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Identification and Characterization of *Rothia amarae* sp. nov. in a Suspension Culture of *Arabidopsis thaliana* (Heynh.) Cells †

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† Presented at the 1st International Electronic Conference on Plant Science, 1–15 December 2020; Available online: https://iecps2020.sciforum.net/.

Published: 1 December 2020

Abstract: We report for the first time that a nonpathogenic bacterial microflora has been found in a suspension culture of *Arabidopsis thaliana* cells. The 16S rRNA gene sequencing showed that the isolate belonged to *Rothia amarae*. Identification was confirmed by microbiological, microscopic, and immunochemical methods. Growth of the isolate on blood agar preserves the morphological and immunochemical properties of the isolate from the plant cell suspension culture. Whether the isolated strain is a contaminant or a true symbiont remains an open question. It is known that *Rothia* bacteria live mostly in oceanic and waste water and in benthos. Members of *Rothia* are part of the normal microflora of the oral cavity, respiratory tract, and stomach of humans. Endophytic *Rothia* are inhibitory against several pathogenic fungi and bacteria. In addition, some actinobacteria, including members of *Rothia*, are nitrogen fixers. It cannot be ruled out that the *R. amarae* strain isolated in this work can be endosymbiotic with a suspension culture of *A. thaliana*. The bacterial "inclusions" found by us in a suspension culture of *A. thaliana* merit further investigation to identify them more deeply and clarify their symbiotic properties.

Keywords: suspension culture of *Arabidopsis thaliana* cells; bacteria; *Rothia amarae*

1. Introduction

Cell suspensions are a convenient model to investigate the interactions of plants and microbes, including those existing for extended periods. Cell suspensions are widely used model systems to examine secondary metabolic pathways, enzyme induction, gene expression, degradation of alien compounds, and problems in cytology. Across the globe, more than a hundred plant species transferred to suspension culture are used in the biosynthetic industry to produce economically important substances [1]. Special requirements are placed on culture conditions, culture purity during transfers, and culture protection against microbial contamination.

It is important to ensure that the growing suspension culture is sterile. However, in the isolation of an explant and in its introduction into culture, bacteria may remain, which initially coexist with the plant as endo- and exosymbionts. The bacteria may be transferred to the suspension culture and may latently coexist with it. The major sources of such contamination are incorrect preparation of material for culturing, errors during transfers, and disruption of culture conditions [2]. Some plant cell suspensions coexist with fungi and bacteria for a long time [3]. In this context, of major importance is the question whether the bacteria found in plant suspension cultures under specific conditions are endo- or exosymbionts.

The making of artificial associations, in which cell systems are prepared by introducing microorganisms into populations of plant cells being cultured, is a comparatively young direction in cell engineering [4]. It is presupposed that cells and their populations should acquire new, useful

properties owing to microbial presence. Such plant–microbe associations can be intercellular (exosymbiotic) as well as intracellular (endosymbiotic).

Arabidopsis thaliana is a model plant used commonly in genetic and molecular biological research. Many studies, especially those reporting the generation of transgenic plants, have used cultured isolated tissues of *A. thaliana* [5]. In the standard maintenance of an *A. thaliana* suspension culture, transfers are made every 10 days, with no visual signs of bacterial or other contamination being observed [6]. However, when growth is extended to 20 and more days or when the culture conditions (temperature, aeration, etc.) are disrupted, the medium thickens and gets turbid, and suspended matter appears in the supernatant liquid. This makes it possible to assume that the culture now contains a bacterial microflora.

Here we tested a suspension culture of *A. thaliana* cells for the presence of bacteria. The tests were followed by the identification of the bacteria.

2. Experiments

Research object. The major research object were microorganisms isolated from a suspension culture of *A. thaliana* (L.) Heynh., wild type (Columbia ecotype, Col-0). The culture was supplied by the All-Russia Collection of Cell Cultures of Higher Plants (Institute of Plant Physiology, Russian Academy of Sciences). The culture was grown in Shenk and Hildebrandt's (1972) liquid medium, with the addition of 1 mg l-1 of 2,4-D, 0.1 mg l-1 of kinetin, and 100 mg l-1 of meso-inositol [7]. Growth was conducted at 25 ± 1 °C in constantly stirred glass flasks in the dark. The passage duration was 10 days. All work with the suspension culture was carried out in a laminar box. Before culturing, the medium was autoclaved at 1.5 atm for 30 min. As the culture grew, growth was controlled visually and the presence of bacteria was checked by microscopy.

