

Characterization of the 1.8-kb plasmid pXV64 from *Xanthomonas campestris* pv. *vesicatoria*

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Abstract. A simple rapid plasmid-screening method was developed and then used on 58 strains of *Xanthomonas campestris* pv. *vesicatoria*. A very small (1.8-kb) cryptic plasmid, designated pXV64, that had a copy number of about 200 to 350 was detected. A physical map of pXV64 was established and a derivative, designated p64PK, was constructed by cloning a kanamycin resistance cartridge into the unique *Pst*I site of pXV64. p64PK had the ability to transform different strains of *Xanthomonas*, could be maintained stably, and was compatible with the broad-host-range IncP plasmid pLAFR1. The results of this study suggest that pXV64 has the potential to be developed into vectors for gene cloning in *Xanthomonas*.

Keywords: Plasmid; Rapid plasmid-screening method; Vector; *Xanthomonas campestris*.

Introduction

Xanthomonas campestris, consisting of more than 123 pathovars, is the major species of the genus *Xanthomonas*, a genus that includes many gram-negative plant-pathogenic bacteria (Vauterin et al., 1990). *Xanthomonas campestris* pv. *vesicatoria* (Xv) is the pathogen for foliage and fruit spot disease in peppers and tomatoes (Minsavage et al., 1990). *Xanthomonas campestris* pv. *campestris* (Xc) is the causal agent of black rot in crucifers (William, 1980).

While plasmids from several pathovars of *Xanthomonas* have been detected, detailed studies on these plasmids are limited (Lin et al., 1979; Tetaz and Luke, 1983; Stall et al., 1984; Civerolo, 1985; Stall et al., 1986; Lazo and Gabriel, 1987; Chen and Tseng, 1988; Xu and Gonzalez, 1991). Efforts to develop plasmids indigenous to *Xanthomonas* into cloning vectors have yet to be reported. In this study, we developed a rapid method for screening plasmids in 58 strains of *X. campestris* pv. *vesicatoria*, which resulted in detection of a miniplasmid pXV64 in strain Xv64. As this plasmid is very small, stably maintained at a high copy number, and is compatible with IncP plasmid, it has good potential to be developed into a shuttle vector. Preliminary tests carried out in this study have demonstrated this possibility.

Materials and Methods

Bacterial Strains, Plasmids and Culture Conditions

Fifty-eight strains of *X. campestris* pv. *vesicatoria*, *X. campestris* pv. *citri*, *X. campestris* pv. *phaseoli*, *X.*

campestris pv. *begoniae*, *X. oryzae* pv. *oryzae*, and *Rhizobium japonica* RJ119 were obtained from S. T. Hsu, National Chung Hsing University. *Xanthomonas campestris* pv. *campestris* 17 (Yang and Tseng, 1988) and *X. campestris* pv. *campestris* P20H (Yang et al., 1988) came from our laboratory collection. Other strains used were *Agrobacterium tumefaciens* LBA4404 (Clontech Laboratories, Inc.), *Azotobacter vinelandii* ATCC478 and *Pseudomonas putida* IF03738 (ATCC8209). Luria broth or L agar (Miller, 1972) was used to grow *E. coli* HB101 (Boyer and Roulland-Dussoix, 1969) at 37°C or all other bacteria at 28°C. Antibiotics used were: ampicillin, 50 µg/ml; chloramphenicol, 34 µg/ml; kanamycin (Km), 50 µg/ml; and tetracycline (Tc), 15 µg/ml.

The plasmids used were pXV64, pXV2 (14.6 kb, our laboratory collection), pRK415 (10.5 kb, Tc^r; Keen et al., 1988), pK701 (15.8 kb, pRK415 carrying a 5.3-kb fragment from Xc17 chromosome), pBR325 (6.0 kb; Bolivar, 1978), pACYC184 (4.2 kb, Chang and Cohen, 1978), and pUC18 (2.7 kb; Yanisch-Perron et al., 1985).

Rapid Screening for Plasmids

A small number of cells were picked by touching a colony with a toothpick. They were then inoculated into a microfuge tube containing 300 µl of L broth. After overnight growth, the cells were pelleted in a microfuge (10,000 rpm, 2 min). The cells were then suspended by vortexing in 20 µl of gel-loading mix (0.25% bromophenol blue and 30% glycerol). Then 40 µl each of chloroform and phenol (saturated with 1.0 M Tris-HCl, pH 8.0) was added. The mixture was vortexed at full speed for 1 min followed by centrifugation for 10 min at 12,000 rpm. Then 10 µl of the aqueous fraction was subjected to electrophoresis on 0.7% agarose minigel (5.2 × 6.0 cm) with TAE buffer (40 mM Tris-acetate, pH 8.0 containing 2 mM Na₂-EDTA) at 100 volts for 30 min. The gel was stained

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with ethidium bromide (0.5 $\mu\text{g/ml}$) and the DNA bands were visualized under a UV transilluminator.

