Plasmid-borne determinants of colony morphology, pigmentation, antibiotic resistance and antibiosis in *Pseudomonas* species antagonistic to bacterial blight of cotton

Sujoy Saha, R. P. Singh, J. P. Verma and J. Jayaraman*

Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi 110 012, India

Plasmids of bacteria are responsible for definite phenotypic traits. The plasmid profile of a cotton phylloplane bacterium belonging to genus *Pseudomonas* and antagonistic to the incidence of bacterial blight of cotton, *Xanthomonas axonopodis* pv. *malvacearum* was studied. The plasmid restriction had restriction sites for *Bam*HI and *Eco*RI. The cured strain of the bacteria lost its original colony characters, pigmentation, antibiotic resistance and antibiosis which were again restored when the cured bacteria was transformed by its plasmid deoxyribonucleic acid (DNA). The investigation confirmed that the above-mentioned phenotypic traits were plasmid-borne.

PLASMIDS are circular, supercoiled DNA molecules present in many strains of bacteria. Most plasmids are small and are about 0.2 to 4 per cent of the size of the bacterial chromosome. A bacterial plasmid was first discovered as the fertility factor (F-factor) in *Escherichia coli*[1]. In *Pseudomonas* sp., plasmids were first discovered by Gonzalez and Vidaver[2]. As genetic material, all the plasmids fall into two groups – the cryptic plasmids (which have no definite function except their own replication and dissemination) and the plasmids which are responsible for definite phenotype. In the present investigation, the plasmid-borne determinants of a *Pseudomonas* sp. isolated from the leaf of cotton infected with bacterial blight caused by *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*) were studied. This *Pseudomonas* sp. which was antagonistic to the bacterial pathogen both *in vivo* and *in vitro* was isolated from the cotton phylloplane.

Plasmids of *Pseudomonas* sp. were isolated by modified alkaline lysis methods[3], unless stated otherwise. Gel electrophoresis of plasmid DNA was carried out using a 0.7% agarose gel in Minnie-Gelcicle, HE-33 (Hoeffer Sci. Inst. USA) at 60 V for 1 h 30 min. Restriction profile of the plasmid was done by digesting it with restriction enzymes like *Eco*RI, *Bam*HI, *Hin*dIII, *Pst*I and *Sma*I (Genei, Bangalore) and then observing the pattern in (0.7%) gel.

Curing of the plasmid was done[4] by exposing the overnight grown cultures to elevated temperature (42°C) and 1% sodium dodecyl sulphate (SDS). For transformation of cured *Pseudomonas*, competent cells were prepared as outlined by Ausubel *et al.*[5]. 15 µl of plasmid DNA (10 ng) was added in a 15 ml test tube and kept on ice. The competent cells were rapidly thawed and 100 µl was quickly dispensed into the test tube. It was kept on ice for 30 min and then the mixture was given a heat shock at 42°C for 2 min, after adding 1 ml of Luria broth in the tube. The tubes were incubated at 28°C in a shaker incubator (200 rpm). Several dilutions were made from this suspension and plated, which were incubated overnight at 28°C.

The *Pseudomonas* sp. was tested for sensitivity to ampicillin rifampicin, streptomycin and tetracycline, at concentrations of 5, 10, 20, 25, 40, 50, 100 and 125 µg/ml.

The plasmid profile of the bacteria exhibited three bands of the plasmid deoxyribonucleic acid (DNA) running parallel to 4 kb, 2 kb and 1.5 kb positions of the 1 kb marker (Figure 1 *a*). The restriction profile showed that the plasmid had no sites for the enzymes *Hin*dIII, *Pst*I and *Sma*I; *Eco*RI had two sites, as exhibited by two fragments corresponding to 4.75 kb and 4.5 kb of the 1 kb ladder (Figure 1 *a*). *Bam*HI had four sites as four bands were observed running parallel to 3, 2.5, 1.5 and 1.3 kb of the ladder (Figure 1 *b*). The aggregate of the fragments in *Eco*RI and *Bam*HI was approximately 8.75 kb and 8.3 kb, respectively. Therefore, the plasmid DNA of *Pseudo-*