Confocal microscopy. Cell suspensions were observed with a TCS SP5 confocal laser scanning microscope (Leica, Germany) by using the fluorescent dye FM1-43 (Invitrogen, USA) [8]. Test tubes containing a 10-day-old culture of *A. thaliana* received a 0.1% FM1-43 solution in 10 mM phosphate-buffered saline (PBS; pH 7.2). After staining for 2 min, the culture was washed with PBS. DNA-containing structures were detected with the fluorescent dye SYTO 17 (Invitrogen, USA).

Transmission electron microscopy. Samples for transmission electron microscopy (TEM) were taken from a 10-day-old *Arabidopsis* culture. For negative staining, a 2% uranyl acetate solution was used. TEM was done with a Libra 120 transmission electron microscope (Carl Zeiss, Germany).

Isolation of bacteria. A 10-day-old plant cell culture was filtered, and bacteria were isolated by centrifugation. After sedimentation with a 5810 R centrifuge (Eppendorf, USA) at 3000× *g* for 30 min, the obtained supernatant was centrifuged again (15,000× *g*, 30 min). The bacteria pellet was resuspended in PBS and kept in 5% formaldehyde solution for further immunization.

Cell size determination. Cell sizes were determined by TEM and dynamic light scattering (Zetasizer Nano-ZS, Malvern, UK). Samples were prepared by stepwise centrifugation of a 10-day-old Arabidopsis culture. After the first centrifugation (3000× *g*, 30 min), the supernatant liquid was passed through a small-pore-size paper filter to clean it free of cellular debris. The culture was then centrifuged again (15,000× *g*, 30 min), and the sediment was resuspended in PBS.

Identification of bacteria. Bacteria were identified on the basis of their 16S rRNA sequence at the "Syntol science and production complex" (Russia) by polymerase chain reaction (PCR) by using a set of universal primers.

Culturing of bacteria. For culturing the isolated cells and examining their properties, we used three media: medium no. 523, Luria–Bertani medium, and blood agar. The cultured cells were stained with Gram and Ziehl–Neelsen stain. The presence of bacterial spores in the suspension culture was checked by the method of Peshkov. Bacteria were viewed with a DMI 3000B inverted microscope (Leica, Germany) fitted with an optical system for integrated modulation contrast.

Immunochemical identification of bacteria. For specific detection of bacteria, we used rabbit polyclonal antibodies raised against the strain under study after fixation with 3% formaldehyde. Rabbits were immunized intramuscularly three times at 2-week intervals with Freund's complete adjuvant (1:1). A week after the last immunization, the animals were bled. The immunoglobulin

fraction of the antiserum was obtained by ammonium sulfate precipitation (30–70% of final saturation); this was followed by ion-exchange chromatography on a Mono-Q anion-exchange column (Pharmacia, Sweden). The performance of the resultant antibodies was tested by dot assay. Two-µl portions of successive twofold dilutions of the bacterial sample were applied to a nitrocellulose membrane. The attachment of the bacteria to the membrane was tested by nonspecific staining with Ponso S. When the result was positive, the nitrocellulose was placed in a blocking buffer (2% skimmed-milk powder in PBS) for 15 min. After blocking, the nitrocellulose strips were washed three times with PBS. Next, the samples were placed in a antiserum (2 µg mL^{-1}) solution and were incubated at 37 ± 2 °C for 1 h. Antigen–antibody binding was detected with colloidal-gold-conjugated goat antirabbit antibodies [9].

3. Results and Discussion

To test the assumption that the suspension culture of *Arabidopsis* contained contaminant bacteria, we used confocal microscopy, because it permits the preparation of thin high-resolution optical sections and the 3D reconstruction of the resultant images. It was found that the plant cells had a prolate shape and a size of about 10–40 µm. In the field of view, there also were smaller objects (about 1 µm in size). In the aging culture at the stage of degeneration and decrease in cytoplasmic volume, these particles moved actively between cell wall and plasmalemma during plasmolysis. Such motility is untypical of plant cells or their organelles. Subsequent staining with SYTO 17 (staining of DNA/RNA) helped to detect, alongside *Arabidopsis* cell nuclei, structures that contained nucleic acids. The structures were untypical of plant cells—they were round and about 1 µm in diameter.

To test for the presence of bacteria, we used several microbiological staining procedures. Peshkov staining enabled the detection of bacterial spores in the culture liquid of *Arabidopsis* cells. Gram staining enabled the bacteria to be characterized as gram-positive. Ziehl–Neelsen staining showed that the bacteria were not acid tolerant.

We next chose nutrient media to culture the bacteria. Bacteria grew only when the *A. thaliana* cell suspension was cultured without stirring or transfers for 20 and more days (stress conditions) and was subsequently plated in liquid 523 medium. After 30 days of culturing, we observed visual signs of bacterial growth: the medium got turbid. Examination of the resultant material by TEM revealed 1-µm round bacteria with a narrow size distribution (Figure 1).

