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Characterization of *Agrobacterium radiobacter,* a new pathogen of pistachio

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Abstract

Crown and stem gall caused by agrobacterial species is one of the most devastating diseases on different crops worldwide. In spring 2018, an alert concerning a disease forming galls on seedlings of pistachio in nurseries was received from Rafsanjan county, Kerman province, Iran. After isolation of bacteria from the galls by using conventional bacteriological methods, bacterial colonies were purified and selected for further analyses including biochemical, pathogenicity and molecular tests. Pathogenicity tests of all isolates on test plants and carrot discs were evaluated positive. Based on the results of biochemical and pathogenicity tests, supported by molecular phylogenies of housekeeping and pathogenicity genes, the isolates were identified as *Agrobacterium radiobacter*. Since agrobacteria could be easily spread through seedlings and agricultural materials between different areas, the identified *Agrobacterium* strains could be considered as a latent threat to the pistachio nurseries of Iran. The present study is the first report on the occurrence of crown and stem gall on pistachio caused by *A. radiobacter* worldwide.

Keywords: *Pistacia vera*, *Agrobacterium*, Crown gall, Molecular diagnostics

24 **Introduction**

25 Pistachio (*Pistacia vera*) is one of the most favorite nuts in the world, and it is widely cultivated in dry and hot areas
26 of the Middle East, the Mediterranean countries, and the United States. This tree is native to Iran and there are many
27 commercial pistachio cultivars like e.g. Akbari and Ahmadaghai, which are originated in Iran (Sedaghat 2006). The
28 World Food and Agriculture Organization (FAO) reported in 2016 that the largest pistachio plantation area in the
29 world was found in Iran, but was ranked as the world's second biggest pistachio producer country (315,151 tons
30 annually) (http://www.fao.org/faostat/en/#data/QC).

31 Also now, crown gall disease has affected a wide range of crops in nurseries, fields and orchards, resulting in serious
32 problems for plant production. Crown gall is an important disease for nursery production, since plants from infected
33 nurseries cannot be marketed (Ganjeh et al. 2020a; Puławska et al. 2010). The spread of the disease has increased due
34 to the extensive exchange of plants between different areas and the financial loss caused by the disease is estimated
35 to millions of dollars a year (Tzfira et al. 2006). Correct diagnosis of disease and identification of the causing agent
36 of a disease are crucial for taking the right decision on disease management.

37 Agrobacteria are ubiquitous soil microorganisms, some of which induce crown gall tumors mostly in roots and on the
38 crown of host plants (Kuzmanović et al. 2019). Tumorigenic agrobacteria of the family *Rhizobiaceae* are commonly
39 accommodated within the genera *Agrobacterium*, *Allorhizobium*, and *Rhizobium*. *Agrobacterium radiobacter* and the
40 former species *A. tumefaciens* are the most well-known agrobacteria, belonging to the family *Rhizobiaceae*
41 (Kuzmanović et al. 2019). The mechanism of inducing galls in wounded plant parts is controlled by a circular plasmid
42 that is called pTi (tumor-inducing plasmid). This plasmid is involved in transfer of oncogenic DNA (T-DNA) to the
43 nuclear DNA of host plant's cells. The genes required for T-DNA processing and transfer are located in virulence
44 (*vir*) regions (*virA*, *virG*, *virB*, *virD*, *virE*, *virF*, *virC*, and *virH*) on pTi (Nabi et al. 2019). The formation of tumor or
45 gall at the site of bacterial infiltration is induced by production of large amounts of plant growth hormones (auxin and
46 cytokinin) followed by overgrowth of plant cells (Mashiguchi et al. 2019). Based on Ti plasmid and the *virD* operon,
47 the primer pair virD2A/virD2C have been designed for the molecular detection and identification of tumorigenic
48 agrobacteria, and separation of pathogenic and non-pathogenic isolates in the populations of *Agrobacterium* biovars
49 I, II and III (Bini et al. 2008; Szegedi et al. 2002; Sawada et al. 1995).

Kerman province is the top producer of pistachio in Iran not only in terms of not only quantity and quality of pistachio, but also in the production of pistachio seedlings (Rostami et al. 2018). In 2018, we observed a disease of pistachio resembling crown gall caused by *Agrobacterium* spp. Due to transfer of pistachio seedlings along with substances such as soil and manure between neighboring regions and provinces, there was a high risk of this disease to spread throughout the whole country as *Agrobacterium* spp. are able to successfully invade cultivated soils and cause epidemics (Gelvin 2018; Bouzar et al. 1987), which would affect adversely the pistachio industry. Thus, we wanted diagnose the cause of this disease of pistachio seedlings using biochemical and pathogenicity tests and molecular techniques.

