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Expression of Plasmid-borne Characters in *Xanthomonas axonopodis* pv. *vignaeradiatae*

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ABSTRACT. *Six isolates of Xanthomonas axonopodis pv. vignaeradiatae (Xav) the incitant of bacterial leaf spot disease of greengram [Vigna radiata (L.) Wilczek], differing in colony characters and pathogenicity were established and studied for their plasmid profile. Plasmids were detected in all the 6 isolates. The highly virulent isolate Xav–1 harboured 3 plasmids of 45, 35 and 1.5 kb (mini plasmid) as compared to least virulent isolate Xav–2 which had only 1 plasmid of 35 kb. Whereas, in the remaining 4 moderately virulent isolates (Xav–3 to Xav–6) only 2 plasmids of 45 and 35 kb in each were observed. However; one 35 kb plasmid was common in all the 6 isolates. Curing of plasmid was done in highly virulent and ampicillin resistant isolate Xav–1 by elevated temperature (42°C) treatment. Cured cells lost 4 traits i.e., ampicillin resistance, exopolysaccharide (EPS) production, pigmentation and virulence. Transformation of plasmids of Xav–1 into cured cells resulted in restoration of antibiotic resistance, EPS and pathogenicity. But pigmentation was not restored probably due to the loss of mini plasmid of 1.5 kb during transformation process. It was concluded that in Xav, plasmid borne genes are responsible for virulence, EPS, antibiotic resistance and pigmentation.*

INTRODUCTION

Greengram [*Vigna radiata* (L.) Wilczek] is an important grain legume in India and other parts of South-East Asia. It has been cultivated since time immemorial and occupies about 3.08 million ha with an annual production of 1.31 million tonnes only (Anon., 1999). Being a rich source of protein (22–24%) it has been recognized as an indispensable constituent of cereal-based diet of vegetarian masses and the sprouted seeds are a rich source of ascorbic acid (Vitamin-C), riboflavin (Vitamin-B_2) and thiamine (Vitamin-B_1). Bacterial leafspot of greengram caused by *Xanthomonas axonopodis* pv. *vignaeradiatae* (*Xav*) (Vauterin *et al.*, 1995) is one of the most important diseases and causes up to 15% losses under favourable environmental conditions (Patel and Jindal, 1970).

The discovery of plasmids in *Agrobacterium tumefaciens* (Zanen *et al.*, 1974) opened up new avenues in the study of plant pathogenic bacteria. It enhanced the genetic study of phytopathogenic bacteria, which is a pre-requisite for understanding pathogenesis and to develop disease control measures. Plasmids have been reported in several phytopathogenic bacteria (Verdier *et al.*, 1998) and code for many important characters such as virulence, resistance to antibiotics and enzyme production *etc.*. So far no work has been done on the role of plasmids in *Xav* and therefore, the present investigations were undertaken to find out the characters determined by plasmids.

MATERIALS AND METHODS

Isolation and establishment of *Xav* cultures

Greengram leaves infected with *Xav* were collected from fields of Indian Agricultural Research Institute, New Delhi and Berhempore (West Bengal) and isolations were made by streak plate method on sucrose peptone agar (SPA) medium (Na_2 HPO_4. $12H_2O$ – 2.0 g, $Ca(NO_3)_2$. $4H_2O$ – 0.5 g, $FeSO_4$–0.5 g, Peptone – 5.0 g, Sucrose – 20.0 g, Agar–20.0 g, Distilled water–1000 ml, pH–6.8). Pathogenicity was tested on susceptible cultivar Pusa Baisakhi using leaf laminar-infiltration method (Klement, 1963). Based on colony characters and degree of virulence (Table 1 and Fig. 1), 6 different isolates (*Xav*–1 to *Xav*–6) were established.

Table 1. Colony characters and virulence of six isolates of *Xav*.

