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Research Article

Molecular diagnosis of bacteria isolated from *Trifolium repens* root nodules

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Abstract
The Fabaceae genus *Trifolium* comprises around 250 species widely distributed worldwide, with the temperate Northern Hemisphere exhibiting the highest variety. The plants in this genus are widely used as livestock fodder crops and are particularly significant economically.This study's objective included isolating bacteria from the root nodules of the *Trifolium repens* plant and diagnosing it at the molecular and microbiological levels. *T. repens* root nodules were used as the source of an endophytic bacteria isolated on Yeast Extract Mannitol (YEM) media that had solidified and diagnosed at the molecular level by DNA Sequencing technique for analysis of the sequence of the nitrogenous bases of 16S rRNA gene with the global database. The isolated bacteria were characteristic of greyish-white color after 48 hours of growth and appeared as a circular shape, slightly convex and gram-negative. The bacteria were resistant to the antibiotics 20µg/ml Aztreonam.The DNA sequencing technique for analysis of the sequence of the nitrogenous bases of 16S rRNA gene with the global database of the National Center for Biotechnology Information (NCBI) showed that the isolated bacteria was at least 96.22% similar to the species *Acinetobacter baumannii* As a result, it was recorded for the first time as *Acinetobacter* sp. AZS1 strain in NCBI.

Keywords: Antibiotic test, *Acinetobacter baumannii,* Sequencing, *Trifolium repens, 16s rRNA*

INTRODUCTION

Endophytic bacteria are extracted from inside plants and do not have any visible harmful effects on the plants (Papik *et al*., 2020) *Acinetobacter baumannii* species belong to the phylum Proteobacteria, the class Gammaproteobacteria, the order Pseudomonodales, family Moraxellaceae and genus Acinetobacter (Liao *et al*., 2020). It is coccobacilli shape, negative for the Gram stain, aerobic, non-fermentation to lactose, immobile, positive for catalase enzyme and negative for oxidase enzyme (Canciello *et al*., 2023). The guanine to cytosine content (G+C) ratio ranges from 39% to 47%, lacking flagella and non-spores formation (Castanheira *et al*., 2023). Its cells are sort (1-1.5) µm, and exist in pairs or chains; they can grow on simple media . (Zhang *et al*., 2022). *A. baumannii is* also isolated from many different sources, such as soil, water, human tissue, and animals. Also is an endophytic bacteria that is isolated from the root of *Oryza sativa* plants (Prajapati *et al*., 2022) and isolated from apoplastic sap of the medullary parenchym of the stem of healthy sugarcane plants (Silva *et al*., 2021), also it isolated from fruit and vegetables (Ababneh *et al*., 2022).

Previous study mentioned that *A. baumannii* LRFN53, isolated from the rhizosphere of wheat, can grow on nitrogen-free media and produce ethylene, which is considered a nitrogen fixer (Mujumdar *et al*., 2023). Another study could isolate bacteria from samples of the soil from Blind mountain's limestone mining region and identification of these isolates based on 16sr RNA showed that isolate (GPC3.7) was closely related to *A. buamannii* and these isolates can fix nitrogen through its growth on Nitrogen free Bromothymol blue medium (NfB) and change color medium from blue to green, In addition, the isolate GPC3.7 could not absorb Conge Red stain which means the isolate it symbiotically bac-

teria to root .

The White clover (*Trifolium repens* L.) belongs to *Leguminoceae* family. The plants of *Leguminoceae* have an important role in increasing soil fertility (Al Manar *et al*., 2023) and nitrogen fixation through their symbiosis relationship with nitrogen-fixing bacteria (Mathesius, 2022). This study aimed to isolate bacteria from the white clover root nodules and their diagnosis at the microbiology and molecular level, with the detection of its family specifically.