Materials and methods

Sampling and isolation

To identify bacteria that cause pistachio crown and stem gall, samples from fresh galls of pistachio seedlings were collected in Kerman province in spring 2018. Each sample was kept in a separate paper bag for delivery to the plant bacteriology laboratory. To isolate the putative causal agent from samples, the galls were washed thoroughly in running tap water to clear any adhering soil particles, then surface-sterilized for two minutes in 1% hypochlorite solution and rinsed in sterilized distilled water. Tissue pieces were crushed using a sterile scalpel in drops of sterilized distilled water (SDW) for 35 min. A loopful of the suspension was spread onto plates containing nutrient agar and potato dextrose agar plus $CaCO_3$ and incubated at 27°C for two to six days. To purify bacterial isolates, white single colonies were selected, re-streaked on nutrient agar (NA) plates and selected for further molecular, biochemical and pathogenicity tests. All bacterial isolates were kept at -80°C in 40% v/v glycerol solution (Basavand et al. 2021a; Borkar 2017). One of the isolates (P1) was deposited in the Agriculture Biotechnology Research Institute of Iran culture collections (Collection number, ABRIICC 20421).

Pathogenicity tests

Pathogenicity of 15 isolated strains were performed under greenhouse conditions by injection of bacterial suspension (c.10^7 CFU/ml) onto stem of indicator plants i.e., Jimsonweed (*Datura stramonium*) and tomato (*Solanum lycopersicum*), using a sterile syringe. The inoculated plants were kept at ambient temperature and the occurrence of symptoms was monitored daily until three weeks post inoculation. Four plants per isolate were used in a separate trial.

76 In addition, pathogenicity of isolated strains was done in Petri dishes on carrot discs (*Daucus carota*) by inoculation
77 of one-day-old bacterial suspensions, 1 ml per plate, c.10^7 CFU/ml. (Basavand et al. 2020b; Ganjeh et al. 2020b). The
78 reference strain *A. radiobacter* ICMP 5856 and SDW used as positive and negative controls, respectively, to mock-
79 inoculate plants and carrot discs (Kuzmanović et al. 2013; Puławska and Kałużna 2012). Pathogenicity tests were
80 repeated two times. Koch's postulates were confirmed by the re-isolation and identification of inoculated bacteria
81 from all plants tested and carrot discs, using phenotypic and biochemical tests and PCR amplification with virD2 and
82 virD2C primers.

83 **Physiological and biochemical tests**

84 Physiological and biochemical characterization of the isolates was performed according to conventional
85 bacteriological methods (Schaad et al. 2001). The utilization of carbon sources was checked using the basal medium
86 according to Ayers et al. (1919) supplemented with 0.1–0.4% of carbon compounds. In all tests, the reference strain
87 *A. radiobacter* ICMP 5856 was used as positive controls. The investigated isolates were deposited in the Culture
88 Collection of Microorganisms at Vali-e-Asr University of Rafsanjan, Iran (VRU 900-VRU 925).

89 **Molecular identification**

90 The Genomic DNA of three bacterial isolates was extracted using a loopful of one-day-old culture suspended in
91 sterilized distilled water (SDW), (A600nm = 0.1, c 10^7 CFU/ml). The next steps of extraction of genomic DNA were
92 performed with a modified CTAB (Cetyl atrimethylAmmonium bromide) method (Basavand et al. 2020b). The DNA
93 was kept at -20°C for further studies.

94 For the molecular identification of the pathogenicity markers, two specific primer pairs
95 (virD2A:ATGCCCGATCGAGCTCAAGT/virD2C: TCGTCTGGCTGACTTTCGTCATAA) and (PGF:
96 GGGGCAGGATGCGTTTTTGAG/PGR:GACGGCACTGGGGCTAAGGAT) were used to amplify *virD* operon
97 and polygalacturonase gene of the isolates (Bini et al. 2008; Sawada et al. 1995). To determine the phylogenetic
98 position of representative isolates, the housekeeping genes 16S rRNA, *atpD* and *gyrB* were amplified using the primers
99 FD1/RP2 (Weisburg et al. 1991), UP-1/UP-2r (Yamamoto and Harayama 1995), and 800F/1350R, respectively
100 (Aujoulat et al. 2011). Ampliqon *Taq* DNA Polymerase Master Mix Red (Ampliqon, Denmark) was used for PCR
101 amplification as recommended by the manufacturer. For each isolate, a 50 µL PCR reaction, including 50 ng total

