Establishing Taxonomy of Pathogenic Bacteria, Causative Agents of Haricot Bacterioses Spread in Different Regions of Georgia by 16S rDNA Fragments


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ABSTRACT. The haricot crop is greatly damaged by bacterial diseases, two of which stand out in Georgia by their harmfulness and wide spread: brown spot and angular leaf spot of haricot caused by the pathogens *Xanthomonas phaseoli* and *Pseudomonas phaseolicola* respectively. In order to determine the taxonomy of pathogenic bacteria causing haricot bacterial disease - brown spot in different regions of Georgia DNA have been isolated and purified. To obtain 16S ribosomal DNA fragments polymerase chain reaction has been carried out with two preliminarily specific primers designed by us, constructed of 20 nucleotides: 5’ TGG CGG ACG GGT GAG GAA TA 3’ (forward) and 5’ CGT CAT CCC CAC CTT CCT CC 3’ (reverse). Selected 16S ribosomal DNA fragments were sequenced. Sequence of PCR fragments and their analysis, using computer program “BLASTA”, allowed to identify two phytopathogenic strains 1466 and 1475 as *Xanthomonas axonopodis* pv. *phaseoli*. A global alignment of 16S rDNA fragments between the strains 1466 and 1475 by means of computer program Lalign has shown 95.8% identity of the fragments. © 2013 Bull. Georg. Natl. Acad. Sci.

**Key words:** bacterial DNA, 16S ribosomal DNA, PCR amplification, DNA sequencing.

Haricot is the most important legume used in human food especially in Georgia. Several diseases caused by bacteria, fungi and viruses are limiting factors of haricot yield. According to existing data, 6 kinds of bacterial diseases are known, two of which stand out in Georgia by their harmfulness and wide spread: brown spot and angular leaf spot of haricot caused by the pathogens *Xanthomonas phaseoli* and *Pseudomonas phaseolicola* respectively [1].

To develop an appropriate disease management strategy, the identification and the genetic diversity of the pathogen populations must be assessed. Current detection and identification procedures consist in biochemical tests [2] and molecular diagnostics by PCR [3].

The genetic diversity of different *Xanthomonas* isolates have been previously characterized by RAPD [4], restriction fragment length polymorphism (RFLP) [5] and amplified fragment length polymorphism analyses (AFLP) [6].

In prokaryotes, the 16S ribosomal DNA genes are essential and occur in at least one copy in a ge-
nome [7]. The universality of the genes makes them an ideal target for phylogenetic studies and taxonomic classification of bacteria [8].

The aim of this work is taxonomic identification of phytopathogenic strains, causing haricot brown spot by using 16S rDNA fragments as a marker, and comparison of partial sequences of the 16S rDNA genes.

**Materials and Methods**

Objects of the study were seventeen isolates (1438, 1466, 1475, 1482, 1513, 1514, 1517, 1617, 1620, 1621, 1467, 1468, 1703, 1708, 1709, 1712, 1713, 1806, 1807, 1814, 1829, 1832, 1853, 1840, 1839, 1858) causing haricot bacterial disease, kindly provided by Dr. Naila Giorgobiani, Kanchaveli Institute of Plant Protection of the Agricultural University of Georgia [1].

For DNA extraction, bacterial cells were grown in 5 ml in Luria–Bertani (LB) with 10% glycerol v/v (suggested by Maringoni et al [9] to avoid xanthane formation) for 48h at 27°C. Cells were centrifuged for 2 min at 13000 x g, the pellet was resuspended in 1.5 ml of lysis buffer (0.5% SDS; 50 mM Tris-HCl pH 7.5-8.0; 400 mM EDTA; 1 mg proteinase K) and incubated at 65°C in water bath for 3-5 h, then deproteinized in 1.5 ml of chloroform/isoamyl alcohol (24:1) v/v, centrifuged 10 min at 13000 x g. To precipitate DNA, 700 µl of cold isopropanol was added and centrifuged. Precipitated DNA was washed in 70% ethanol, dried at room temperature and suspended in 50 µl of TE buffer 10 mM Tris-HCl, 1 mM EDTA, pH 8.0. The samples were electrophoresed on 0.8% agarose gel, stained with ethidium bromide and photographed under UV light.

Primers for 16S rDNA amplification were selected and designed from database of phytopathogenic bacteria [10]

**bacteria cpgr.tigr.org** based on *Xanthomonas* ribosomal DNA gene sequence using computer program oligo 4.1.

Amplification of 16S ribosomal DNA fragments was carried out using primers 5’ TGG CGG ACG GGT GAG GAA TA 3’ (forward) and 5’ CGT CA T CCC CAC CTT CCT CC 3’ (reverse) by PCR (Thermocycler, Techne TC412). All PCRs were performed in a total volume of 25 µl using 50 ng DNA, 25 mM MgCl₂, 100 µM dNTPs, 0.5 mM each primer and 0.05U/µl Taq polymerase (Sigma). Cycling conditions were: one cycle of 94°C for 1 min and 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 1 min. Obtained PCR products were studied by horizontal electrophoresis on agarose gel [10]. 16S rDNA gene sequences were conducted commercially at The Laboratory Services Division of the University of Guelph, (ON, Canada). Obtained sequencing results were analyzed, i.e. resemblances of nucleotide sequences of 16S ribosomal DNA with data base analogues were established by computer program BLASTA. The 16S rDNA sequences of different strains of *Xanthomonas phaseoli* were aligned by using sequence alignment program (Clustal W, Lalign).

