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Rapid Mini-Prep Isolation of High Quality Small and Large Plasmids from Phytopathogenic Gram Negative Bacteria

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A simple and cheap method of plasmid DNA preparation from phytopathogenic Gram-negative bacteria (Xanthomonad, *Erwinia stewartii*) is presented here. In this method, in place of the high-priced chemicals and commercial kits, available and cheap chemicals were used for rapid isolation of small and large plasmids from different Gram negative bacteria. The time also was reduced by using this method giving a high quality plasmid production as demonstrated on the agarose gel, which make this method be used on a preparative scale to isolate sufficient quantities of plasmid DNA required for restriction analysis, cloning, or transformation experiments. A down scaled- protocol is also very useful for rapidly screening the wild plasmids in a large numbers of bacterial isolates in the experiments where hundreds of colonies should be screened for their plasmid contents such as in studying plasmid curing; antibiotic and heavy metal resistant bacteria or; xenobiotic compound degrading bacteria.

Key words: Plasmid, DNA, *Erwinia*, *Xanthomonas*, Curing.

The preparation of plasmid DNA is one of most used techniques in the field of molecular biology. Screening and studying of wild plasmids is the most used application in this field; for example, screening of the presence of plasmids in antibiotics resistant bacteria, heavy metal tolerant bacteria, or xenobiotic compound degrading bacteria. Studying of plasmid loss in plasmid cured isolates needs also screening of hundreds of treated colonies. Screening of plasmids in these experiments should be reliable, fast, and applicable in each laboratory like the routine essays in laboratories.

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Off course, there are different methods for plasmid isolation which are suitable for the general conditions of various laboratories. However, the method developed in this study will enable the workers to perform and screen plasmids in a large number of samples within 45 min by using only bench-top centrifuge. In addition, the reagents described in this study are easy to be used and should be available in most laboratories.

Most of isolation protocols for Gram-negative bacteria are based upon the alkaline denaturation methods described by Kadoand Liu (1981) and; Brimbion and Doly (1979). In case of small plasmids, although these methods are still useful, problems exist in sharing of high molecular weight plasmids that constitutes a barrier for using these methods in extraction of the large plasmids. In addition to their relatively high cost, and low yield, the extraction of plasmid DNA using the

commercial plasmid preparation kits may not be really suitable for screening of a large number of bacterial strains. This is because the methods producing a clear plasmid patterns was only suitable to gain a high quantities of pure plasmid DNA or genetics manipulations techniques and may not be suitable for screening purpose. As well, these methods may require a large volume of cultivated bacteria (e. g., 500 ml for low copy number plasmid and 2.5 L for very low copy number plasmid).

MATERIALS AND METHODS

Bacterial strains

Different Gram negative bacterial strains belonging to *Xanthomonas* and *Erwinia* spp were used in this study and obtained as lyophilized samples from the GSPB bacterial collection (Göttinger Sammlung phytopathologener Bakterien) (Table 1).

The strains were grown in Luria-Bertani (LB) medium [Bacto Tryptone 10 g/L; Bacto Yeast Extract 5 g/L; NaCl 10 g/L; pH 7.2] at 28 ºC (Sambrook*et al.,* 1989).