102 DNA and 2 µL of each pair primer (10 pmoles). PCR was performed in the T-100 (Applied Biosystem) thermal cycler
103 using the following protocol: initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for
104 1 min, annealing at 50-58°C for selected primers (50°C for virD2A/virD2C; 54°C for PGF/PGR; 58°C for UP-1/UP-
105 2r, 58°C for 16S rRNA) and extension at 72°C for 1 min, with a final elongation step for 10 min at 72°C. PCR products
106 (4 µl) were separated by electrophoresed in 1.5% agarose gels (wt/vol) cast in TEB buffer at 80V for 60-90 minutes
107 and stained with Red Safe Nucleic Acid Staining Solution (iNtRON Biotechnology, South Korea). The gels were
108 documented under UV light. The 16S rRNA amplicon was partially sequenced at Macrogen Corporation, South Korea
109 via Sanger sequencing technology. The sequences were edited using BioEdit v.7.0.5.2 and compared with those
110 deposited in GenBank. The sequences of the test and reference agrobacterial strains were aligned by ClustalW
111 (Thompson et al. 1994). A maximum-likelihood phylogenetic tree of two combined protein- coding housekeeping
112 genes (*gyrB*-*atpD*) was constructed using MEGA7 (Kumar et al. 2016). Tamura-Nei 93 model with a gamma
113 distribution and invariant sites (T93+ G+ I) was selected the best-fit model for *gyrB* and *atpD* genes based on Akaike
114 information criterion (AIC).

115 **RESULTS**

116 In this study, 15 isolates resembling *Agrobacterium* on PDA containing $CaCO_3$ plates, with characteristic white,
117 slightly mucoid and convex colonies after 2 days at 27ºC were isolated from symptomatic crown tissue of pistachio
118 seedling. Fresh galls observed on naturally infected pistachio were distinct from other possible lumps at the crown
119 and were soft and dark and with a spongy surface, up to 8 cm in diameter (Fig. 1).

120 Among isolates that showed typical colonies on PDA containing $CaCO_3$, all test isolates along with reference strain
121 were positive in tumorigenicity test on jimsonweed (*Datura stramonium*) and on carrot discs after two weeks
122 incubation. Positive responses were indicated by formation of galls with different appearances and sizes on the stem
123 of test plants and the expansion of callus symptoms on carrot discs (Fig. 2). However, none of the agrobacterial test
124 isolates induced tumor symptoms on tomato. Isolates P1, P2 and P6 were able to induce more callus on carrot discs
125 than the other isolates. No symptoms were observed in the negative controls (SDW inoculation). To complete Koch's
126 postulates, the pathogen was routinely re-isolated from tumor tissue of test plants and carrot discs and re-identified by
127 biochemical tests and PCR amplification with virD2 and virD2C primers.

128 The biochemical and physiological tests showed that the isolates were Gram negative and displayed negative reactions
129 in oxidase, starch hydrolysis, production of fluorescent pigment on King's B medium and arginine dihydrolase
130 activity. Positive reactions were observed in catalase and urease tests and they could grow in 4% NaCl. They used D-
131 lactose, D-xylose, D-fructose, glycerol, sucrose, adonitol and D-ribose as sources of carbon for growth but none
132 utilized malonic acid, maltose, L-lysine, D-tartrate, and L-tartrate. The biochemical and nutritional characteristics of
133 the isolates are summarized in Table 1. The isolates were phenotypically similar to reference strain *A. radiobacter*
134 ICMP 5856 (Table 1).

135 The result of multigenic identification by specific primers showed that a 224 fragment was amplified by the primer
136 pair virD2A/virD2C in PCR. The successful amplification of this region of the *virD* gene indicated the presence of Ti
137 plasmid in 15 test isolates. These isolates were consequently identified as agrobacteria. In order to separate the two
138 species of *Allorhizobium vitis* and *A. radiobacter*, PGF/PGR primers were used in PCR. In the reference strain *Al.*
139 *vitis* ICMP 10752 the expected length (466bp) of polygalacturonase-encoding enzyme was amplified; however, this
140 specific region was amplified neither in our isolates nor in *A. radiobacter* strain ICMP 5856 (data not shown).

