Identification of *Agrobacterium tumefaciens* Strains by PCR-RFLP Analysis of the 16S-rDNA

*Nihayah Al-Karablieh, Hamed Khlaiif, and Lama Al-Banna*

**ABSTRACT**

16S-rDNA analysis of 30 *Agrobacterium tumefaciens* Jordanian isolates belonging to 1, 2, 3 and intermediate biotypes, were characterized by the restriction fragment length polymorphism analysis of PCR product. Two reference cultures of *A. tumefaciens* were used for comparison. The profile obtained by the analysis formed three main clusters confirmed the biotyping of the strains, and allocated intermediate biotype with biotype 1 strains as a typical biotype 1 strains, also, higher genetic distances were found within biotype 1 strains.

**KEYWORDS:** *Agrobacterium*, 16S-rDNA, PCR-RFLP, Dendrogram, Electrophoresis.

1. INTRODUCTION

Crown gall induced by *Agrobacterium tumefaciens* (Smith and Townsend Conn., 1983). The disease is characterized by the formation of tumors at the wounded sites (Pionnat et al., 1999), systematic infection in the xylem, or could be found as latent infection (Marti et al., 1998).

*Agrobacterium tumefaciens* strains survive in plants, soils or other reservoirs, where the pathogen can remain undetected in these reservoirs mainly in the soils, where nurseries will be established and seedlings produced in nurseries. Also, the genetic structure of agrobacterial population is very complex because it could contain both pathogenic and non-pathogenic strains (Ponsonnet and Nesme, 1994; and Hass et al., 1995).

Restriction Fragment Length Polymorphism (RFLP) analysis of the target PCR product DNA fragment has been developed, to identify and discriminate the phylogenetic relatedness among many microorganisms (Vilgalys et al., 1990; Ponsonnet and Nesme, 1994; Nesme, et al., 1995; Lin et al., 1996; Liu et al., 1997; and Mougel, et al., 2001). No published information is available about the possible variation among the different Jordanian isolates of *A. tumefaciens* so far, therefore, analysis of 30 *Agrobacterium tumefaciens* isolates collected from different hosts and locations in Jordan were characterized by the restriction fragment length polymorphism analysis of the 16S rDNA fragment.

2. MATERIAL AND METHODS

Thirty Jordanian isolates belonging to different biovars of *A. tumefaciens*, biotyped by the method of Moore et al. (1988) (Table 1), representing different locations, and hosts were analyzed by PCR-RFLP of the 16S-rDNA (16S8-16S1467 region). Two reference bacterial isolates C58 and B6 (kindly provided by R. Ivaly and M. Lopez from Spain) and water were used as positive and negative controls, respectively.

**Genomic DNA Extraction.**

Genomic DNA purification kit (Promega) was used for the extraction of genomic DNA as described by the manufacturer instructions. Briefly, 1ml of 10^7 CFU/ml...
bacterial suspension was prepared from 24hrs old cultures of each tested bacterial isolate or control was placed in a microcentrifuge tube, centrifuged at 13000rpm for 2mins, supernatants were discarded and the pellets were re-suspended in 600µl of nuclei lysis solution. Tubes were incubated at 80°C for 5mins to lyse the cells, then cooled at room temperature. Three microliters of RNase solution were added to the cell lysate and incubated at 37°C for 30mins. After cooling to room temperature, 200µl of protein precipitation solution was added, vortexed for 20 seconds, and incubated in an icebox for 5mins. Tubes were centrifuged at 13000rpm for 3mins, and 600µl of the supernatants were filled into new microcentrifuge tubes containing 600µl of 99% isopropanol and gently mixed until visible mass of thread-like strands of DNA formed. After centrifugation at 13000rpm for 2mins, the supernatant fluid was discarded and the tubes were blotted on sterile filter paper. Pellets were re-suspended in 600µl of 70% ethanol to wash the DNA pellets, followed by centrifugation at 13000rpm for 2mins, the supernatant fluids were discarded and the tubes were blotted on sterile filter paper and allowed to air-dry for 15mins. A total of 100µl DNA dehydration solution was added to the tubes, and were incubated at 65°C for 1hr for dissolving DNA. The extracted DNA was kept at 4°C until used for PCR.

