger quantity for characterization. Similarly in case of high copy *E. coli* vectors, where several samples need to be processed routinely at the same time and when resources are limited, high throughput yet cost-effective protocols capable of yielding high concentrations of plasmid DNA, amenable to molecular manipulations are important. Though several protocols are available for extraction of low and high copy plasmid DNA from plant pathogenic or non-pathogenic bacterial strains, including strains of *X. campestris* and *E. coli* (4-7), they often suffer from one or the other limitations. Most of these methods are lengthy, time-consuming, used costly chemicals, yet often less productive. Here we described two simple, rapid and economical miniprep methods for extraction of low as well as high copy plasmid DNA, amenable to molecular manipulations from strains of *Xam* and *E. coli,* respectively.

Races of *Xam* were identified as per Hunter et al 1968 (8). Cultures of *Xam strains* were grown in nutrient broth (4 ml) from a single colony of the bacterium grown on YGCA (9). The culture was incubated at 30°C in orbital shaker at 180 rpm. The bacterial cells were harvested from overnight culture in 2.0 ml micro centrifuge tube, washed with 0.7% aqueous solution of NaCl to remove EPS layer encapsulating the bacterial cells. The washed cells were thoroughly suspended by vortexing well in 300 µl of suspension buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 100 µg/ml RNase A). Cell lysis was done at room temperature by gentle agitation in 300 µl of lysing buffer (10% SDS, 200 mM NaOH) until the entire cell-mass melted into a clear viscous lysate. To the lysate, 300 µl of chilled neutralization solution (3.0 M potassium acetate, pH 5.2) was added and mixed thoroughly to avoid localized precipitation. The mixture was left on ice for 5 min to complete precipitation of SDS along with cell debris

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Abbreviations: PCR - Polymerase Chain Reaction; EPS - Exopolysaccharide; YGCA - Yeast glucose calcium carbonate agar.

followed by centrifugation at 13,225g for 10 min. The clear supernatant was transferred to a fresh microcentrifuge tube and extracted once each with equal volumes of phenol: chloroform: isoamyl alcohol (25:24:1) and chloroform: isoamyl alcohol (24:1). The contents of the tube were mixed by inverting the tube each time thoroughly well. The sample was centrifuged at 13,225 g for 5 min, each time transferring the aqueous layer containing DNA to a fresh tube. DNA in the sample was precipitated at room temperature with equal volume of isopropanol. The plasmid DNA was pelleted by centrifugation at 13,225 g for 5 min. The DNA pellet was washed with 70% ethanol and resuspended in 30 µl of sterile distilled water or suspension buffer without RNase.

In order to evaluate comparative efficacy of protocols plasmid DNA from four strains of *Xam* was also extracted by alkaline lysis methods of Kado and Liu (4), Gabriel and Defeyter (5) and Weng et al (6).

Liquid culture of *E. coli* (XL1 blue) harbouring recombinant plasmid pPKCCP16 (10) was initiated from a single colony grown on Luria agar (Luria broth, LB+15g agar/L), in 3 ml LB (10g tryptone, 5g yeast extract, 10g NaCl, distilled water, pH7.0), supplemented with ampicillin (70 µg/ ml). The culture was incubated overnight at 37°C in orbital shaker at 300 rpm. Bacterial cells in 1.5 ml culture were centrifuged in a microcentrifuge tube at 2817 g for 1 min. Most of the supernatant following centrifugation was discarded by inverting the tube. The cell pellet was resuspended by vortexing in remaining amount (about 100 µl) of culture filtrate to which was added 300 µl of lysing solution (Dissolve 0.2 g NaOH in 39 ml of water and add 1 ml of 20% SDS), prepared fresh before use. The suspension was gently mixed by inverting the tube 5-6 times, until a clear viscous lysate was formed. Cell debris along with SDS was precipitated with 175 µl of neutralising solution (3M Sodium acetate, pH 5.2), mixing the contents by inverting the tube few times. The precipitate was removed by centrifugation at 13, 225 g for 5 min. The supernatant was transferred to a fresh tube and plasmid DNA was precipitated with 1 ml of 95-100 % ethanol. Optional storage of the tube at –20°C for several minutes enhanced precipitation of plasmid DNA. The plasmid DNA was centrifuged at 13,225 g for 5 minutes and DNA pellet was rinsed briefly in 70% alcohol. High copy plasmid DNA was dissolved in 60 µl sterile distilled water or suspension buffer without RNase.

Purity of the plasmid DNA extracted using the two protocols was estimated by measuring the absorbance at A260/280 and A260/230.

The native plasmid DNA of *Xam* and recombinant plasmid extracted from *E. coli* (pPKCCP16) were used to amplify pathogenicity gene *pth*N (11) and coat-protein (CP) gene of CLCuV respectively, using gene-specific primers (10).