141 To determine the phylogenetic position of the test strains at genus level, the 16S rRNA gene of isolate P1 was
142 sequenced partially. Based on Blast results, the sequence of 16S rRNA (MK863506) of isolate P1 showed 99% identity
143 with *A. radiobacter* strain JZY4-60. Moreover, the genes *gyrB* (DNA gyrase subunit B) and *atpD* (ATP synthase beta
144 subunit) were sequenced for three representative isolates P1, P2, and P6. Blast analysis of the *gyrB* and *atpD* sequences
145 of isolates P1, P2, P6 showed 98-99% identity with *A. radiobacter* strains NCPPB1641, and 1D1609 for the *gyrB*, and
146 *atpD* sequences, respectively. Phylogenetic analysis based on the *gyrB* and *atpD* gene sequences showed that
147 representative isolates grouped with the reference/type strains of *A. radiobacter* (Fig. 3). The gene sequences were
148 deposited in the GenBank database and assigned the following accession numbers: MT648827, MT648828,
149 MT648829, and MT678488, MT678489, MT678490 for the sequences of the genes encoding 16S rRNA *gyrB*, and
150 *atpD*, respectively.

151 **Discussion**

152 In the present study, we isolated and studied *Agrobacterium* isolates from samples which showed crown gall disease
153 symptoms on pistachio seedlings (Badami cultivar) in Rafsanjan county, Kerman province in 2018. The isolates were
154 identified by carrying out pathogenicity, morphological, biochemical and molecular tests.

155 The morphological, biochemical and physiological properties showed that the test isolates resembled agrobacteria
156 based on most characteristics. In recent decades, methods based on PCR have been used for the detection of
157 *Agrobacterium* strains (Makarkina et al. 2019; Cubero et al. 1999). Based on Ti plasmid several primer pairs such as
158 virD2A/virD2C have been designed and reported for the molecular detection and identification of tumorigenic
159 members of *Rhizobiaceae*. These primers are recommended for the separation of pathogenic and non-pathogenic
160 isolates in the populations of *Agrobacterium* biovars I, II and III (Bini et al. 2008; Szegedi et al. 2002; Sawada et
161 al.1995). In our molecular assays, 224 bp fragments were amplified by virD2A/virD2C primers in PCR for 15 test
162 isolates and the reference strain.

163 Pathogenicity evaluation was performed in order to select the tumorigenic isolates. The test isolates harboring Ti
164 plasmid did not induce any tumor symptoms on tomato. Differences in the ability of inducing gall on plants by isolates
165 probably depend on several factors such as bacterial genetics, plant genetics and genetic compatibility between
166 agrobacteria and their hosts plants, mechanisms of plasmid linkage with plant genome, and expression of pathogenic
167 bacterial genes and Ti plasmid (Ganjeh et al. 2016). Therefore, using multiple host plants is recommended for
168 pathogenicity tests (Ganjeh et al. 2016; Knauf et al. 1982). According to previous studies (Ganjeh et al. 2016), a
169 pathogenic isolate was not necessarily able to induce gall on all tested plants. Accordingly, in a study reported by Ridé
170 et al. (2000), about 60% of *A. radiobacter* isolates were able to induce gall on tomato and jimsonweed plants, and
171 65% of *Al. vitis* isolates were able to induce gall on Kalanchoe (*kalanchoe blossfeldiana*).

172 Sequencing of the 16S rRNA gene is considered as a standard and simple method for the description of bacterial taxa
173 (Basavand et al. 2021b). The analysis of 16S rRNA sequence showed that strain P1 was 99% similar to *A. radiobacter*,
174 which is in agreement with the results of the biochemical and physiological tests. Two protein-coding housekeeping
175 genes, *gyrB* and *atpD,* were sequenced for strains P1, P2 and P6. Based on BLAST of the sequences of *gyrB* and *atpD*,
176 the three test strains were identified as *A. radiobacter* (with 98-99% similarity). The comparison of the *gyrB* and *atpD*
177 gene sequences could be considered as a straightforward and rapid approach for identification of bacterial species,
178 including members of the family *Rhizobiaceae*, , since these genes could allow separation of closely related species
179 (Basavand et al. 2021c; Pulawska and Kaluzna 2012). In the phylogenetic tree based on *gyrB* and *atpD* sequences,
180 isolates P1, P2, and P6 recovered from the crown and stem gall of pistachio grouped with *A. radiobacter* (Fig. 3). The
181 results obtained in this work in the identification of *A. radiobacter* isolates using classical and molecular methods

182 concur with those reported by other researchers (Basavand et al. 2020b; Ganjeh et al. 2020b). Based on the findings
183 pertaining to the morphological and biochemical characteristics, pathogenicity, and identification achieved using
184 molecular methods, it was concluded that the crown and stem gall symptoms causing pistachio in Iran were caused
185 by the *A. radiobacter*.