**PCR Amplification for 16S rDNA.**

The primers for the amplification reactions, were F63r16S (5’GGAGAGTTAGATCTTGGCTCAG3’) and F153r16S (5’AAGGAGGGGATCCAGCCGCA3’), were used to detect the presence of 16S rDNA region (Ponsonnet and Nesme, 1994).

PCR reaction was performed in a total volume of 50µl containing: 2µl genomic DNA, 1X PCR (5µl of 10X) buffer (which has 50mM KCl, 10mM Tris-HCl, 1.5mM MgCl2, 0.1% Triton X-100, pH 9.0 at room temperature), also, 0.5mM of MgCl2 (1µl of 25mM) was added, until the final concentration of MgCl2 reached 2mM, 0.2mM (1µl of 10mM) of d-NTPs mixture, 0.2µM (1µl of 10µM working dilution) of both F63r16S and F153r16S primers and 2.5U (0.5µl of 5U/ µl) of Taq DNA polymerase (Promega).

Amplification reaction was performed in a thermal cycler (9700 Perkin-Elmer), using the following protocol: Initial denaturation at 94°C for 3mins, followed by 35 cycles of 1min at 94°C for denaturation, 45 seconds at 60°C for annealing, and 1min at 72°C for extension, and an additional extension at 72°C for 4mins. After the amplification reaction, samples were stored at 4°C.

**Analysis of the PCR Products.**

The PCR products were separated by horizontal electrophoresis in 1X TBE buffer; on 1% (W/V) LE agarose gel contains 1µg/1ml ethidium bromide. The 1kb ladder was used as a standard molecular marker (Promega). A total of 3µl of PCR products mixed with 7µl loading dye were electrophoresed at 100V for 60 min. The gels were photographed under UV light (302 nm).

**RFLP Analysis of the PCR Products of the 16S rDNA.**

Ten microliters of the PCR products were digested for 3hrs with the following restriction endonucleases; *TaqI, NdeII*, and *HaeIII* (Promega). The reactions were in a final volume of 15µl, with 10U (1µl of 10U/µl) of each enzyme, 1.0X (1.5µl of 10X) of each specific enzyme buffer was added to the digestion mix, 10mM (1.5µl of 100mM) of Dithiothreitol (DTT) were added only to the digestion mix of *NdeII*. The assay temperature used for *NdeII* and *HaeIII* was 37°C and 65°C for *TaqI*.

Digested products were separated by horizontal electrophoresis in 1X TBE buffer using a 3% low melting agarose containing 1µg/ml ethidium bromide. In each well, 10µl of each digested product was mixed with 2µl of 6X blue orange loading dye. The 100bp ladder was used as a standard molecular marker (Promega). Gels were run at 75V for 150mins, and immediately photographed under UV light (302nm).

**3. DATA ANALYSIS**

For RFLP bands, presence or absence of each site resulted from digestion with different enzymes was scored as 1 and 0, respectively. Phylib (Phylogeny Inference Package) v. 3.6 (a2)/ Restdist, was used to
estimate the number of nucleotide substitution per site according to Nei et al. (1985),
\[ D = \frac{-\ln S}{r}. \]

Where \( S = 2 \frac{m_{xy}}{m_x + m_y} \).

Here, \( m_x \) and \( m_y \) are the number of restriction sites for isolate \( x \) and \( y \), respectively, \( m_{xy} \) is the number of restriction sites shared by the two isolates, and \( r \) is the number of restriction nucleotides in the recognition sequence of the restriction enzymes (\( r = 4 \) in all cases in the study). The distance matrix (D value) was used to construct dendrograms using methods of UPGMA.

4. RESULTS

PCR-RFLP Analysis of the 16S rDNA.

All the tested isolates formed a single band of 1479bp when F63r16S and F153r16S primers were used for PCR amplification of the 16S-rDNA region (Fig. 1). Digestion of this region with restriction enzymes \( TaqI \), \( NdeII \), and \( HaeIII \) for strains belonging to the three biotypes and intermediate biotype formed different fragment patterns for the 16S-rDNA region. The fragment patterns produced by C58 and B6 were similar and identical fragment patterns described by Ponsonnent and Nesme (1994) for the 16S-rDNA region. Whereas the digestion of the 16S-rDNA region of the tested isolates, produced different band sizes. Digestion of the product with \( TaqI \) produced band sizes of 913, 455, 355, 266, and 218 bp, while with \( NdeII \) it produced band sizes of 945, 725, 474, 355, 272, 248, and 175bp, and with \( HaeIII \) band sizes of 738, 492, 385, 307, 246, 184, and 153bp were produced. The presence or absence of certain fragment varied according to the isolates (Fig 2-Fig9).