DNA Techniques

Plasmid was prepared by the alkaline lysis method of Birnboim and Doly (1979). Plasmid DNA bands in the agarose gel were eluted by using a GeneClean kit (Bio101 Inc., La Jolla, CA). Preparation of bacterial total DNA, transformation of *E. coli*, agarose gel electrophoresis (0.7%), preparation of ^{32}P -labeled probe, and DNA hybridization were carried out as described by Maniatis et al. (1982). Plasmids were transformed into the bacterial cells by electroporation (Wang and Tseng, 1992).

Stability Test

The stability of plasmids was tested as previously described (Fu and Tseng, 1990), except that the subculturing were continued for over 80 generations and the antibiotic used was kanamycin.

Incompatibility Test

To test for plasmid incompatibility, Xc17 and Xv65 containing p64PK were transformed by electroporation with the IncP plasmid pLAFR1 (Friedman et al., 1982), selecting for Tc^r conferred by pLAFR1. After verifying the retention of both the resident and the incoming plasmids in the same cells by plasmid extraction, the cells were subcultured by diluting them in fresh L broth with tetracycline, favoring the retention of the exogenous plasmid. The subculturing were repeated to allow the cells to grow for about 50 generations. Then the cells were spread on L agar with tetracycline. Two hundred colonies were randomly picked and tested for tetracycline and kanamycin resistance using L agar plates.

Estimation of Copy Number

Cells of Xv64 containing pXV64 and *E. coli* HB101 containing pUC18, at mid-log phase, were harvested and diluted with distilled water to obtain several concentrations ranging from 1 to 8×10^8 cells ml^{-1} , as estimated by reading OD_{550} and OD_{600} for Xv64 and HB101, respectively. To achieve better accuracy, each suspension was subjected to plate count on L agar plate. The rapid-screening method was followed for plasmid extraction, gel electrophoresis, and gel staining. The bands were scanned with Foto Analyst II (Fotodyne, Wisconsin, USA). pUC18 extracted from a similar amount of cells was employed as the standard for comparison.

Results

Rapid Screening for Plasmids

Cells of *Xanthomonas* produce large amounts of exopolysaccharide, which interferes with the extraction of plasmid DNA. In this study, a simple and rapid method as described in "Materials and Methods" was developed and used to screen for the presence of plasmids. The re-

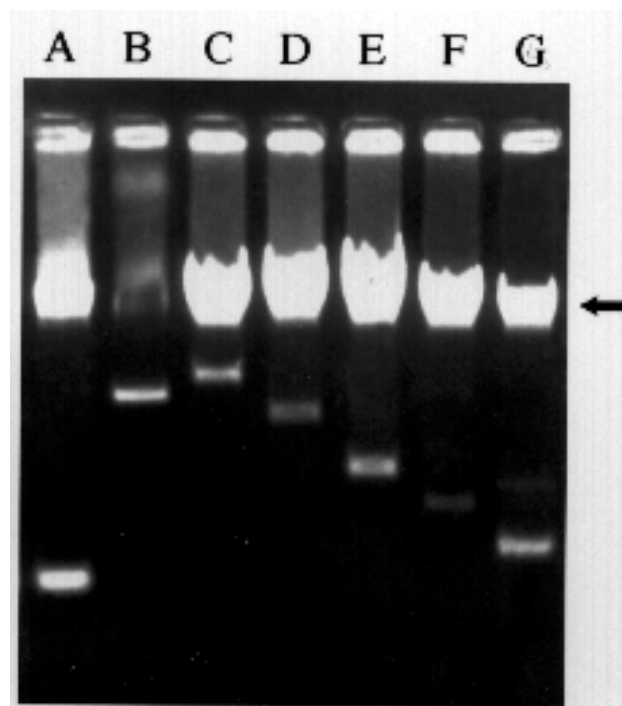


Figure 1. Agarose gel electrophoresis of the plasmids extracted by the rapid method from Xv64 (A), Xv36 (B), Xc17 (C), and *E. coli* HB101 (D to G). Lanes: A, pXV64 (1.8 kb); B, pXV2 (14.6 kb); C, pK701 (15.8 kb); D, pRK415 (10.5 kb); E, pBR325 (6.0 kb); F, pACYC184 (4.2 kb) and G, pUC18 (2.7 kb). The chromosomal DNAs are indicated by an arrow.