Plasmid DNA extracted from six strains of *Xam* was used to detect plasmid-borne pathogenicity genes using *pth*N (11) as DNA probe. Plasmid DNA (3 µg) from each strain of *Xam* was digested for 2h with 10U *Eco*RI (MBI, Fermentas) at 37°C. Following digestion and electrophoresis, Southern blotting of fragmented plasmids was done on positively charged nylon membrane, (Biodyne B, Pall Gelman, USA) by alkaline transfer using 0.4 N NaOH as transfer solution (12). Digoxigenin labelled *pth*N, was hybridized to *Xam* plasmid DNA and immunodetected using DNA-labelling and detection kit (Roche, Germany), following manufacturer's protocol. Recombinant plasmid pPKCCP16, was similarly subjected to Southern hybridization using DIG-labelled CLCuV-CP gene as DNA probe.

The comparative efficiency of three methods in extracting plasmid DNA from four independent strains of *Xam* was evaluated (Fig. 1). Besides, plasmid was also extracted from one of the strains of *Xam* by the protocol of Weng *et al* (6) (data not shown). All four methods yielded one or more bands of plasmid DNA from each of the four strains of *Xam*. The three methods showed identical plasmid

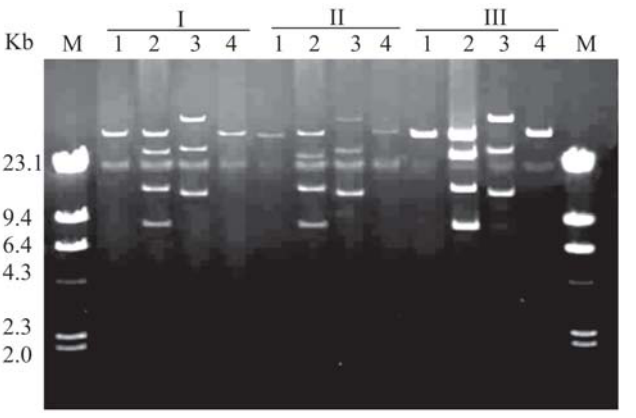

Fig. 1. Plasmid DNA (5 µl) extracted from *Xanthomonas axonopodis* pv. *malvacearum (Xam)* strains by methods of Kado and Liu, 1981 (I), Gabriel and DeFeyter, 1992 (II) and New protocol (III). M, λ/ *Hin*dIII; 1-4, plasmids of race 18 strains 53, 13, 32 and 43.

profile for the same strain. Nevertheless, the yields of plasmids varied with different methods used, which ranged from 0.8-1.5µg /ml culture (Fig.1). The new protocol yielded highest concentration of plasmid DNA, > 1.5 µg/ml compared to that when extracted by the method of Kado and Liu (4) where the yield was 1.0 µg/ml. The concentration of plasmid DNA though good was however lowest (0.7 µg/ml) when extracted using method of Gabriel and DeFeyter (5), under uniform conditions. Protocol of Weng *et al*. (6) is more of a qualitative nature which allowed general screening of plasmids. Most procedures for plasmid extraction rely on the principle of alkaline lysis of bacterial cells. The method of Kado and Liu, has been used widely for isolation of plasmids from several *Xanthomonads* including *Xam* (4). Small-scale extraction of plasmids from several species and pathovars of *Xanthomonas* has also been done by method of Gabriel and DeFeyter (5). Both these methods however, are lengthy and used some expensive chemicals. The new protocol neither uses chemicals like CAPS (5), nor is so lengthy like the method of Kado and Liu (4). Extraction of plasmid DNA can be accomplished within one hour in the new protocol compared to more than two hours when extracted with the method of Gabriel and DeFeyter (5). The method utilizes simple solutions without any complicated procedural step. The $A_{260/280}$ ratio of the plasmid preparation ranged between 1.8-1.9 suggesting that DNA preparation was sufficiently pure, free from protein and other cellular contaminations. The method yielded comparatively higher quantity of DNA in a shorter time and with negligible genomic DNA contamination. The step involving heating the lysate to denature DNA in Kado and Liu (4) procedure has been omitted. Although all protocols gave good yield of plasmids, the new protocol yielded highest concentrations of plasmid DNA with minimum contamination of genomic DNA (Fig 1).

Alkaline extraction procedure of Birnboim and Doly (7) is widely used for DNA extraction and screening recombinant plasmids from *E. coli*. However, the protocol takes enormously long time, more than 2.30 h. For making the DNA suitable for transformation and restriction digestion, further manipulations of more than 40 minutes are required. The protocol developed by us for extraction of high copy recombinant plasmid from strains of *E. coli* is simple and can be accomplished within 30-35 min. The procedure neither involves lengthy steps nor does it employ expensive chemicals like lysozyme for cell-lysis or hazardous chemicals liked phenol. Besides being rapid and throughput, the protocol yields high concentration of plasmid DNA of more than 3 µg/ml culture (Fig. 2).

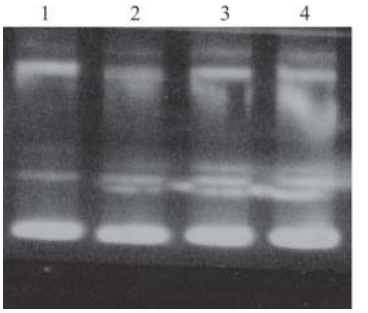

Fig. 2. Recombinant plasmid pPKCCP16 (5 µl) extracted from four independent colonies of *Escherichia coli* (XL1 Blue) by rapid method.