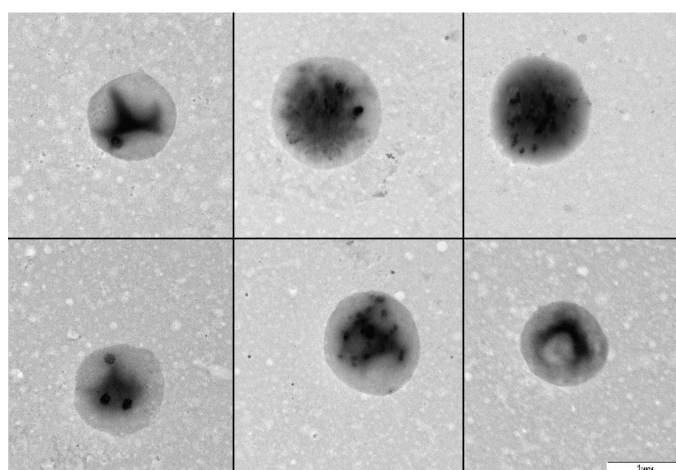

Figure 1. TEM of cells isolated from the *Arabidopsis* suspension culture. Contrasting with 2% uranyl acetate.

Using dynamic light scattering, we found that 80.7% of all particles in the cuvette with the material being examined were composed of 1130-nm particles, 11.4% were composed of 150-nm particles, and 7.9% were made up of 5560-nm particles (Figure 2).

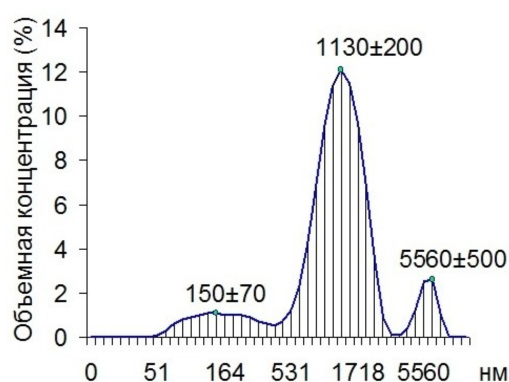

Figure 2. Size distribution of particles in the sample obtained from the *Arabidopsis* suspension culture.

The identification was conducted by PCR on the basis of 16S rRNA by using a set of universal primers. The resultant sequence was as follows:

TGGGTCTAATACCGGATATGACAAGGAACCGCATGGTTTTTGTGGAAAGGGTTTGTA
CTGGTTTTAGATGGGCTCACGGCCTATCAGCTTGTTGGTGGGGTAATGGCTCACCAAGGCGA
CGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCARAC
TCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACG
CCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAG
TGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTA
GGGCGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGGCGGTTTGTTGCGTCTG
CTGTGAAAGACCGGGGCTTAACCCCGGTATTGCAGTGGGTACGGGCAGACTAGAGTGCAG
TAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCG
ATGGCGAAGGCAGGTCTCTGGGCTGTAACTGACGCTGAGAAGCGAAAGCATGGGGAGCGA
ACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCACTAGGTGTgGGGGGACA
TTCCACGTTTTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCA
AGGCTWAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAA
TTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATATACTGGATCGCCTCAGAGATGG
GGTTTCCCTTCGGGGCTGGTATACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGAT
GTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTYTATGTTGCCAGCACGTTATGGTGGG
GACTCATAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCA
TGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAAAGGGTTGCGATACTG
TGAGGTTGAGCTAATCCC.

On the basis of its 16S rRNA sequence (identity, 99.73%), the isolate corresponded to the species *R. amarae*. The strain used for comparison was strain J18, which, according to GenBank, is a type strain of *R. amarae* [10].

After the isolate was identified, it was cultured on blood agar—the optimal medium for *R. amarae* growth [10]. The obtained isolate was loop seeded on blood agar and was placed in a thermostat set at 25 ± 1 °C (a temperature necessary for plant cell growth) and also at 30 ± 2 °C [10]. In either case, the result was positive—the bacteria grew.

TEM showed that the bacteria grown on blood agar were morphologically similar to those isolated directly from the *Arabidopsis* suspension culture (Figure 3). But the electron densities of the bacterial surfaces differed somewhat. The approximate size of the bacteria (about 1 μm) was preserved, as was their aggregation as dyads and tetrads. The obtained results check nicely with the literature data that describe *Rothia* cells as being gram-positive, round, and dyad- and tetrad-forming and their colonies as being milky white, slimy, and having a smooth concave surface [11]. Thus, we have shown that it is possible to grow the isolated bacteria outside a plant suspension culture, specifically on blood agar in Petri dishes.