Extraction of plasmid DNA

Plasmid DNA was extracted by five different methods: Kadoand Liu (1981), Sathyanarayana and Verma (1993), two different commercial ready kits (Maxi and Mini Prep. Qiagen), and the new protocol developed in this study. The details of the plasmid mini-prep procedure used in this study are outlined in Fig. 1. Cells from 1.5 ml of an overnight shaken culture were harvested by centrifugation at 7000Xg in a microcentrifuge tube. The bacterial pellet was then washed in 2% NaCl followed by 30 mMTris HCL (pH 8.0). The pellet was resuspended in 250 µl of resuspension buffer (50 mMTris HCl, pH 8.0, 10 mM EDTA and 100 μgml^{-1} RNase A, stored at 2-8 ºC and followed by adding 250 µl of lyses buffer (200 mM NaOH, 1% SDS; stored at room temperature not longer than one week). The cell mixture was mixed gently but thoroughly by inverting 4-6 times, and incubated at room temperature for 5 min. Three hundred fifty microliters (350µl) of neutralization buffer (3.0 M potassium acetate pH 5.5) was added and the solution was mixed immediately but gently by inverting 4-6 times. The mixture was centrifuged at 13000 X g for 10 min and then a compact white pellet was obtained. The clear solution was then transferred into a new microfuge tube. An equal volume of 1:1 phenol:chloroform solution was added to the microfuge tube containing the clear solution and the mixture was shaked by gently inverting the tube. The solution was centrifuged at 13000 X g for 15 min at 4 ºC. The aqueous fraction was carefully transferred to a fresh tube without disturbing the bulky interphase. The clear supernatant was taken and the plasmid DNA was precipitated by adding 0.7 volume of room temperature isopropanol followed by immediate centrifugation at 13000 X g for 15 min at 4 ºC. The formed pellet was washed by 70% ethanol and precipitated by centrifugation at 13000 X g for 2 min at 4 ºC. The pellet was then dried and resuspended in 50 µl Tris HCl, pH 8.5 buffer or in bidest H_2O.

Agarose gel electrophoresis of plasmid DNA.

Once plasmid DNA has been precipitated, it should be visualized before analysis. This step is normally achieved by electrophoresis through an agarose gel (Towner and Cockayne, 1993) such as horizontal slabagarose gel with a concentration of 0.7% in 1 x TAE buffer.

RESULTS AND DISCUSSION

To examine the plasmid profiles, firstly the plasmid has to be separated from the chromosomal DNA. The earliest plasmid isolation procedures depended on the separation of covalently closed circles of plasmid DNA from chromosomal DNA fragment by ultracentrifugations on cesium chlorid gradients containing high concentrations of ethidium bromide (Currier and Nester, 1976). This procedure is still being used in order to prepare large quantities of pure plasmid DNA but not practically suitable for screening of plasmids in large bacterial numbers.

Afterward, numerous rapid mini-prep methods for isolation of plasmid DNA on a small scale have been developed that can be applied to large numbers of bacterial isolates for typing purposes and epidemiological studies (Towner and Cockayne, 1993). The best yields are obtained with smaller plasmids, partly because of their large copy numbers and because they are less prone to physical damage during the isolation process. But

Table 1. Bacterial strains used in this study

GSPB Nr.	Race	Origin and date of isolation	Plasmid content Mdal.
*Xcm*1252	18	USA	45.2 Mdal, 28.3 Mdal
Xcm 1384	18	Nicaragua 1986	42.4 Mdal, 35.1 Mdal, 28.3 Mdal, 16.5 Mdal, 11.7 Mdal, 5.3 Mdal.
Xcm 1385	18	Nicaragua 1986	42.4 Mdal, 35.1 Mdal, 28.3 Mdal, 16.5 Mdal.
Xcm 1386	18	Nicaragua 1986	42.4 Mdal, 35.1 Mdal, 28.3 Mdal, 16.5 Mdal.
Xcm 1429	18	Nicaragua 1986	42.4 Mdal, 35.1 Mdal, 28.3 Mdal, 16.5 Mdal.
Xcm 1432	18	Nicaragua 1986	42.4 Mdal, 35.1 Mdal, 28.3 Mdal, 16.5 Mdal.
Xcm 1435	18	Nicaragua 1986	42.4 Mdal, 35.1 Mdal, 28.3 Mdal, 16.5 Mdal, 3.9 Mdal)
Xcm 3012	18	Sudan 1991	54.6 Mdal, 28.3 Mdal, 25.6 Mdal.
Erwinia stewartii SS104 2628		Obtained from Prof. K. Geider, Heidelberg 1996	

also plasmids > 100kb in size can be visualized if appropriate care in handling is taken (Towner and Cockayne, 1993). By using the Maxi Prep., commercial ready kits (Qiagen), sharp and clear patterns were obtained, but in more than 2 hr using large volumes of cultivated bacteria (500 ml) which recompensed plasmid yield loses and high costs as well (Fig. 4).