Large number of polymorphism was generated by PCR-RFLP of the tested isolates. The genetic distance between the 32 Agrobacterium isolates ranged from 0.0 for B6, A275, and A360; A419, A575, A1068, and A1087; A1230, and A1037; A509 and A1294; A510 and A1302; A291 and A299; A1108, and A145; A202, and A186, and for A933, and A1046 to 0.4031 for A1215 and both A202 and A186 isolates.

However, the genetic distance between B6, A275, and A360, and A419, A575, A1068, and A1087 was 0.0297 that indicated close relationship between them, also A510 and A1302 and A1215 and A208 were very close in genetic distance which is 0.0297, and A1230, A1037 was related to A1074 in genetic distance which is 0.0566, and A1251 was more related to A152 in genetic distance which is 0.1041.
Table (1): Source and biotyping of Jordanian *A. tumefaciens* isolates used for PCR-RFLP analysis.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Location</th>
<th>Plant species</th>
<th>Biotype</th>
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<tr>
<td>A275</td>
<td>Al-Mafraq</td>
<td>Rose</td>
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</tr>
<tr>
<td>A360</td>
<td>Al-Mafraq</td>
<td>GF677</td>
<td>1</td>
</tr>
<tr>
<td>A419</td>
<td>Al-Mafraq</td>
<td>GF677</td>
<td>Intermediate</td>
</tr>
<tr>
<td>A575</td>
<td>Al-Mafraq</td>
<td>Mahaleb</td>
<td>1</td>
</tr>
<tr>
<td>A1230</td>
<td>Al-Mafraq</td>
<td>Quince</td>
<td>2</td>
</tr>
<tr>
<td>A1037</td>
<td>Al-Salt</td>
<td>Apple</td>
<td>2</td>
</tr>
<tr>
<td>A509</td>
<td>Al-Shobak</td>
<td>Myrobalan</td>
<td>2</td>
</tr>
<tr>
<td>A510</td>
<td>Al-Shobak</td>
<td>Myrobalan</td>
<td>Intermediate</td>
</tr>
<tr>
<td>A1302</td>
<td>Al-Shobak</td>
<td>GF305</td>
<td>1</td>
</tr>
<tr>
<td>A1294</td>
<td>Al-Tafila</td>
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<tr>
<td>A291</td>
<td>Al-Yadoda</td>
<td>Bitter almond</td>
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</tr>
<tr>
<td>A152</td>
<td>Al-Yadoda</td>
<td>Bitter almond</td>
<td>2</td>
</tr>
<tr>
<td>A299</td>
<td>Al-Yadoda</td>
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<tr>
<td>A323</td>
<td>Al-Yadoda</td>
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<tr>
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<td>Rose</td>
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</tr>
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<td>A933</td>
<td>Amman</td>
<td><em>Cichorium pumilum</em></td>
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<td>Amman</td>
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<td>A1215</td>
<td>Irbid</td>
<td>Olive</td>
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<tr>
<td>A344</td>
<td>Jerash</td>
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<tr>
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<tr>
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<tr>
<td>A1251</td>
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<td>A1074</td>
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<tr>
<td>A148</td>
<td>Madaba</td>
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<td>A145</td>
<td>Madaba</td>
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<td></td>
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</tr>
<tr>
<td>B6</td>
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5. DISCUSSION

The results of PCR-RFLP of 16S-rDNA indicated the occurrence of genetic relationship among the tested *A. tumefaciens* isolates mainly those isolated from the same location, but regardless of the plant species from which they were isolated. Some of the tested isolates were identical, since the genetic distance between them were 0.0 and the same banding pattern was formed when their 16S-rDNAs were digested with different restriction enzymes.