sults of agarose gel electrophoresis on the plasmids extracted by this method are shown in Figure 1. Plasmids with sizes up to 15 kb were detectable in *X. campestris* pv. *vesicatoria*, *X. campestris* pv. *campestris*, and *E. coli* as well, whereas some of the larger ones might have been masked by the smearing of the chromosome. In addition, each plasmid formed a single band in the agarose gel. Among 58 strains of *X. campestris* pv. *vesicatoria* screened, most were found to harbor plasmids with various sizes. One small plasmid, designated pXV64, was identified in strain Xv64 (Figure 1).

Physical Mapping of Plasmid pXV64

To construct a physical map, pXV64 DNA was cut with different restriction endonucleases and subjected to agarose gel electrophoresis. The results showed that it carried single restriction sites for *Cla*I, *Hinc*II, *Pst*I, *Sal*I, *Xho*I, and *Xho*II and double sites for *Mlu*I. It was not cleaved by *Bam*HI, *Eco*RI, *Hind*III, *Pvu*II, or *Sma*I. The molecular size of this plasmid was estimated to be 1.8 kb based on the sum total of the restriction fragment lengths. The physical map is shown in Figure 2.

Cloning of a Kanamycin-Resistance Gene into pXV64

Xanthomonas campestris pv. *vesicatoria* carrying pXV64 was sensitive to kanamycin, indicating that Km^r could be used as a selective marker after cloning into an

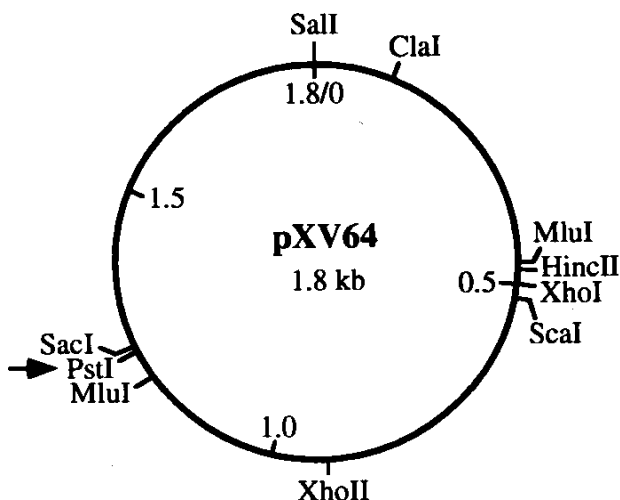


Figure 2. Physical map of pXV64. The arrow stands for the site into which a Km^r cartridge was inserted to form p64PK.

appropriate site. To achieve this end, the steps used were as follows. The 1.3 kb *Pst*I fragment containing Km cartridge from plasmid pUC4K (Vieira and Messing, 1982) was cut down with *Pst*I, electrophoresed, and eluted from the agarose gel. After modification of the ends with appropriate linkers, except for those to be cloned into the *Pst*I site, the fragments were ligated, respectively, with the pXV64 DNA cut with *Pst*I, *Sal*I, or *Xho*II. Then the ligation mixtures were electroporated into *X. campestris* pv. *campestris* 17 (Xc17) and Xv65, the strains which have no indigenous plasmid. Transformants were selected in L agar containing kanamycin. More than 30 clones were obtained, all of which had the Km cartridge inserted in the *Pst*I site, whereas insertion into the *Sal*I or *Xho*II site was not successful.

Host range of pXV64

To test for host range, p64PK was transformed into each of the gram-negative bacteria described in Materials and Methods, selecting for kanamycin resistance. To ensure the efficiency of electroporation, experiments were repeated five times with triplicate samples in each repeat. The plasmids were then extracted from the transformants, digested with *Pst*I, subjected to agarose gel electrophoresis, and examined by Southern hybridization. The results showed that the plasmid was maintained without change in all *Xanthomonas* strains tested (data not shown). Since no kanamycin resistant transformants of other bacteria were obtained, pXV64 appeared to be a narrow-host-range plasmid.

Stability of p64PK in Xc17 and Xv64

Stability tests on p64PK in Xc17 and Xv65 showed that about 96% of the colonies of both hosts retained the plasmid after 80 generations of growth without the selective pressure of kanamycin, indicating good stability in both *X. campestris* pv. *campestris* and *X. campestris* pv. *vesicatoria*. It was noted that, in the presence of

kanamycin, the resident pXV64 in strain Xv64 was excluded by the presence of p64PK after about 30 generations.