The elution of the large plasmid molecules from the commercial ready columns is not easy and need large quantities of DNA solution resulting from lyses of a large volume of bacterial cultures as mentioned before. While the kits used for Maxi, Mega, and Giga preparations were successful in obtaining of considerable plasmid yield, the high cost and the long time of the plasmid preparation are still a barrier to use these methods in screening of large numbers of bacterial strains.

The plasmid profiles of the selective strain(s) of race 18 *Xcm* using both of the modified method by Sathyanarayana and Verma (1993), and Kado and Liu (1981) were not clear enough to evaluate the results in the means of plasmid screening from different strains (Fig.2 and 3, respectively) as the long incubation in alkaline medium (20 min.) results in damage of high molecular weight/low copy numbers plasmids which become irreversibly denatured.

In Fig. 5, the plasmid profiles of different strains of race 18 *Xcm* extracted with mini prep Qiagen ready kits are shown. The advantages of this method are the rapid proceeding to obtain the plasmid DNA from large number of bacterial strains. However, the low plasmid yield, the not satisfying quality as well as the high costs are still the barriers to use this method in screening of wild plasmids from large numbers of bacterial strains.

For these reasons, the need of a fast and save method was required to screen the plasmid from large numbers of bacterial cells thus some of

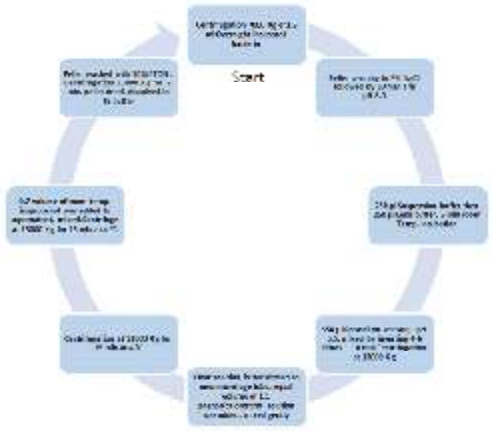

Fig. 1. Step-by-step outline of the plasmid mini-prep procedure deveoped in this study

Fig. 2. Plasmid profiles of *Erwiniastewartii,* GSPB 2628 (lane 1) and strain of race 18 *Xcm*, GSPB 1386 (lane 2) by using Sathyanarayana and Verma (1993) modified method for plasmid isolation

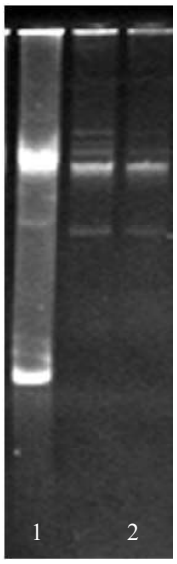

Fig. 3. Plasmid profiles of *Erwiniastewartii,* GSPB 2628 (lane 1) andstrain of race 18 *Xcm*, GSPB 1386(lane 2 + 3) by using Kado andLiu (1981) method for plasmidisolation

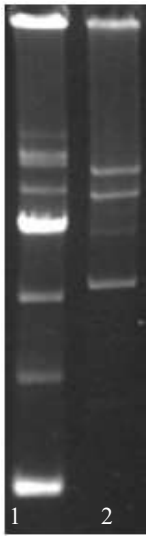

Fig. 4. Plasmid profiles of *Erwinia stewartii,* GSPB 2628 (lane 1) and strain of race 18 *Xcm*, GSPB 1386 (lane 2) by using a commercial kits for plasmid isolation (Maxi Prep.)