Digestion of the 16Sr-DNA region of the different isolates with the restriction fragment length polymorphism separated the different tested isolates according to their biotypes except for intermediate biotype isolates. The genetic distance between intermediate biotype and biotype 1 isolates were 0.0 and they formed the same banding pattern as isolates belonging to biotype 1. Therefore, these isolates could be considered as a typical biotype 1 depending on biochemical and physiological tests. These results are in agreement with the results of Ride *et al.* (2000) and Cubero and Lopez (2001), where a typical biotype 1 was recorded as a result of bacterial isolates adaptation to their host. On the other hand, the polymorphisms found within the tested biotype 1 and biotype 2 indicated the occurrence of at least two genomic subspecies among each tested biotype, since the main group which corresponded to the biotypes was also separated to two sub-groups. These results are in agreement with the results of Ponsonnet and Nesme (1994), who detected at least two genomic subspecies in the biotype 1 strains. In most cases, the isolates that have been isolated from the same geographic location were clustered together, which indicated the relatedness of *A. tumefaciens* populations within the same geographic region. Biotype 3 isolate was found to be more related to biotype 2.

![Agarose gel electrophoresis of 16S-rDNA region of *Agrobacterium* isolates.](image)

*Fig. (1): Agarose gel electrophoresis of 16S-rDNA region of *Agrobacterium* isolates.*

*Lane M*, 1kb molecular size marker; *Lane 1*, water control added to the PCR mix in the PCR workstation; *Lane 2*, water control added to the PCR mix where genomic DNA templates were added; *Lane 3 + 4*, DNA from *Agrobacterium* C58 and B6 respectively; *Lane 5*, DNA from Jordanian isolate A275 (Biotype 1); *Lane 6*, DNA from Jordanian isolate A1037 (Biotype 2); *Lane 7*, DNA from Jordanian isolate A1251 (Biotype 3).
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Fig. (2): Restriction pattern of the amplified 16S-rDNA after digestion with discriminating enzymes for reference *A.tumefaciens* C58 and B6, *A.tumefaciens* isolates A275 (Biotype 1) and A360 (Biotype 2) and water. *Lane M*, 100 bp molecular size marker; *Lane 1-3*, water control added to mix of *TaqI, NdeII*, and *HaeIII*, respectively; *Lane 4-6*, C58 digested with *TaqI, NdeII* and *HaeIII*, respectively; *Lane 7-9*, B6 digested with the same enzymes, respectively; *Lane 10-12*, A275 digested as mentioned above; *Lane 13-15*, A360 digested as mentioned above.

Fig. (3): Restriction pattern of the amplified 16S-rDNA after digestion with discriminating enzymes for *A.tumefaciens* isolates A419 (Intermediate biotype), A575 (Biotype 1), A1230 (Biotype 2), and A1037 (Biotype 2).  
*Lane M*, 100 bp molecular size marker; *Lane 1-3*, A419 digested with *TaqI, NdeII* and *HaeIII*, respectively; *Lane 4-6*, A575 digested with the same enzymes, respectively; *Lane 7-9*, A1230 digested as mentioned above; *Lane 10-12*, A1037 digested as mentioned above.
Fig. (4): Restriction pattern of the amplified 16S-rDNA after digestion with discriminating enzymes for *A. tumefaciens* isolates A509 (Biotype 2), A510 (Intermediate biotype), A1302 (Biotype 1), and A1294 (Biotype 2).

Lane M, 100 bp molecular size marker; Lane 1-3, A509 digested with *Taq*I, *Nde*II and *Hae*III, respectively; Lane 4-6, A510 digested with the same enzymes, respectively; Lane 7-9, A1302 digested as mentioned above; Lane 10-12, A1294 digested as mentioned above.

Fig. (5): Restriction pattern of the amplified 16S-rDNA after digestion with discriminating enzymes for *A. tumefaciens* isolates A291 (Biotype 1), A344 (Biotype 1), A299 (Intermediate biotype, and A323 (Biotype 1).