Copy Number of pXV64

The results of copy number estimation showed that the band of pXV64 extracted from 4.92×10^8 cells of Xv64 had an intensity slightly stronger than that of pUC18 extracted from 2.34×10^8 cells of *E. coli* HB101 (Figure 3). Since the Xv64 cell number was about twice that of the HB101, it seems safe to claim that pXV64 had a copy number of 200 to 350 per cell, which was about half of 400 to 700, the copy number estimated for pUC plasmids by Chambers et al. (1988) and Minton et al. (1988). The copy number of p64PK in Xv65 and Xc17 was found to be similar to that of pXV64.

Sequence Homology Between pXV64 and Other Replicons

To detect DNA homology to pXV64, total DNAs from Xc17 and Xv65 were digested with *Sal*I, electrophoresed

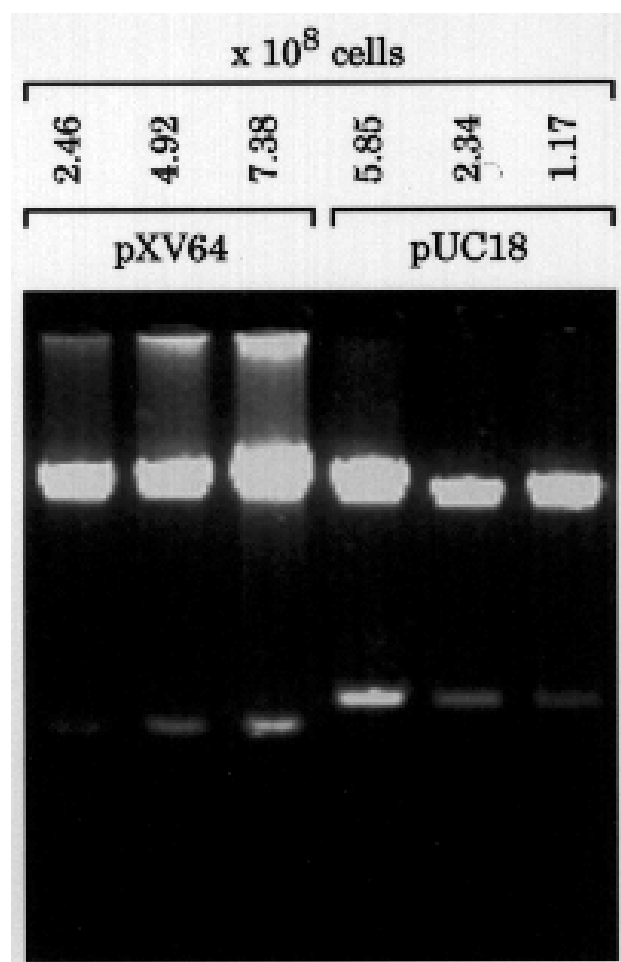


Figure 3. Estimation of copy number of pXV64 by comparing the DNA bands with that of the pUC18. The procedure is described in "Materials and Methods." The cell numbers are denoted on top of each lane.

in agarose gel, transferred to a nitrocellulose filter and then subjected to hybridization using pXV64 as a positive control and ^{32}P -labeled pXV64 DNA as the probe. Hybridization tests were also run with RF DNAs of the *Xanthomonas* filamentous phages ϕLf , ϕXv , and ϕXo (Lin et al., 1994). No sequence homology between pXV64 and these replicons was observed (data not shown).

Compatibility of pXV64 with Commonly Used Broad-Host-Range Vector

Incompatibility group P plasmid pLAFR1 (Friedman et al., 1982) is an useful broad-host-range vector. This plasmid was found to be compatible with p64PK in both Xc17 and Xv65. In both hosts, after 50 generations of growth, 98% of the cells retained both p64PK and pLAFR1.

Transformation Efficiency of p64PK

To test for transformation efficiency of p64PK, the plasmid was isolated from *X. campestris* pv. *campestris* P20H and transformed by electroporation into P20H, and *X. campestris* pathovars *vesicatoria*, *phaseoli*, *begoniae*, and *citri*. Data obtained from four independent experiments showed that the efficiency ranged from 2.7×10^5 to 8.0×10^6 transformants/ μg DNA, similar to the electroporation efficiency by the filamentous phage ϕLf RF DNA (Wang and Tseng, 1992). The results indicate that the *Xanthomonas* strains tested here can be used as cloning hosts and electroporation is a feasible method to deliver pXV64 derivatives into these bacteria.

