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Full Length Research Paper

Identification of indigenous *Xanthomonas* isolates through 16S rRNA gene amplification and SDS PAGE analyses

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Characterization of seven *Xanthomonas* strains comprising four species was done using molecular techniques, that is, 16S rRNA gene amplification and SDS-PAGE analysis. 16S rRNA gene amplification was performed to confirm the identity of strains. The primer sets used in the present study amplified 0.352 Kb and 1.3 Kb sized fragments of rRNA gene in all the strains. The species of the genus *Xanthomonas* exhibited relatively high levels of overall similarity. SDS-PAGE analysis was done to check the protein profile of the isolates and homogeneous banding pattern was observed that confirmed the authenticity of strains as *Xanthomonas.* A database of SDS-protein patterns provides a valuable tool for the identification of unknown *Xanthomonas*.

Key words: *Xanthomonas*, 16S rRNA gene amplification, SDS PAGE.

INTRODUCTION

As sequencing entire genomes on a routine basis for taxonomic purposes is still impractical, the sequencing of “molecular chronometers” like rRNA genes has been developed during last couple of decades (Woese, 1987). A considerable part of the 16S rRNA gene is conserved in all bacterial genera, whereas a smaller part is variable and this enable workers to estimate genealogical distances, from which phylogenies are derived (Hauben et al., 1997).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is an important molecular technique used for the identification at species level of whole cell proteins and it has the advantage of being fairly simple and rapid to perform. However for identify-cation this technique requires extensive data to cover all known target species (Leisner et al., 1994). In SDS-polyacrylamide gel electrophoresis, proteins are separated largely on the basis of polypeptide length, and so their molecular weight can also be estimated.

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MATERIALS AND METHODS

Procurement of bacterial cultures

Seven isolates of *Xanthomonas* representing four different species including *X. campestris*, *X. maltophilia, X. axonopodis* and *X. nematophilus* were procured from First Fungal Culture Bank of Pakistan (FCBP), Institute of Plant Pathology, University of the Punjab, Lahore. List of these isolates along with their substrate sources is given in Table 1.

Isolation of genomic DNA

DNA was extracted from bacterial cells by following the method of DNA isolation described by Newman (1998) with slight modifications. A single colony from a fresh Luria-Bertani agar (LBA) plate was used to inoculate 10 ml LB broth which was grown to saturation. An aliquot of 1.5 ml of the culture was centrifuged at maximum speed (14000 rpm for 5 min), the supernatant was discarded and pellets were resuspended by vigorous vortexing in 500 µl TE buffer to which 60 µl 10% SDS and 60 µl proteinase K (20 mg/ml) were added. After 1 h incubation at 37ºC, DNA was extracted twice with phenol/chloroform (1:1) and twice with chloroform. Nucleic acids were precipitated using absolute ethanol and sodium chloride. The resulting pellet was resuspended in TE buffer containing RNase A and incubated at 37ºC for 30 min. DNA

Table 1. List of isolates of *Xanthomonas* species used for molecular characterization.

FCBP Accession #	Strain	Substrate
1	*Xanthomonas axonopodis* (I)	Citrus fruit
186	*Xanthomonas axonopodis* (II)	Rhizospheric soil
8	*Xanthomonas campestris* (III)	*Mangifera indica*
118	*Xanthomonas maltophilia* (IV)	*Brassica campestris*
132	*Xanthomonas maltophilia* (V)	*Pisum sativum*
98	*Xanthomonas nematophilus* (VI)	Root nodules
115	*Xanthomonas nematophilus* (VII)	*Brassica campestris*

Table 2. List of Oligonucleotide primers used for 16S rDNA amplification.

Primer	Sequence	Tm	nmole
16F27	5'AGAGTTTGATCMTGGCTCAG3'	48	74.5
16R343	5'ACTGCTGCCTCCCGTA3'	48	100.3
16R1389	5'ACGGGCGGTGTGTACAAG3'	52	60.9

was finally precipitated with 5M ammonium acetate and isopropanol. After centrifugation, the pellet was washed twice with 70% ethanol, air dried and dissolved in TE buffer.

PCR amplification of 16S rRNA Gene from *Xanthomonas* isolates

As the cultures of *Xanthomonas* species were collected from FCBP therefore targeting of 16S rRNA gene was used to confirm the identity of strains. The 16S rRNA gene is most typically used for classification of bacteria. However, the classification method is still a matter of controversy. The accuracy of phylogenetic analysis using a single gene depends on the selected gene that may not truly reflect the whole evolutionary history of organism in question. Moreover, the 16S rRNA classification has been useful only for taxa above the rank of species (Woese et al., 1990).