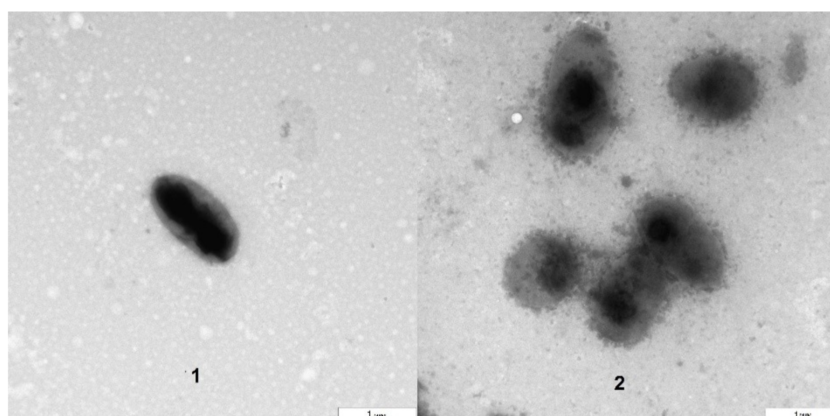

Figure 3. TEM of bacteria isolated from the *Arabidopsis* suspension culture (1) and obtained from colonies grown on blood agar (2).

The correspondence between the bacteria grown on blood agar and those isolated from the suspension culture was confirmed by raising polyclonal antibodies in rabbits. For immunization, we used a suspension of fixed cells that had been washed free of formaldehyde.

The bacteria were immunochemically identified by indirect dot assay with colloidal-gold-labeled antirabbit immunoglobulins as the secondary antibodies. Both fixed bacteria, used for immunization, and bacteria grown on blood agar were included in the assay. Figure 4 shows that the antibodies bound to the bacteria grown on blood agar, whereas the formaldehyde-fixed bacteria had a less pronounced coloration in the dot assay.

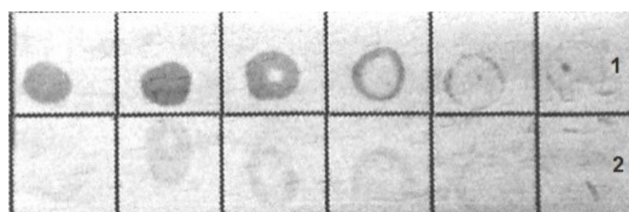

Figure 4. Dot assay of *R. amarae*. (1) Bacteria grown on blood agar. (2) Bacteria fixed with formalin.

Thus, the bacteria grown on blood agar and those obtained directly from a living suspension culture of *Arabidopsis* were found to be morphologically and immunochemically identical.

4. Conclusions

This is the first time that a nonpathogenic bacterial microflora has been found in a suspension culture of *A. thaliana* cells. The 16S rRNA gene sequencing showed that the isolate belonged to *R. amarae*. Growth of the isolate on blood agar preserves the morphological and immunochemical properties of the isolate from the plant cell suspension culture.

Whether the isolated strain is a contaminant or a true symbiont remains an open question. It is known that *Rothia* bacteria live mostly in oceanic and waste water and in benthos. Members of *Rothia* are part of the normal microflora of the oral cavity, respiratory tract, and stomach of humans [12]. *Rothia* bacteria have also been detected as endophytes in association with associated with Magellan sphagnum (*Sphagnum magellanicum* Brid.), stellate pohostemon (*Dysophylla stellata* (Lour.) Benth.), pointed banana (*Musa acuminate* Colla), alder (*Alnus*) and *Seidlitzia rosmarinus* Ehrenb. ex Boiss. [13–17]. Endophytic *Rothia* are inhibitory against several pathogenic fungi and bacteria. Notably, *Rothia* was detected in both roots and shoots, indicating possible migration of some species from roots to shoots. The root-associated bacteria showed higher levels of indole-3-acetic acid synthesis compared with those isolated from the shoots, as well as the higher production of 1-aminocyclopropane-1-carboxylate deaminase [17]. In addition, some actinobacteria, including members of *Rothia*, are nitrogen fixers [18]. It cannot be ruled out that the *R. amarae* strain isolated in this work can be endosymbiotic with a suspension culture of *A. thaliana*. The bacterial "inclusions" found by us in a

suspension culture of *A. thaliana* merit further investigation to identify them more deeply and clarify their symbiotic properties.

Author Contributions: A.S. and O.S. conceived and designed the experiments; A.S. and L.D. performed the experiments; A.G., L.D. and O.Y. analyzed the data and wrote the paper. All authors have read and agreed to the published version of the manuscript.

Acknowledgments: We are grateful to M.K. Sokolova and to A.M. Burov for help with microscopy. This work was supported in part by the Russian Foundation for Basic Research (grant no. 18-04-00469).

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

PBS phosphate-buffered saline
TEM transmission electron microscopy

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