Isolates	Colony characters	Virulence
Xav–1	Big, raised, mustard yellow, mucoid, opaque and glistening	Highly virulent
Xav–2	Small, raised, deep yellow, mucoid, opaque and glistening	Least virulent
Xav–3	Big, raised, lemon yellow, mucoid, opaque and glistening	Moderately virulent
Xav–4	Small, raised, lemon yellow, mucoid, opaque and glistening	Moderately virulent
Xav–5	Medium size, raised, lemon yellow, mucoid, opaque and glistening	Moderately virulent
Xav–6	Medium size, raised, albino, mucoid, opaque and glistening	Moderately virulent

Plasmid isolation

Plasmids of *Xav* were isolated by the method of Birnboim and Doly (1979) with 2 modifications; (1) washing with 2% NaCl to remove excess exopolysaccharides, (2) freezing and thawing at 70°C and 37°C respectively for 10 min each to achieve complete cell lysis. Plasmid DNA was precipitated with ethanol and resuspended in double distilled water. Gel electrophoresis of the plasmid DNA was conducted on a horizontal slab gel for 4 h at 50 v using Tris acetate EDTA buffer (Sambrook *et al.*, 1989).

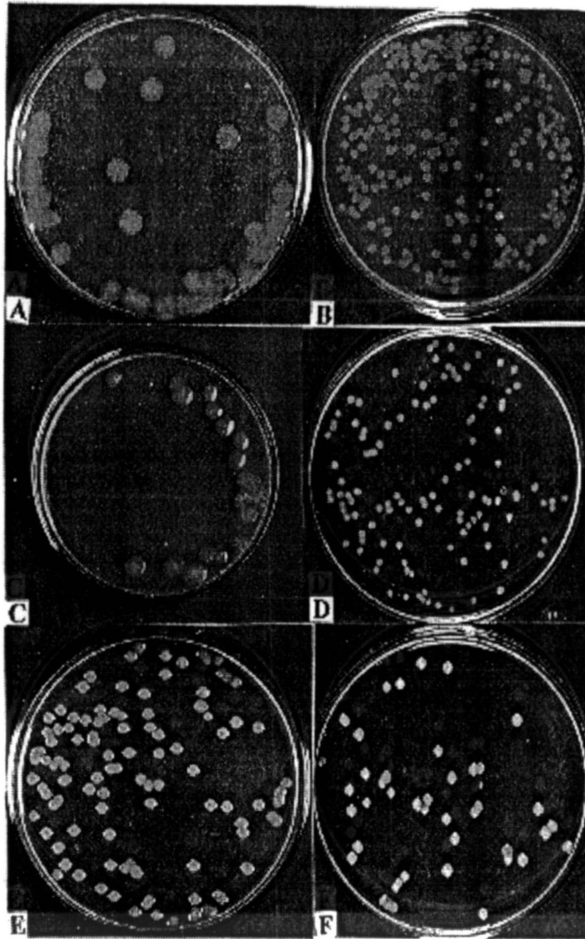


Fig. 1. Colony characters of *Xav* isolates.
[Note: A - *Xav*–1, B - *Xav*–2, C - *Xav*–3, D - *Xav*–4, E - *Xav*–5, F - *Xav*–6].

Curing of plasmid

For plasmid curing, heat-curing method described by Mahadevan and Ulaganadhan (1992) was adopted. Single colony of the wild isolate (*Xav*–1) was inoculated in 50 ml Luria Broth (LB) medium and incubated at 42^{0}C (a temperature about 15^{0}C higher than the normal) for 48 h in rotor shaker. After suitable dilutions, 50 μl of the cell suspension was plated on Luria Agar (LA) medium and incubated at 26±1^{0}C for 48 h.