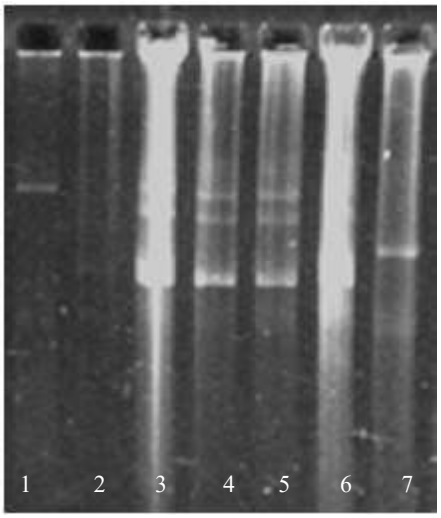

Fig. 5. Plasmid profiles of different strains of race 18 *Xcm.* lane 1: GSPB 1252; lane 2: GSPB 1385; lane 3: GSPB 1386; lane 4: GSPB 1429; lane 5: GSPB 1432; lane 6: GSPB; lane 7: 3012 by using a commercial kit forplasmid preparation(Mini Prep)

the *Xcm* strains including high molecular weight plasmid [up to 60 kb (Sathyanarayana and Verma, 1998); up to 73.4 Mdal. (AbdelRehim, 2005)].

The plasmid profiles obtained from different strains of *Xcm* using our method are shown in Fig. 6. The plasmid yield and the quality were easily observed. In addition to the low costs of this method for their plasmids screening, 24 samples could be screened for its plasmid within about 45 min. Furthermore, only a bench

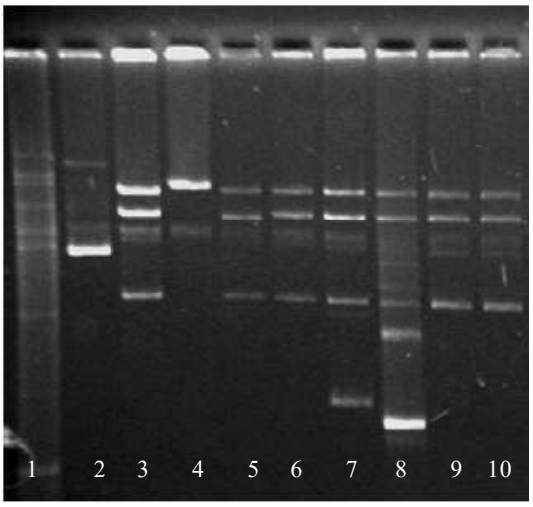

Fig. 6. Plasmid profiles of different strains of races 4 and 18 *Xcm.* lane 1: *Erwiniastewartii,* Lanes 2-9, strains of race 18, lane 2: strain GSPB 3012; lane 3: GSPB 1429; lane 4: GSPB 1252; lane 5: GSPB 1385; lane 6: 1432; lane 7: 1435; lane 8: 1384; lane 9: 1386; lane 10: race 4, strain 1430 using our improved method for mini-preparation of plasmids

microcentrifuge is needed to perform this experiment.

In this method, the use of NaCl in the first washing of bacterial pellet help the elimination of the macromolecules (exopolysacchrides) which disturbing the plasmid extraction (Das and Verma, 1996). The incubation of bacterial pellet with NaOH and SDS for only 5 min but no longer denatures the genomic DNA which becomes linearized and the strands are separated. Plasmid DNA is circular and remains topologically constrained. Adding of the potassium acetate allows circular DNA (plasmid) to renature. Sheared genomic DNA remains denatured as single stranded DNA (ssDNA). The ssDNA is precipitated, since large ssDNA molecules are insoluble in high salt. Adding potassium acetate to the SDS solution forms KDS (Potassium Dodocyle Sulphate), which is insoluble. This will allow the easy removal of the SDS from the plasmid DNA by centrifugation.

For the precipitation of the plasmid, absolute ethanol is often used. In this procedure, isopropanol was used which also gives a good and high yield of plasmid. It is worthy to note that isopropanol is much cheaper than ethanol.

The results of this study showed that the continuous freezing and re-melting of the extracted plasmids are not recommended as this method cause shearing of the plasmid molecules especially those with high molecular weights and therefore producing undesired patterns on the gel. In addition the method newly described in this investigation enables several trials to be carried out within the day, thus no long storage and refreezing of the samples is no longer required.

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