Lane M, 100 bp molecular size marker; Lane 1-3, A291 digested with *Taq*I, *Nde*II and *Hae*III, respectively; Lane 4-6, A344 digested with the same enzymes, respectively; Lane 7-9, A299 digested as mentioned above; Lane 10-12, A323 digested as mentioned above.
Fig. (6): Restriction pattern of the amplified 16S-rDNA after digestion with discriminating enzymes for *A.tumefaciens* isolates A1215 (Biotype 1), A152 (Biotype 2), A246 (Biotype 2), and A208 (Intermediate biotype).

*Lane M*: 100 bp molecular size marker; *Lane 1-3*, A1215 digested with *Taq*I, *Nde*II and *Hae*III, respectively; *Lane 4-6*, A152 digested with the same enzymes, respectively; *Lane 7-9*, A246 digested as mentioned above; *Lane 10-12*, A208 digested as mentioned above.

Fig (7): Restriction pattern of the amplified 16S-rDNA after digestion with discriminating enzymes for *A.tumefaciens* isolates A1074 (Biotype 2), A1068 (Biotype 1), A342 (Biotype 2), and A1087 (Biotype 1).

*Lane M*, 100 bp molecular size marker; *Lane 1-3*, A1074 digested with *Taq*I, *Nde*II and *Hae*III, respectively; *Lane 4-6*, A1068 digested with the same enzymes, respectively; *Lane 7-9*, A342 digested as mentioned above; *Lane 10-12*, A1087 digested as mentioned above.
Fig (8): Restriction pattern of the amplified 16S-rDNA after digestion with discriminating enzymes for *A.tumefaciens* isolates A1108 (Biotype 1), A1251 (Biotype 3), A148 (Biotype 1), and A145 (Intermediate biotype).

*Lane M*, 100 bp molecular size marker; *Lane 1-3*, A1108 digested with *TaqI*, *NdeII* and *HaeIII*, respectively; *Lane 4-6*, A1251 digested with the same enzymes, respectively; *Lane 7-9*, A148 digested as mentioned above; *Lane 10-12*, A145, digested as mentioned above.

Fig (9): Restriction pattern of the amplified 16S-rDNA after digestion with discriminating enzymes for *A.tumefaciens* isolates A202 (Biotype 1), A186 (Biotype 1), A933 (Biotype 1), and A1046 (Biotype 1).

*Lane M*, 100 bp molecular size marker; *Lane 1-3*, A202 digested with *TaqI*, *NdeII* and *HaeIII*, respectively; *Lane 4-6*, A186 digested with the same enzymes, respectively; *Lane 7-9*, A933 digested as mentioned above; *Lane 10-12*, A1046 digested as mentioned above.
Based on the genetic distance between the different isolates, a dendrogram has been constructed (Fig 10). According to the dendrogram, the isolates could be grouped into the following groups:

**Group 1:** includes members of the isolates forming four clusters as follows: cluster I, C58, A291, A299, A344, and A323; cluster II, A419, A575, A1068, A1087, B6, A275, A360, A1108, A145, and A148; cluster III, A202 and A186; and cluster IV, A510, A1302, A1215, A208, A933, and A1046. These were biotyped as biotype 1 according to biochemical and physiological tests except the A419, A510, A299, A208, and A145 which biotyped as intermediate biotype.

**Group 2:** includes two clusters of the isolates: Cluster I, A1230, A1037, and A1074; and Cluster II, A509, A1294, A246, A342, and A152. These isolates were biotyped as biotype 2 according to both the biochemical and physiological tests.

**Group 3:** includes only A1251 isolate that biotyped as biotype 3 according to biochemical and physiological tests.
Fig. (10): Dendrogram showing genetic relationships between *A. tumefaciens* strains at chromosomal level based on PFLP analysis of the 16S-rDNA region. The tree was constructed by using the UPGMA method with table 3 data. The scale indicates the number of restriction site change between two consecutive nodes.
REFERENCES


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PCR-RFLP analysis of Agrobacterium tumefaciens

16S- rDNA

Osmały Lebzi u, Jabarin, desks, some elements of the work have been copied, translated and adapted from other works. The use of PCR-RFLP technique for the identification of Agrobacterium tumefaciens has been studied. Thirty isolates of the pathogen were analyzed by 16S-rDNA PCR-RFLP. The results showed that the pathogen can be identified by comparing the restriction patterns obtained from the PCR products. The results indicated that the pathogen can be identified by comparing the restriction patterns obtained from the PCR products.