Plasmid DNA extracted from strains of *Xam* and *E. coli* using the new methods when used as template in PCR, resulted in amplification of a 0.4 kb *pth*N gene fragment in former (Fig. 3) and 0.7 kb CP gene (Fig. 4) in latter, as expected.

Native plasmid of *Xam* and recombinant plasmid pPKCCP16 of *E. coli* extracted was completely digested

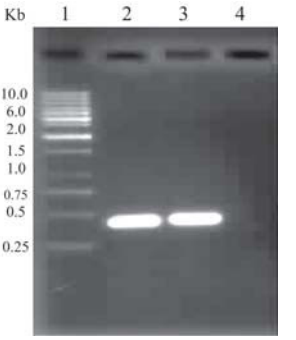

Fig. 3. PCR amplification of 0.4 kb *pth*N gene using plasmid DNA extracted from Xam strains. Lane 1, 1 kb DNA ladder; lane 2, strain 20 (race 18), lane 3, strain 24 (race 3), lane 4, negative control.

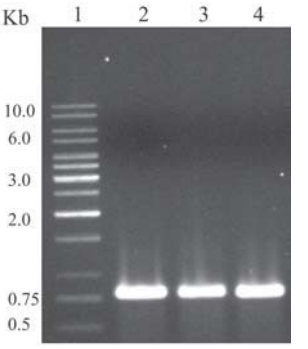

Fig. 4. PCR amplification of 0.7 kb Coat protein gene from plasmid pPKCCP16. Lane 1, 1kb DNA ladder; lanes 2-4, colonies of *E. coli* pPKCCP16.

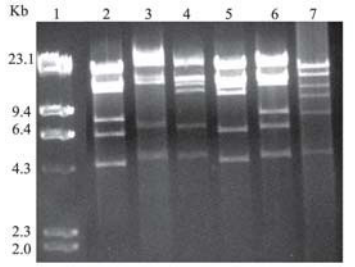

Fig. 5. *Eco*RI digested plasmid DNA of *Xam* strains. Lane 1, λ/*Hin*dIII; lanes 2-7, *Xam* strains, 20 (race 18), 24 (race 3), 40 (race 16), 86 (race 5), 102 (race 18) and 137 (race 9).

with the restriction enzymes used, showing that the DNA extracted using the new protocols were sufficiently pure and amenable to molecular manipulations. Plasmids of six strains of *Xam* yielded distinctly variable DNA profile with *Eco*RI except strains *Xam* 20 and *Xam* 102, which had identical fragmentation pattern (Fig. 5). The DIG-labelled *pth*N when used as DNA probe hybridised strongly to plasmid-borne pathogenicity (*pth*) genes possessed by all strains of *Xam*, yet exhibiting distinct polymorphism (Fig. 6). Members of *avr/pth* gene family exist in several plant pathogenic strains of *Xanthomonas* (13). Southern hybridization of *Eco*RI-*Bam*HI digested plasmid pPKCCP16 (Fig. 7) with DIG-labelled CP gene showed that the probe bound specifically to 0.7 kb CP gene cloned in 3.0 kb plasmid pGEMT (Fig. 8).

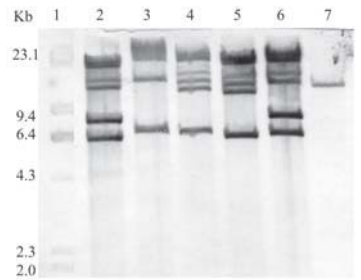

Fig. 6. The *Eco*RI digested plasmid DNA was hybridized with DIG-labelled *pth*N as DNA probe. Lane 1, λ/*Hin*dIII; lanes 2-7, *Xam* strains, 20, 24, 40, 86, 102 and 137.

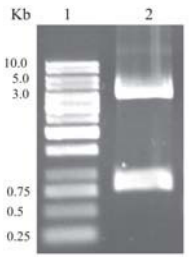

Fig. 7. Restriction digestion of pPKCCP16 with *Eco*RI/*Bam*HI. The 0.7 kb coat protein gene was released from plasmid pGEMT of 3.0 kb (lane 2); 1 kb DNA ladder (lane 1).

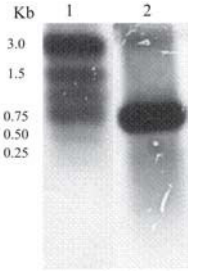

Fig. 8. Southern hybridization of *Eco*RI/*Bam*HI digested plasmid pPKCCP16 with DIG labeled CP gene. Probe hybridized specifically to 0.7 kb CP gene (lane 2); 1 kb DNA ladder (lane 1).

Improved protocols developed for extraction of low copy plasmid DNA from strains of *Xam* and high copy plasmids from *E. coli* were not only simple and rapid but also yielded higher concentrations of quality plasmid, free from protein and cellular contaminants. Plasmids extracted by both the protocols were suitable for various molecular procedures.

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