186 *A. radiobacter* is a Gram-negative soil bacterium that has worldwide distribution. It causes crown gall disease on a
187 wide range of plants including nut, stone and pome-fruit trees, and some ornamental plants in many places in the
188 world. Presently, crown gall is regularly observed in orchards on stone fruit trees and nuts in several countries such
189 as Iran, Greece, Tunisia, Spain and France. Crown gall is a problem in walnut production in the United States,
190 negatively influencing their productivity (Puławska 2010).

191 Due to an outbreak of crown gall disease in Iran, the ability of *Agrobacterium* species to infect a wide range of plant
192 species, and the high amount of damage to agricultural crops, the identified *Agrobacterium* in this study could be
193 considered as a latent threat to pistachio orchards and nurseries across the country. It is worth noting that the transfer
194 of young seedlings of pistachio from nurseries or greenhouses to the orchards is the main method for cultivation of
195 pistachio in Iran. Further studies are recommended to be conducted on sanitation principles that can prevent the
196 outbreak of the pathogen elsewhere. To the best of our knowledge, the present study is the first report of the occurrence
197 of crown and stem gall of pistachio and identification of the *A. radiobacter* species as pathogenic agent in the world.

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200 **Compliance with ethical standards** The result of this study has not been published previously and is not under
201 consideration elsewhere.

202 **Conflict of Interest** The authors declare that they have no conflict of interest.

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309 universal primers and their application to the detection and taxonomic analysis of *Pseudomonas*
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315 Fig 1. Naturally infected pistachio seedlings showing characteristic symptoms of crown and stem gall
316 caused by *Agrobacterium* isolates. **A** The internal tissue of the gall, **B-F** Infected crown and stem of
317 seedlings with formed galls at the different sizes
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321 Fig 2. **A** Symptomatic carrot disc showing callus symptoms, **B** Gall symptoms on the stem of Jimsonweed