Transformation of plasmid DNA

Plasmids were transformed into the cured cells according to the method described by Atkins *et al.* (1987). The competent cells of cured cells were prepared by the method

described by Mandel and Higa (1970). Fifty ml of LB was inoculated with overnight grown *Xav* culture and incubated at 26±1°C for 2 h with constant shaking at 110 rpm on a shaker. The cell suspension was aseptically transferred to sterile screw cap tubes and left on ice for 10 min. The suspension was centrifuged at 5000 rpm for 10 min at 4°C to pelletize bacterial cells. The pellet was resuspended in 10 ml ice cold 0.1 M $MgCl_2$ and centrifuged at 5000 rpm for 10 min at 4°C. The pellet was again resuspended in 10 ml ice cold 0.1 M $CaCl_2$ and kept on ice for 1 h. The cells were recovered by centrifuging at 5000 rpm for 10 min at 4°C and the pellet was resuspended in 2 ml chilled 0.1 M $CaCl_2$ and used for transformation after keeping it on ice for 1 h. Competent cell suspension (200 µl) was added to 2 µl of plasmid DNA and kept on ice for 1 h. The mixture was given a heat shock at 42°C for 2 min after adding 1 ml LB, incubated at 26±1°C for 1 h in a shaker incubator (110 rpm). The cell suspension was spread on LA plate containing ampicillin (50 µg/ml). The plates were incubated at 26±1°C for 3 days.

Method of inoculation

Injection-infiltration method of Klement (1963) was used. A 22 gauge hypodermic needle was placed between the lower surface of the leaf and the thumb (opening face of the needle facing towards the leaf) and desired amount of bacterial suspension (10^7 cells/ml) was forcibly injected into the intercellular space to produce water soaked areas (2 cm) on leaflets. The inoculations were made between 9 am to 12 noon, when the stomata are fully open.

Assay for antibiotic resistance

Stock solution of the antibiotics were prepared by dissolving 100 mg in 20 ml of sterilised distilled water and were filter sterilized with Acro-disc. Antibiotics which did not dissolve in water were dissolved in 0.5 ml methanol and the volume was made 20 ml by adding sterilised distilled water. This stock solution contained 5 µg/ml antibiotic. For each antibiotic, different concentrations (5, 10, 25, 50 and 100 µg/ml) in 25 ml melted and cooled (45°C) SPA medium was prepared and poured in petridish. After solidification of the medium, the plates were streaked with 24 h old bacterial growth. For each concentration, 3 replicates were maintained. After 48 h of incubation at 26±1°C, the plates were examined for bacterial growth.

RESULTS AND DISCUSSION

Antibiotic resistance

For the detection of antibiotic resistance markers, 6 isolates of *Xav* were tested against 4 antibiotics (ampicillin, rifampicin, kanamycin and streptomycin) at different concentrations. Except *Xav*–6 (albino), all other isolates showed high degree of resistance against ampicillin, but they were susceptible to rest of the 3 antibiotics (streptomycin, kanamycin and rifampicin). Isolate *Xav*–6 (albino) behaved differently from other isolates and showed resistance to streptomycin.

Plasmid profile

Plasmids were detected in all the 6 isolates. The highly virulent isolate *Xav*–1 harboured 3 plasmids of 45, 35 and 1.5 kb (mini plasmid) as compared to least virulent isolate *Xav*–2 which had only 1 plasmid of 35 kb. Whereas, in the remaining 4 isolates (*Xav*–3, *Xav*–4, *Xav*–5, *Xav*–6) only 2 plasmids of 45 and 35 kb in each were observed. However, in all the 6 isolates of *Xav*, 1 plasmid of 35 kb was ubiquitous (Fig. 2). Nevertheless, a mini plasmid of 1.5 kb was observed only in *Xav*–1 which can be used for construction of indigenous vectors by introducing 1 or more specific markers. In *Xav*, there is a direct correlation between the number of plasmids and the degree of virulence. However, Sathyanarayana (1992) reported only 1 plasmid of 50 kb in *Xav*.