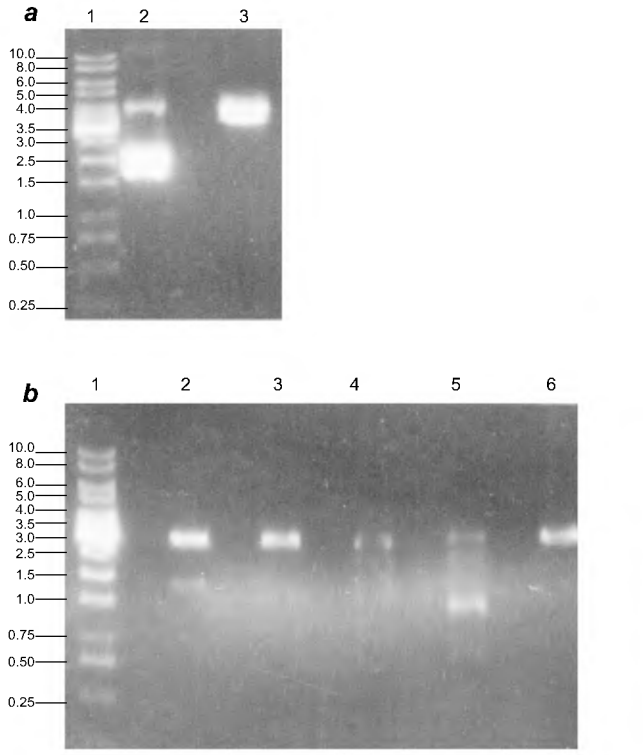

Figure 1 *a*, *b*. Plasmid profile of *Pseudomonas* sp. ***a***, Lane 1, 1 kb DNA ladder (marker); lane 2, Plasmid of *Pseudomonas*; lane 3, Restriction with *Eco*RI; ***b***, Lane 1, 1 kb DNA ladder (marker); lane 2, Plasmid of *Pseudomonas*; lane 3, Restriction with *Hin*dIII; lane 4, Restriction with *Pst*I; lane 5, Restriction with *Bam*HI; lane 6, Restriction with *Sma*I.

*For correspondence. (e-mail: jaya@bic.iari.ren.nic.in)

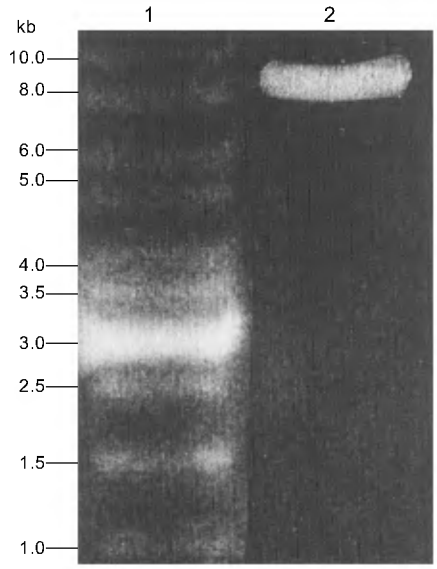

Figure 2. Plasmid profile of *Pseudomonas* sp. Lane 1, 1 kb DNA ladder (marker); lane 2, Plasmid of *Pseudomonas* sp.

monas sp. (Figure 2) was approximately 8.5 kb (mean of the two).

The bacteria was cured by both SDS and elevated temperature. Curing by SDS (40%) was more efficient than elevated temperature (28%). Ruiz Barba *et al.*[6] reported that SDS gave 10 to 50% plasmid cured strains of *Lactobacillus plantarum*, which is in accordance with the present findings. It was also found to be naturally resistant to ampicillin as it could withstand a concentration of 125 µg.