MATERIALS AND METHODS

Isolation and purification of the bacteria

Seeds of the White clover, *Trifolium repens* L. were procured from the Mosul City/Iraq's local markets and cultured in the house garden soil. After germination with 30 days, they were completely uprooted the roots of plant. The fresh nodules were removed with part of the root and washed several times with running water. Then 8-10 nodules were picked up and the surface outer sterilized by soaking in 70 % (v/v) ethanol alcohol for 3min. Subsequently, three rinses were done in sterile water in 1.5% (v/v) sodium hypochlorite solution (NaOCl) for 15 minutes. The nodules with surface sterility were transferred to the surface of solid Nutrient Agar medium and incubated at a degree 28±2°C (Tokgöz *et al*., 2020 ;(after confirming the efficiency of the surface sterilization taken 6-8 nodules and crushed with 3.0 ml of liquid YEM medium used sterile glass rod and then streaked onto sterile plates of solid YEM medium with one loop full with nodular suspension. Samples were incubated at 28±2°C. A single well-isolated colony was selected to solid YEM medium in a petri plate and incubated at 28C° after the purity and uniformity of colony type were thoroughly evaluated through several re-streaked experiments.

Preparation cell suspension of isolated bacteria

Cell suspension was prepared to transfer a colony of bacteria within 48 hours, put in a flask containing 20 ml from liquid YEM medium, and incubated at 28 °C for 48 hrs. in a shaker at1 20 rpm/min. rotational speed (Godschalx *et al*., 2017).

Characterization of isolated bacteria

Colony morphology was visually characterized for bacteria isolated from root nodules of white clover. They were observed for cell colony color, shape, and Gram stain.

Sensitive test for antibiotics

1.0 ml from 48 old of isolated bacteria suspension was cultured by streaking on all the 25 ml of solid YEM medium surface in the petri dish 9.0 cm containing antibiotics: Amoxicillin 15µg/ml, Azithromycin 20µg/ml, Aztreonam 20µg/ml, Cefixime 10µg/ml, Cefotaxime 20µg/ml, Ceftriaxone 5µg/ml, samples incubated for 48 hrs. at 28±2ºC.

Sequencing of 16S rRNA detection

Deoxyribonucleic acid (DNA) was isolated using a DNA purification kit (Geneaid, Korea). 16S rRNA gene was amplified using this primer For=5´-GTCATGAAGCATACCGTGGT-3´, Rev=5´- CATAA-GAGTTTGATCCTGGCT-3´ (Volokhov *et al*, 2023). Mixed the reaction components in pre-mix PCR tubes. Amplification of DNA was performed in a Thermocycler (T100 ^{TM} Thermal Cycler, BioRad, USA) using the following sequence**:** initial denaturation at 95 °C for 6 min**,** followed by 35 cycles**,** each cycle consisting of (denaturation at 95 °C for 45 sec**,** annealing at 56 °C for 1 min and 72 °C for 1 min) followed by a final extension at 72 °C for 5 min. Amplification products were visualized on a gel stained with 1.0 % (w/v) Agarose with 3.0 µl safe red stain. The purified PCR products were sequenced at 3130 genetic analyzer (Japan). The nucleotides sequence was compared with the nucleotide database available at the Gene Bank, using BLAST tool at the National Center for Biotechnology Information (NCBI).

RESULTS AND DISCUSSION

Cultural characterization

The colonies of a novel isolated strain of the endophytic bacteria isolated from *Trifolium repens* L. root nodules were white to grey color after 48 hours of incubation and took a circular shape, slightly convex and mucous (Fig. 1). The results showed a negative reaction to Gram's staining. The results agreed with Alshelawy (2014), whose results showed *Acinetobacter* is now defined as Gram-negative nonfermenting coccobacilli, strictly aerobic, no motile, catalase positive, and oxidase negative.