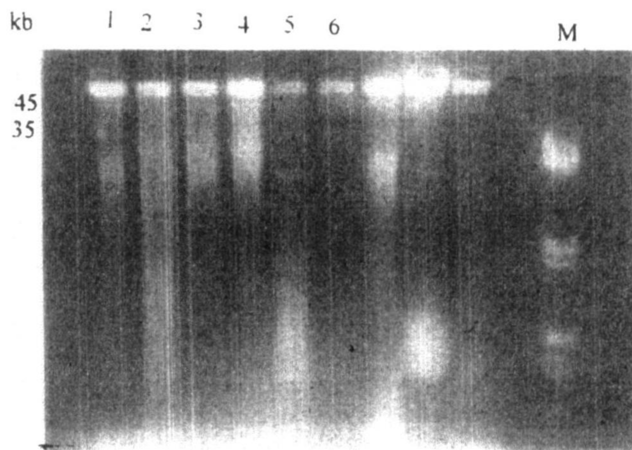


Fig. 2. Plasmid profiles of six isolates of *Xav*.
[Note: 1 - *Xav*–1, 2 - *Xav*–2, 3 - *Xav*–3, 4 - *Xav*–4, 5 - *Xav*–5, 6 - *Xav*–6, M - Marker (λ DNA digested with *Eco* R I + *Hind* III)].

Plasmid curing

In order to assess the plasmid borne characters, isolate *Xav*–1 was used for plasmid curing since it contains maximum number of plasmids, resistant to ampicillin and is highly virulent. The colonies of plasmid cured cells lost 4 characters *i.e.*, ampicillin resistance, EPS production, pigmentation and virulence. The colonies of cured cells became flat, non-mucoid and lost pigment as against raised, mucoid and yellow colonies of the wild type. Similar changes in colonies formed by cured cells were recorded by Ulaganathan (1989) in *X. axonopodis* pv. *vignicola*. Similar findings of plasmid-encoded other characters in plant pathogenic bacteria have been reported, *e.g.*, virulence (Nester and Kosuge, 1981; Sathyanarayana and Verma, 1993; Chakrabarty *et al.*, 1995) and resistance to antibiotic (Amuthan and Mahadevan, 1994; Canteros *et al.*, 1995). However, in contrast

to this, plasmid cured strains were found virulent to some extent in *X. oryzae* pv. *oryzae* (Amuthan and Mahadevan, 1994), *X. axonopodis* pv. *vignicola* (Ulaganathan and Mahadevan, 1988). Choi *et al.* (1989) also did not find any correlation between the presence of plasmids in 3 strains of *X. oryzae* pv. *oryzae* and phenotypes such as virulence, antibiotic resistance, pigmentation and EPS.

Transformation

After transformation of plasmid DNA into cured cells, the transformants have restored EPS production (Fig. 3) and antibiotic resistance marker for ampicillin but not the pigmentation. On screening for the presence of plasmids, the transformed cells were able to restore only 2 plasmids of 45 and 35 kb, but not the mini plasmid of 1.5 kb.

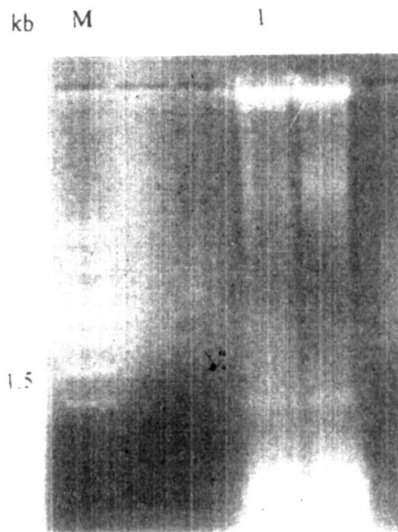


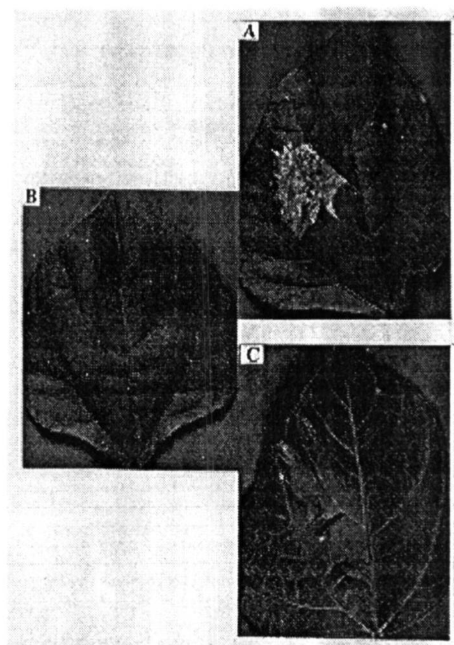
Fig. 3. Bacterial growth on slants.
[Note: A - wild, B - cured, C - transformed].