The colonies of the cured strain were white compared to the yellowish-white colour of the wild type strain. The colonies were also smaller than the wild type strain. Moreover the colonies of the wild type strain were raised but the cured strains exhibited flat colonies. When tested for antibiotic resistance, it was observed that the cured strain of *Pseudomonas* was susceptible to an ampicillin concentration of 5 µg/ml, whereas the wild strain was naturally resistant to it. *In vitro* antibiosis of the wild and cured strains were tested against *Xam* by the dual culture method and it was found that the wild strain gave a clear halo zone depicting antibiosis, while the cured strain could not exhibit a similar property. *In vivo* antibiosis was also checked against the same pathogen and it was found that the wild type strain (in the presence of *Xam*) gave a protection which was exhibited by a hypersensitive reaction (HR) while the cured strain did not protect the cotton plants from *Xam* infection, i.e. there was no HR but a clear water-soaking characteristic of the disease on leaves, indicating a susceptible reaction (SR). The comparative characters of wild type and cured strain are briefly summarized in Table 1. Similar results of plasmid encoded

Table 1. Comparison of phenotypes of wild *Pseudomonas* and its plasmid cured strain

Character	Wild strain	Plasmid cured strain
Plasmid profile	Three bands of plasmids were seen	All the bands were absent
Colony	Yellowish white; raised and smooth; 2–3 mm in diameter	White; flat and rough smaller size ~ 1 mm in diameter
Antibiotic resistance	Amp^r (normal growth up to 125 µg/ml)	Amp^r (no growth at 5 µg/ml)
Antibiosis		
In vivo	Present	Absent
In vitro	Present	Absent

characters were reported on plant pathogenic bacteria only. The present study highlights similar properties of a phylloplane non-pathogenic bacterium.

The colony characters of the transformed cells of cured bacteria were identical to the wild type strain of *Pseudomonas*. The transformants also regained the attribute of antibiotic resistance. Amuthan and Mahadevan[7] obtained similar results when they transformed pMA36 of *Xanthomonas oryzae* pv. *oryzae* into a cured strain to get ampicillin resistance. The antibiosis studies were done by dual culture method using the transformant as well as the wild strain. Halo zones were observed in both the cases, clearly signifying that the transformant could give protection against the pathogen *in vitro*. Finally *in vivo* protection was also ascertained by the transformant when it exhibited HR (in the presence of *Xam*) in susceptible cotton cultivars.

The transformation studies confirm that those properties which were lost from *Pseudomonas* after curing, i.e. colony morphology, pigmentation, antibiotic properties and antibiosis properties, were restored after transformation and these traits were plasmid-borne.

1. Lederberg, J., Cavali, L. L. and Lederberg, E. M., *Genetics*, 1952, **37**, 720–730.
2. Gonzalez, C. G. and Vidaver, A. K., *J. Gen. Microbiol.*, 1979, **110**, 161–170.
3. Birnboim, H. C. and Doly, J., *Nucleic Acids Res.*, 1979, **7**, 1515–1523.
4. Marcelo, G., Tolmasky, E., Actis, L. A. and Crosa, J. H., *Plasmids – A Practical Approach* (ed. Hardy, K. G.), IRL Press, Oxford University Press, 1998, p. 252.
5. Ausubel, F., Brent, R., Kingston, R. E., Moore, R. D., Seedman, J. G., Smith, J. A. and Struhl, K., *Short Protocols in Molecular Biology*, John Wiley, 1995, pp. 2–11.
6. Ruiz Barba, J. L., Piard, J. C. and Jiminez Diaz, R., *J. Appl. Bacteriol.*, 1991, **71**, 417–421.
7. Amuthan, G. and Mahadevan, A., *J. Appl. Bacteriol.*, 1994, **76**, 529–538.

Received 13 January 2000; revised accepted 27 July 2000