Fig. 1. *Endophytic bacteria isolated form the root nodules of white clover grown on the YEM medium after 48 hour of incubations*

Table 1. Susceptible antibiotic test

Antibiotics (µg/ml)	Results
Amoxicillin (15)	S
Azithromycin (20)	S
Aztreonam (20)	R
Cefixime (10)	S
Cefotaxime (20)	S
Ceftriaxone (5)	S

Susceptible test of bacteria

The results of the grown isolate on a solid YEM medium containing different types of antibiotics showed its ability to resist Aztreonam 20µg/ml and was sensitive to other antibiotics used in this study. The reason for bacterial resistance to antibiotics may be due to their ability to adapt to live in the presence of many antibiotics in the soil produced by fungi and actinomycetes, which has stimulated these bacteria to increase their ability to resist antibiotics by developing specialized self-mechanisms to maintain their growth and reproduction (Cochrane and Lohans, 2020), and the sensitivity of bacteria to other antibiotics may explain the process of stopping protein inhibition that these antibiotics stimulate inside the bacterial cell or affecting the construction of nucleic acids and the cell wall, or it may be due to an imbalance in the transfer of plasmid DNA during the process of cell division, which It leads to the formation of cells lacking the plasmid DNA containing the gene responsible for antibiotic resistance (Weldrick *et al*., 2021), (Table 1).

16s rRNA sequencing

The concentration of chromosomal DNA isolated from the endophytic bacteria reached to 234 µg/ml and its purity was 1.8. The results of the electrophoresis in 1.5% agarose gel of the DNA amplified product by PCR using the specific primer of 16s rRNA gene showed the single band separation with a molecular weight of 1355 bp (Fig. 2) matching with molecular weight of the specific primer used.

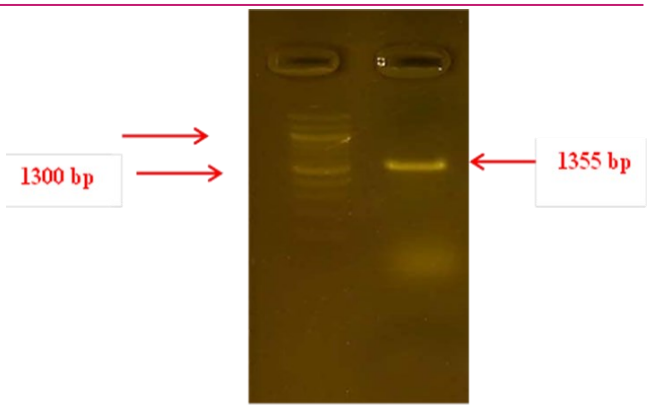

Fig. 2. *Electrophoresis of the DNA amplified to the 16s rRNA region by PCR and isolated from the studied bacteria in 1.0% agarose*

The analysis results of the 16S rRNA gene sequence of nucleotides in the DNA Blast program showed there is a 96.22% similarity between this sequence and the sequences of a number *Acinetobacter baumannii* registered with the Gene bank in NCBI (Table 2).

After sending the results of nitrogenous bases sequences analysis to the gene bank at the NCBI, the study was able to register it for the first time as a new strain and was given its code ON076417 and was known as *Acinetobacter* sp. AZS1 (Fig. 3).

The results correspond with many studies with the probability of finding the genetic variation between the studied isolated bacteria through the technique of stud-

Table 2. Convergence ratio of genetic diagnosis of isolate bacteria with some of reference isolates registered in National Center For Biotechnology Information (NCBI)

	Microbacterium genus registered in NCBI	Identities %	Number of nitrogenous bases
Isolate Bacteria	Acinetobacter baumannii strain MC.W.KOL.NR.P1.C1	96	744
	Acinetobacter baumannii strain SKJ12	96	745
	Bacterium strain BS1726	96	765
	Acinetobacter baumannii strain TWV102	96	768
	Acinetobacter baumannii OIFC137 clone 1061064327438	96	776
	Acinetobacter baumannii Naval-18 clone 1061064264480	96	777
	Acinetobacter baumannii Canada BC-5 clone 1124747678390	96	776
	Acinetobacter baumannii strain AB5116 chromosome, complete genome	96	Length: 3907300
	Acinetobacter baumannii strain DSM30011-VUB chromosome, complete genome	96	Length: 3996233
	Acinetobacter baumannii strain ATCC19606-VUB chromosome, complete genome	96	Length: 3980154
	Acinetobacter baumannii strain ATCC17978-VUB chromosome, complete genome	96	Length: 3901878