This could be the reason for non-restoration of pigmentation. Ulaganathan and Mahadevan (1988) reported that the genes encoding pigmentation in *X. axonopodis* pv. *vignicola* are located on a plasmid of 95 Mda. Chen and Tseng (1988) also reported that pigmentation in *X. oryzae* pv. *oryzae* is a plasmid borne character.

To prove the role of plasmids in pathogenicity, susceptible greengram cultivar (Pusa Baisakhi) was inoculated with wild, cured and transformed cell suspension using leaf injection-infiltration method of Klement (1963). The wild and transformed cells produced symptoms whereas cured cells did not show any symptoms (Fig. 4). The plasmid-borne characters expressed by wild, cured and transformed cells are summarized in Table 2. The

Table 2. **Phenotypic characters of wild, cured and transformed cells.**

Cells	Antibiotic resistance	EPS production	Pathogenicity	Pigmentation
Wild	+	+	+	+
Cured	-	-	-	-
Transformed	+	+	+	-



ig. 4. **Symptoms producd by wild, cured and transformed strains of *Xav*–1 on greengram.**
[Note: A - wild (symptoms), B - Cured (no symptoms), C - transformed (symptoms)].

irulence of *X. axonopodis* pv. *malvacearum* has been reported to be associated with EPS roduction (El-Banoby and Rudolph, 1979; Chowdhury and Verma, 1980). Verma (1986) iggested that the EPS in *X. axonopodis* pv. *malvacearum* was needed for permanent water oaking which favours multiplication and spread of the pathogen. Collmer *et al.* (1991) ported that EPS facilitate the infection process and acted as secondary disease eterminant in some plant pathogens. Denny and Back (1991) concluded that EPS acted s an aggressive factor rather than as virulence factor.

CONCLUSIONS

The rôle of plasmids (generally considered to be cryptic) was demonstrated in the virulence of *X. axonopodis* pv. *vignaeradiatae*, the inducer of bacterial leaf spot disease of greengram (*Vigna radiata*) through plasmid curing and reintroduction of the plasmids in plasmid cured strain through transformation. The plasmid borne genes were responsible for virulence, EPS production and antibiotic resistance.

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REFERENCES

Amuthan, G. and Mahadevan, A. (1994). Plasmid and Pathogenicity in *Xanthomonas oryzae* pv. *oryzae*, the bacterial blight pathogen of *Oryza sativa*. J. Appl. Bacteriol. 76: 529-538.

Anonymous. (1999). Sector-2. Pulses: A little impetus needed. Survey of Indian Agriculture, National Press, Chennai-600 002.

Atkins, D.T., Barber, C.E. and Daniels, M.J. (1987). Transformation of *Xanthomonas campestris* pv. *campestris* with plasmid DNA. J. Gen. Microbiol. 13: 2727-2731.

Birnboim, H.C. and Doily, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513-1523.

Canteros, B.I., Minsavage, G.V., Jones, J.B. and Stall, R.E. (1995). Diversity of plasmids in *Xanthomonas campestris* pv. *vesicatoria*. Phytopathol. 85(12): 1482-1486.

Chakrabarty, P.K., Mahadevan, A., Raj, S., Meshram, M.K. and Gabriel, D.W. (1995). Plasmid-borne determinants of pigmentation exopolysaccharide production and virulence in *Xanthomonas campestris* pv. *malvacearum*. Can. J. Microbiol. 41: 740-745.