Ribotyping was carried out as described by Saiki et al. (1988). The conditions of rRNA gene amplification were optimized on all the isolates of *Xanthomonas* species by using different bacterial specific rRNA primers. The rRNA gene amplification was targeted by regulating different temperature, that is, 45, 50 and 55°C during polymerase reaction and the optimized melting temperature for rRNA gene amplification was 55°C. The reaction mixture for rRNA gene amplification is given in appendix I. List of *Xanthomonas* specific rRNA primers are given in Table 2.

Temperature cycling condition of rRNA gene amplification

The samples were processed through 35 amplification cycles consisting of 45 sec of denaturation at 94°C, 45 s of annealing at the primer specific temperature (55°C), and 2 min of primer extension at 72°C.

Analysis of amplified rRNA gene fragment

The standard reagents were supplied by Fermentas. The amplification product was analyzed by 1% agarose gel electrophoresis. Before loading the amplified rRNA gene in the wells of gel, 5 µl of 6X gel loading dye was added. DNA ladder of 100 bp was loaded on one side of the gel. The gel was run at 100 volts for 45 min at room temperature. The bands were examined under UV transilluminator (WISEDoc MUV-M 20) and photographed by using the gel Documentation system.

Total protein estimation through SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is an important molecular technique used for the identification at species level of whole cell proteins and it has the advantage of being fairly simple and rapid to perform. However for identification this technique requires extensive data to cover all known target species (Leisner et al., 1994). Preparation and electrophoresis of whole-cell protein extracts was performed by the SDS-PAGE method of Laemmli (1970), using a slightly modified procedure.

Cultures were grown on LBA slants for 48 h at 28°C. Cells were suspended in 0.01 M-potassium phosphate buffer with pH 7.0 and transferred to flasks containing 150 ml LB broth medium. Flasks were incubated for 48 h at 28°C. Cells were harvested from the flasks with 20 ml phosphate-buffered saline solution (4.26 g Na_2HP0_4, 2.27 g KH_2P0_4, 8 g NaCl, in distilled water), and washed and centrifuged at 15000 rpm twice. A pellet of about 70-80 mg was suspended in sample treatment buffer (7.5 g Tris, 50 ml β-mercaptoethanol, 100 ml glycerol, in double-distilled water; final pH 6.8, adjusted with HCl) in an Eppendorf tube with 0.14 ml of a 20% (w/v) SDS solution, suspension was mixed and heated at 95°C for 10 min. Tubes were cooled on ice and centrifuged at 10,000 rpm. Supernatant was divided into two portions, one of which was stored at - 20°C for immediate use and the other at - 80°C for long storage. Electrophoresis was performed in a 12% (w/v) gel slab, run vertically until the bromophenol blue tracking dye had migrated 95 mm (about 15 h). Gels were stained for 1 h in a solution containing 0.25% (w/v) Coomassie Blue G250, 50% (v/v) ethanol and 10% (v/v) acetic acid, and destained in a solution containing 25% methanol and 10% acetic acid. The reproducibility of the electrophoresis technique was monitored by preparing protein extracts of strains in duplicate and running reference samples on each of the slab gels.

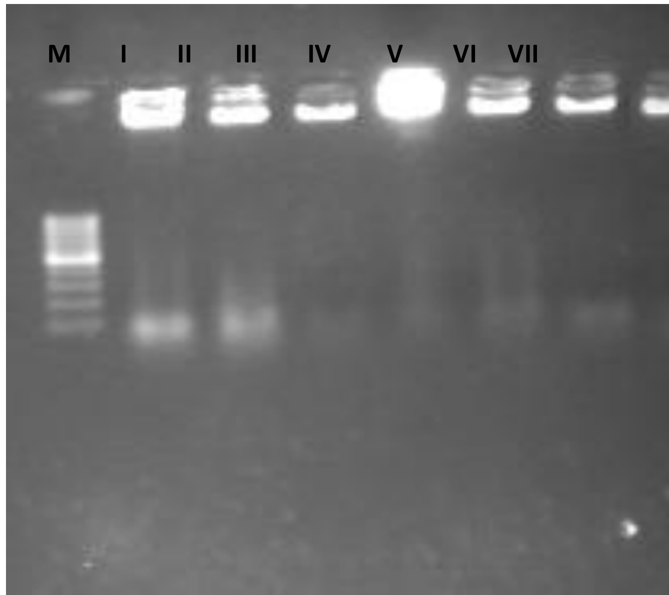

Figure 1. Genomic DNA isolation from different isolates of *Xanthomonas* species. M: marker (100 bp). I: *X. axonopodis* (citrus fruit) II: *X. axonopodis* (Rhizospheric soil) III: *X. campestris* (*Mangifera indica*) IV: *X. maltophilia* (*Brassica campestris*), V: *X. maltophilia* (*Pisum sativum*) VI: *X. nematophilus* (Root nodules) VII: *X. nematophilus* (*Brassica campestris*).