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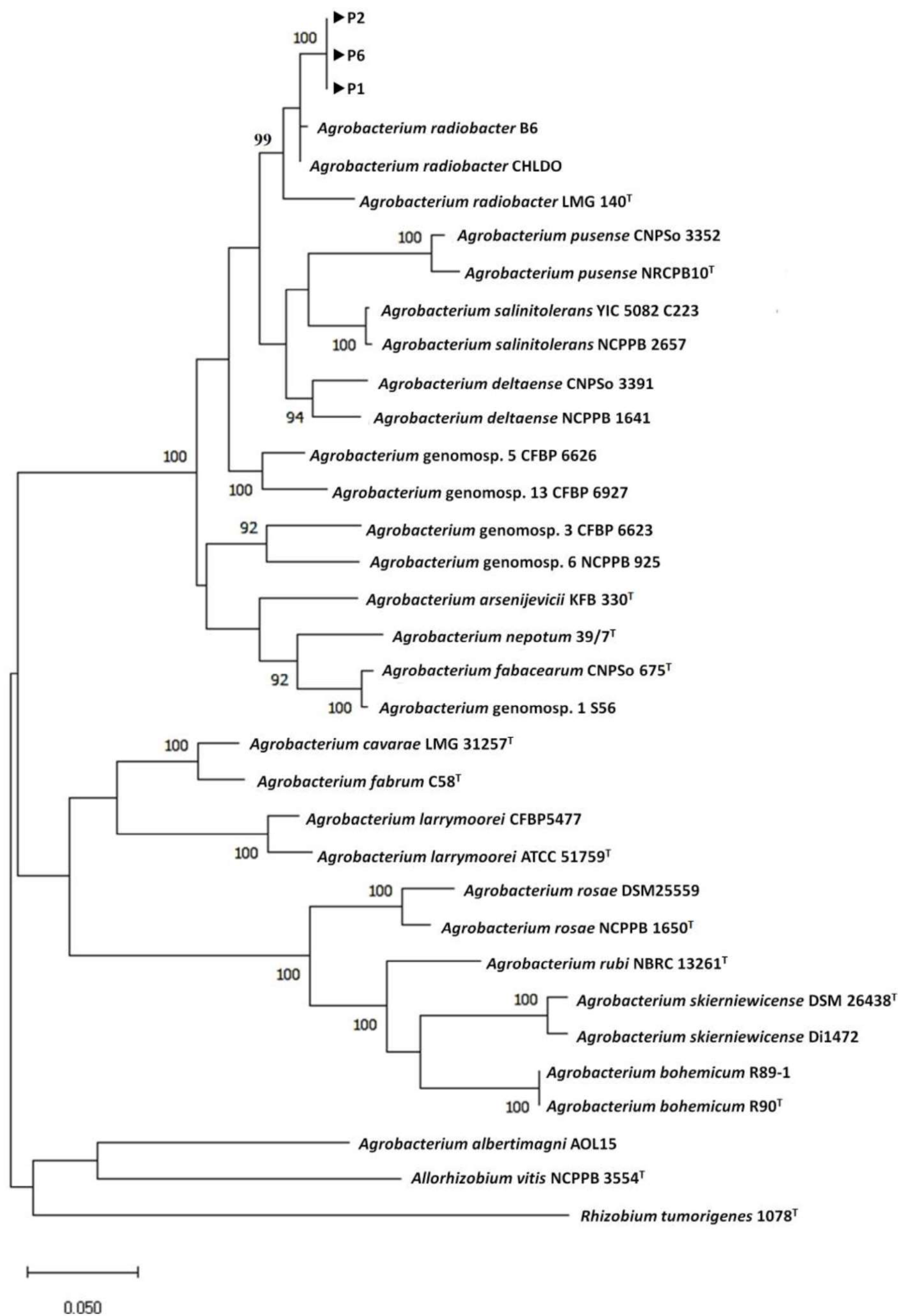

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325 Fig 3. Maximum likelihood phylogenetic tree based on the *gyrB* and *atpD* sequences showing the
326 phylogenetic position of gall inducing isolates of *Agrobacterium radiobacter* (P1, P2 and P6). The
327 tree was constructed using Tamura-Nei 93 model with a gamma distribution and invariant sites
328 (T93+ G+ I). Bootstrap values calculated for 1,000 replications are indicated. *Rhizobium*
329 *tumorigenes* 1078^{T} was used as the outgroup organism

330 Table 1. Biochemical and phenotypic characteristics of 15 *Agrobacterium* isolates from crown and stem
331 galls of pistachio in Iran

Characteristics	Reaction		Characteristics	Reaction	
	Pistachio isolates	ICMP 5856[a]		Pistachio isolates	ICMP 5856
Gram stain	–[b]	–	Production acid from:		
Oxidative/fermentative	+[c]	+	Erythritol	–	–
Oxidase	–	–	Cellobiose	+	+
Catalase	+	+	Galactose	+	+
Arginine dihydrolase	–	–	Glucose	+	+
Phenylalanine deaminase	–	–	Xylulose	+	+
Fluorescent pigment	–	–	Maltose	–	–
3'-Ketolactose	+	+	Mannitol	+	+
Levan formation	+	+	Sorbitol	–	–
Indole production	–	–	Lactose	+	+
Hydrolysis of:			Sucrose	+	+
Gelatin	–	–	Trehalose	–	–
Arbutin	+	+	Adonitol	+	+
Esculin	+	+	Arabitol	+	+
Starch	–	–	Dulcitol	+	+
Tween 80	–	–	Inositol	–	–
Urease	+	+	Rhamnose	+	+
Production of H_2S from:			Utilization of:		
thiosulfate	–	–	Malonic acid	–	–
peptone	–	–	Citrate	+	+
Methyl red	–	–	Ribose	+	+
Growth on 4% NaCl	–	–	Melezitose	+	+
Growth at 37°C	–	–	Lysine	–	–
			Glycerol	+	+
			D- Tartrate	–	–
			L- Tartrate	–	–
			Fructose	+	+
			L-Ornithine	–	–
			L-Arginine	–	–

332 Note: (a) *Agrobacterium radiobacter* ICMP 5856, (b) Negative reaction, (c) Positive
333 reaction

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