Chen, L.J. and Tseng, Y.H. (1988). Cryptic plasmids of *Xanthomonas oryzae* pv. *oryzae*. Plant Prot. Bull. Taiwan. 30: 78-85.

Choi, S.H., Ardales, E.Y., Leung, H. and Lee, E.J. (1989). Characterization of indigenous plasmids of *Xanthomonas oryzae* pv. *oryzae*. Korean J. Pl. Pathol. 5: 223-229.

Chowdhury, H.D. and Verma, J.P. (1980). Exopolysaccharide production by pathogenic and non-pathogenic bacteria associated with leaves of cotton. Indian Phytopathol. 33: 304-307.

Collmer, A., Beuer, D.W., He, S.Y., Lindeberg, M., Kelemu, S., Rodriguez-palenzuala, P., Burr, T.J. and Chatterje, A.J. (1991). Pectic enzyme production and bacterial plant pathogenicity. Adv. Mol. Gen. Pl. Microbe Interact. 1: 65-72.

Denny, T.P. and Back. S.R. (1991). Genetic evidence that extracellular polysaccharide is a virulence factor of *Pseudomonas solanacearum*. Mol. Pl. Microbe Interact. 4: 198-206.

El-Banoby, E.E. and Rudolph, K. (1979). Induction of water soaking in plant leaves by extracellular polysaccharide from phytopathogenic bacteria *Pseudomonas* and *Xanthomonas*. Physiol. Plant Pathol. 15: 341-349.

Klement, Z. (1963). Rapid detection of the pathogenicity of pathogenic pseudomonads. Nature. 199: 299-300.

Mahadevan, A. and Ulaganathan, K. (1992). Techniques in molecular plant pathology. Sivakami Publications, Madras, India.

Mandel, M. and Higa, A. (1970). Calcium dependent bacteriophage DNA infection. J. Mol. Biol. 53: 159-162.

Nester, E.W. and Kosuge, T. (1981). Plasmids specifying plant hyperplasia. Annual Rev. Microbiol. 35: 531-565.

Patel, P.N. and Jindal, J.K. (1970). Bacterial diseases in seed legumes in 1968 and 1969. Paper presented at 4th Annual Workshop Conference on Pulse Crops, April 1970, Ludhiana.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning a laboratory manual. 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sathyanarayana, N. (1992). Characterization of plasmids in *Xanthomonas campestris* pv. *malvacearum*. Ph.D. thesis, IARI, New Delhi.

Sathyanarayana, N. and Verma, J.P. (1993). Possible role of plasmids in the virulence of *Xanthomonas campestris* pv. *malvacearum*. Indian Phytopath. 46(2): 165-166.

Ulaganathan, K. and Mahadevan, A. (1988). Indigenous plasmids of *Xanthomonas campestris* and characters encoded by a plasmid of *Xanthomonas campestris* pv. *vignicola*. Indian J. Expt. Biol. 29: 1022-1026.

Ulaganathan, K. (1989). Plasmid screening and characterization in plant pathogenic bacteria with special reference to *Xanthomonas campestrix* pv. *vignicola*. Ph.D. thesis, University of Madras, Chennai.

Vauterin, L., Hoste, B., Kersters, K. and Swings, J. (1995). Reclassification of *Xanthomonas*. Intn. J. Syst. Bacteriol. 45: 472-489.

Verdier, V., Assigbeste, K., Chhetri, G.K., Wydra, K., Rudolph, K. and Geiger, J.P. (1998). Molecular characterization of the incitant of cowpea bacterial blight and pustule, *Xanthomonas campestris* pv. *viginicola*. European J. Pl. Pathol. 104: 595-602.

Verma, J.P. (1986). Bacterial blight of cotton. CRC Press, Boca Raton, Fla., USA. Pp. 278.

Zanen, I., Vanlarbeke, N., Teuchy, H., Van Montagu, M. and Schell, J. (1974). Super coiled circular DNA in crown gall inducing *Agrobacterium* strains. J. Mol. Biol. 86: 109- 127.