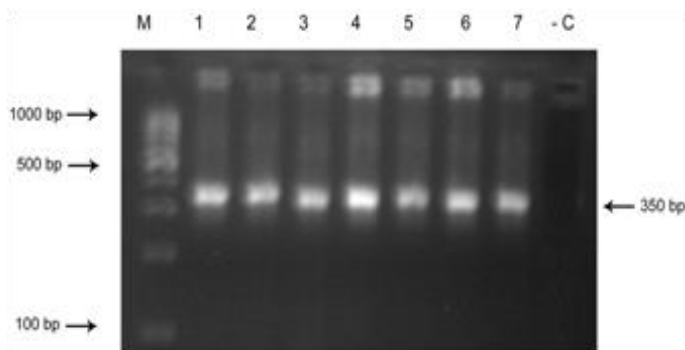

Figure 2a. 16S rRNA gene amplification (350bp) of seven *Xanthomonas* isolates by using gene specific primers. M: DNA marker (100bp) 1: *X. axonopodis*(citrus fruit) 2: *X. axonopodis* (Rhizospheric soil) 3: *X. campestris* (*Mangifera indica*) 4: *X. maltophilia* (*Brassica campestris*) 5: *X. maltophilia* (*Pisum sativum*) 6: *X. nematophilus* (Root nodules), 7: *X. nematophilus* (*Brassica campestris*) -C: Negative control.

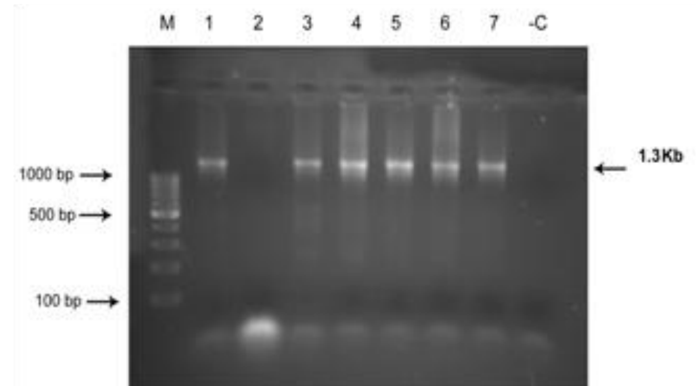

Figure 2b. 16S rRNA gene amplification, (1.3 Kb fragment) of seven *Xanthomonas* isolates by using gene specific primers. M: DNA marker (100bp) 1: *X. axonopodis*(citrus fruit) 2: *X. campestris* (Mangifera indica) 3: *X. axonopodis* (Rhizospheric soil) 4: *X. maltophilia* (*Brassica campestris*) 5: *X. maltophilia* (*Pisum sativum*) 6: *X. nematophilus* (Root nodules) 7: *X. nematophilus* (*Brassica campestris*) -C: Negative control.

RESULTS

Genomic DNA extraction of Xanthomonas Isolates

Total seven isolates of Xanthomonas representing four species were subjected to DNA extraction and the samples were run on 0.8% agarose gel. Results of all these isolates are shown in Figure 1.

PCR amplification of 16S rRNA Gene for *Xanthomonas* Isolates

Specifically designed primers for *Xanthomonas* 16S rRNA gene were used to confirm the strains identity. Different sets of primers amplified various conserved regions of the gene. The primer sets used in the present study amplified 0.352 Kb and 1.3 Kb sized fragments of rRNA gene in all the strains (Figure 2a and 2b). In negative control no amplification was observed indicating the validity of this assay. A band of 0.352 Kb was observed by using the primer set i.e. 16F27 (5' AGAGTTTGATCMTGGCTCAG 3') and 16R343 (*5'* ACTGCTGCCTCCCGTA 3'), whereas larger fragment of 16S rRNA gene (1.3Kb) was amplified by using the primer set i.e. 16F27 (5' AGAGTTTGATCMTGGCTCAG 3') and 16R1389 (5' ACGGGCGGTGTGTACAAG 3'). In all the isolates 1.3 Kb band was amplified except for *X. campestris* (*Mangifera indica*). Two sets of primers used for rRNA gene amplification were previously described in Table 2.

#Total protein estimation through SDS-PAGE

Protein bands obtained from each isolate were compared with standard SDS-PAGE Low range molecular weight marker (97 Kda). Homogenous protein profiles with slighter variations were observed in all the isolates showing that they belonged to the genus *Xanthomonas* (Figure 3).