![Electrophoregram of 16S ribosomal DNA fragments of phytopathogenic bacteria, causing haricot brown spot, in Georgia on 1%-agarose gel (C- sample without DNA).](image)

**Fig. 1.** Electrophoregram of 16S ribosomal DNA fragments of phytopathogenic bacteria, causing haricot brown spot, in Georgia on 1%-agarose gel (C- sample without DNA).
Results and Discussion

Amplification of 16S ribosomal DNA has been conducted by PCR. DNA of pathogenic bacterial strains (1438, 1466, 1475, 1482, 1513, 1514, 1517, 1617, 1620, 1621, 1467, 1468, 1703, 1708, 1709, 1712 and 1713), causative agents of haricot bacterioses, isolated from different regions of Georgia were applied as matrices for amplification.

The following strains: 1466, 1475 1482, 1514, 1517, 1709 and 1703 gave fragments of 16S ribosomal DNA.

As is seen from the electrophoregram (Fig.1), the fragments of 16S ribosomal DNA obtained by PCR are of equal size on agarose gel. The tested restriction endonuclease Xba I was ineffective. Selected 16S rDNA fragments were sequenced. Successful

Table 1. Resemblance of analyzed 16S rDNA sequences with the analogues available in database of computer program “BLAST”

<table>
<thead>
<tr>
<th>Strain</th>
<th>Fragments size, bp</th>
<th>BLAST results</th>
<th>Identity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1466</td>
<td>16S rDNA</td>
<td>Xanthomonas axonopodis pv. phaseoli strain IAC13755 16S ribosomal RNA gene, partial sequence</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xanthomonas axonopodis pv. punicae strain AP-4 16S ribosomal RNA gene, partial sequence</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xanthomonas axonopodis pv. punicae strain AP-3 16S ribosomal RNA gene, partial sequence</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xanthomonas axonopodis strain KSI 1432 16S ribosomal RNA gene, partial sequence</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xanthomonas axonopodis strain KSI 1432 16S ribosomal RNA gene, partial sequence</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xanthomonas sp. EECC-449 16S ribosomal RNA gene, partial sequence</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xanthomonas sp. EECC-446 16S ribosomal RNA gene, partial sequence</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xanthomonas sp. EECC-365 16S ribosomal RNA gene, partial sequence</td>
<td>89</td>
</tr>
</tbody>
</table>

| 1475   | 16S rDNA           | Xanthomonas sp. EECC-263 16S ribosomal RNA gene, partial sequence | 90 |
|        |                    | Xanthomonas axonopodis pv. phaseoli strain IAC13755 16S ribosomal RNA gene, partial sequence | 89% |
|        |                    | Xanthomonas axonopodis pv. punicae strain 82 16S ribosomal RNA gene, partial sequence | 89% |
|        |                    | Xanthomonas axonopodis pv. punicae strain 81 16S ribosomal RNA gene, partial sequence | 89% |
|        |                    | Xanthomonas axonopodis pv. punicae strain 80 16S ribosomal RNA gene, partial sequence | 85% |
|        |                    | Xanthomonas sp. EECC-365 16S ribosomal RNA gene, partial sequence | 85% |
|        |                    | Xanthomonas sp. EECC-263 16S ribosomal RNA gene, partial sequence | 85% |
|        |                    | Xanthomonas sp. EECC-201 16S ribosomal RNA gene, partial sequence | 85% |
sequences results were obtained for fragments, belonging to strains – 1466 and 1475.

Resemblance of obtained 16S rDNA sequences with the analogues available in database of computer program “BLASTA” is presented in Table 1.

As seen from the Table 1, 16S rDNA fragments of the strain 1466 reveal 92%, and of the strain 1475 – 90% of identity with that of *Xanthomonas axonopodis pv. phaseoli*.

A global alignment of 16S rDNA fragments between the strains 1466 and 1475 by means of computer program *Lalign*, version 2.2u [11] has shown 95.8% identity of the fragments (Table 2).

Thus, two strains 1466 and 1475 isolated from brown leaf diseased haricot are identified as *Xanthomonas axonopodis pv. phaseoli* with 95.8% identity of 16S rDNA.

### Table 2. Sequence alignment of the strains 1466 and 1475 by computer program *Lalign*.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Identity %</th>
<th>Global Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1466</td>
<td>92%</td>
<td>1863</td>
</tr>
<tr>
<td>1475</td>
<td>90%</td>
<td>1863</td>
</tr>
</tbody>
</table>

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საჯაროსფერო ხელფრთული ხეობის მუშაობა

სასჯელი მხრივ გაბატონებული როლის მცენარეთა თავითმუშაობის საშუალებით პათოგენური მანძილგარების შეწვრთნის განვითარება 16S რიბოსომის ჰემ-ის დინამიკური შემკულობა

D. Gaganidze, T. Sadunisvili, N. Amashukeli

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