Other Properties

Xanthomonas campestris pv. *vesicatoria* strain Xv64, carrying pXV64, could not grow in L broth containing chloramphenicol, kanamycin or tetracycline, indicating that plasmid pXV64 does not confer resistance to these antibiotics. In addition, no difference was observed between Xv65 and the Xv65 carrying pXV64 in colony morphology, pigmentation, growth in M9 medium (Neidhardt et al., 1974) containing sucrose, amounts of exopolysaccharide produced, or sensitivity to the filamentous phage ϕXv . Therefore, pXV64 is a cryptic plasmid.

Discussion

In this study, a simple and rapid method for screening plasmids with sizes up to 15 kb has been developed. It is especially useful when sample sizes are large. This method can be achieved in one step simply by phenol/chloroform treatment of the cells pelleted from a volume as small as 300 μl . The experiment from growing cells to the completion of plasmid extraction can be carried out using the same microfuge tube. The whole procedure takes about 40 min from harvesting the bacterial cultures to visualization of the DNA bands in agarose gel. Using this method, we have detected plasmids in *Pseudomonas*, *Aeromonas*, *Rhizobium*, and *Agrobacterium*, as well as

replicative form (RF) DNAs of two different filamentous phages of *Xanthomonas* (unpublished results).

pXV64 appears to be cryptic since no known function has been assigned. With a size of 1.8 kb, pXV64 is very small compared to the previously reported *Xanthomonas* plasmids which are mostly over 70 kb in size (Chen and Tseng, 1988; Lazo and Gabriel, 1987; Lin et al., 1979; Kado and Liu, 1981). Interestingly, it is only about 400 bp larger than the smallest plasmid reported, pAP12875 (Fomenkov et al., 1995). pXV64 was found to be maintained at very high copy number in the hosts. Since it is cryptic, the significance of its existence at a high copy number remains to be elucidated.

The regions near the *Pst*I site appear to be non-essential, since insertion of foreign DNA fragment did not affect plasmid replication. Furthermore, copy number control was not disrupted by the insertion, since the copy number of p64PK was as high as that of the pXV64. In contrast, the regions spanning the other unique sites appear to be indispensable which might contain elements such as *ori* or the Rep protein gene, since cloning of the Km cartridge into these sites was not successful. These observations are useful for future study in identification of the minimal region for autonomous replication of pXV64. For such a study, the *Pst*I site should be an appropriate point to linearize the plasmid for subsequent deletion of the non-essential sequences.

A good cloning vector should be small in size, multiple in copy number, stably maintained, and bear unique restriction sites for commonly used enzymes, especially inside the antibiotic resistance genes. This study has shown that pXV64 possesses most of these properties. It is very small and maintained stably at a high copy number without the selective pressure of antibiotic. In addition, it has no sequence homology with other replicons in Xc17 and Xv65, suggesting that a pXV64 derivative would not undergo homologous recombination when introduced into these strains. After appropriate modification, e.g., by fusion with an *E. coli* vector such as pUC18 and insertion of a Km cartridge, it may become a shuttle vector. With its high copy number, pXV64 has the advantage of exerting gene dosage effects, so that high level expression of cloned genes can be obtained. In addition, pXV64 is compatible with the commonly used cloning vector pLAFR1, so that it can be used as a member of a binary system to express cloned genes in combination with a vector from IncP group.

In this study, success in construction of p64PK has shown that pXV64 allows insertion of foreign genes, demonstrating the potential of pXV64 being developed into a vector for gene cloning in *Xanthomonas*. In addition, we have shown that several *Xanthomonas* strains can be used in conjunction with pXV64 to constitute vector/host systems in which recombinant plasmid can be delivered by means of electroporation.

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茄科點斑病菌之質體 pXV64 之特性研究

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經以一簡單、快速篩選質體之方法偵測 58 個茄科點斑病菌的分離株後，發現一約 1.8 kb 大小之質體，命名為 pXV64。質體 pXV64 之拷貝數目約為 200 至 350，其鑑識圖譜已建立完成。且得到一衍生質體 p64PK，帶有一抗康黴素的基因插入於 pXV64 的單一 *Pst*I 切位上。p64PK 可以轉殖於不同的 *Xanthomonas* 菌株中並穩定存活。又 p64PK 能與泛寄主 IncP 質體 pLAFR1 互容。本研究結果顯示值得將 pXV64 改造成為一適用於 *Xanthomonas* 之選殖載體。

關鍵詞：茄科點斑病菌；質體；拷貝數；鑑識圖譜；選殖載體。