DISCUSSION

Molecular techniques like 16S rRNA and SDS-PAGE

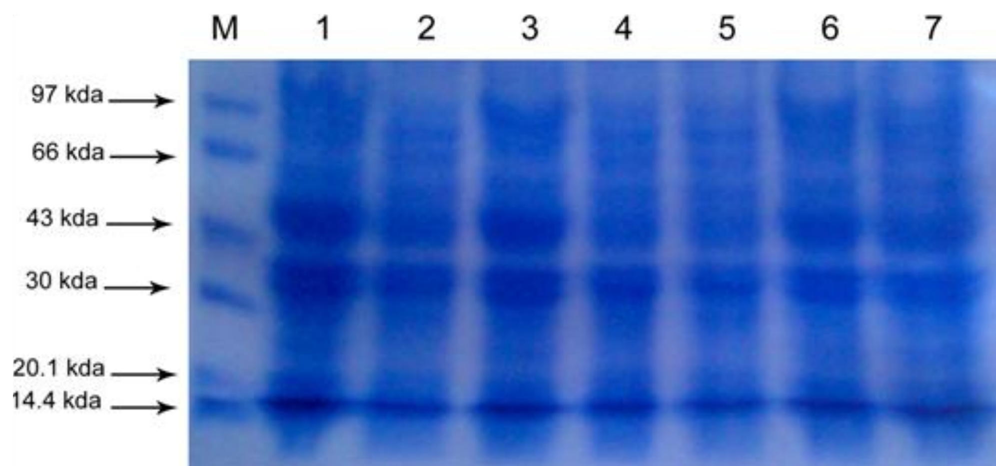

Figure 3. Total protein profile of *Xanthomonas* isolates through SDS-PAGE. M: Protein marker (SDS-PAGE low molecular range marker) 1: *X. axonopodis* (Citrus fruit) 2: *X. campestris* (*Mangifera indica)* 3: *X. axonopodis* (Rhizospheric soil) 4: *X. maltophilia* (*Brassica campestris)* 5: *X. nematophilus* (Root nodules) 6: *X. nematophilus* (*Brassica campestris*) 7: *X. maltophilia* (*Pisum sativum*).

were employed to confirm the identity and authenticity of strains. Two rRNA gene fragments amplified were of 0.350 and 1.3 Kb size.

The 16S rRNA genes have become the standard for the determination of phylogenetic relationships, the assessment of diversity in the environment, and the detection and quantification of specific populations. Gene encoding the small ribosomal subunit (16S rRNA gene in bacteria) was selected because this gene contains both conserved and variable region.

In the current study, partial amplification of 16S rRNA gene (0.352 and 1.3 Kb) was carried out by using the method of Saiki et al. (1988). Specific primers were used for amplification of 16S rRNA to confirm the identity of strains as *Xanthomonas*. All the strains showed similar bands on 1% agarose gel because only a small part of 16S rRNA gene is variable which can only be detected through sequencing. Sequence analyses of these amplified bands will further aid in determining variation among different *Xanthomonas* species. Our findings resemble the results reported by Hauben et al. (1997) who revealed a very small degree of divergence of 16S rRNA gene sequences among the *Xanthomonas*, with just three phylogenetic lineages being detected. These lineages included *X. campestris* (cluster1), *X. sacchari* (cluster2) and *X. albilineans* (cluster3). Mean similarity value of 98.2% was exhibited by species of the genus *Xanthomonas* which corresponds to an average of 14 mutual nucleotide differences.

The structure of the whole cell proteins as revealed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of seven *Xanthomonas* isolates showed a very uniform pattern. A database of SDS-protein patterns used as a tool for the identification of unknown *Xanthomonas*. Our results showed similar pattern of protein bands among two isolates of *X. axonopodis* on different substrates, that is, citrus fruit and rhizospheric soil and were supported by the findings of Ogunjobi et al. (2007), who observed homogeneous profile of whole cell protein patterns of *X. axonopodis* pv *manihotis* strains. Goncalves and Rosato (2000) reported a similar observation for *Xanthomonas* strains isolated from passion fruit plants and concluded that the *passiflorae* group was closely related and different from the species from other *Xanthomonas*.

The protein-banding pattern was not discriminatory enough within the same species and could not reveal minute differences in the bacterial strains. Our results also in line with Vauterin et al. (1991), they analyzed protein electrophoregrams of 307 *Xanthomonas* strains comprising all species and 27 *X. campestris* pathovars. From the fairly high similarity values between clusters of *Xanthomonas* species and *X. campestris* pathovars they revealed that most of the *Xanthomonas* display a common SDS-PAGE protein profile. Highly distinct profiles were only produced by *X. albilineans* and *X. maltophilia.*

16S rRNA gene amplification was the initial step of ribotyping that can further be proceeded for sequence analyses to acquire a new picture of the genus *Xanthomonas* and further helps in taxonomic characterization. SDS-PAGE analysis will help to identify any unknown *Xanthomonas* and this work will further be processed towards expression analysis of different proteins in order to distinguish pathogenic and non-pathogenic isolates.

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