GenBank ▾ Sen

Acinetobacter sp. strain AZS1 16S ribosomal RNA gene, partial sequence

GenBank: ON076417.1
FASTA Graphics

Go to:

```
LOCUS       ON076417                687 bp    DNA     linear   BCT 31-MAR-
2022
DEFINITION  Acinetobacter sp. strain AZS1 16S ribosomal RNA gene, partial
            sequence.
ACCESSION   ON076417
VERSION     ON076417.1
KEYWORDS    .
SOURCE      Acinetobacter sp.
  ORGANISM  Acinetobacter sp.
            Bacteria; Proteobacteria; Gammaproteobacteria; Moraxellales;
            Moraxellaceae; Acinetobacter.
REFERENCE   1  (bases 1 to 687)
  AUTHORS   Qaddawi,Z.T., Mohammed,A.A. and Sultan,S.J.
  TITLE     Molecular and family specifically diagnosis of bacteria
isolated
            from Trifolium repens root nodules
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 687)
  AUTHORS   Qaddawi,Z.T., Mohammed,A.A. and Sultan,S.J.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-MAR-2022) Biology, University of Mosul, Al-
majmoaa
            street, Mosul 41002, Iraq
COMMENT     Sequences were screened for chimeras by the submitter using
            lasergene DNA star.

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       61 gtcctacggg agaaagcagg ggatcttcgg accttgcgct aatagatgag cctaagtcgg
      121 attacctact tggcggggta aaggcctacc aaggcgacga tctgtcccgg gtctgacagg
      181 atgatccgcc acactgggac tgagacacgg cccacactcc tacgggaggc accagtgggg
      241 aatattgcac aatgggggga accctgatcc agccatgccg cgtgtgtgaa gaaggcctta
      301 tggttgtaaa gcactttaag cgaggaggag gctactttag ttaataccta cagatagtgg
      361 acgttactcg cagaataagc accggctaac tctgtgccag cagccgcggt aatacagagg
      421 gtgcgagcgt taatcggatt tactgggcgt aaagcgtgcg tacgcggctt attaactcgg
      481 atgtgaaatc cccgagctta acttgggaat tgcattcgat actggtgagc tagagtatgg
      541 gagaggatgg tagaattcca cgtgtaccgg tgaaatgcgt agagatctgc aggaataccg
      601 atggcgaacg cacccatctg gcctactact gacgctgccc accaaagcat ggcgagcaaa
      661 caggattacc taccctggta gtccatg
//
```

Fig. 3. *Novel Acinetobacter sp. AZS1 with code ON076417 in NCBI*

ying nitrogenous bases sequences conducted by the researcher (Sun *et al*., 2023) to isolate 56 isolates of rhizobia bacteria from the leguminous host *Anthyllis vulneraria* in southern France, where the researchers used the technology DNA sequencing for 16S rRNA region. The results showed that the strain ChimEc512T belongs to the genus *Rhizobium*, and is closer to the standard strain *Rhizobium endophyticum* CCGE2052T (NR-116477.1) with a percentage of 98.4%. The results of another research project by (El-Zanaty *et al*., 2014) on the genetic diversity using the molecular diagnosis of the 16s rRNA region between eleven isolates of *R. leguminosarum symbiovar*. In the Arab Republic of Egypt, *Rhizobium* bacteria were isolated from the *Vicia faba L*. root nodes grown in eleven fields from various governorates. It was split into two groups of isolates, and the genetic distances between the isolated strains had various values, with the greatest genetic distance between isolate RL10 isolated from Sharkia and RL6 from North Sinai Governorate.

Conclusion

The present study succeeded in isolating bacteria from *T. repens* root nodules and its diagnosis at the microbiology and molecular level.The analysis results of the 16S rRNA gene sequence of nucleotides in the DNA Blast program showed a 96.22% similarity between this sequence and the sequences of a number *Acinetobacter baumannii* registered with the Gene bank in NCBI.

Conflict of interest

The authors declare that they have no conflict of interest.

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