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Presence of *Nifh* Gene in Fast Growing *Klebsiella* Like Bacteria Isolated From Groundnut Root Nodules in the Sudan

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***Abstract*: In this study three bacteria were isolated from groundnut root nodules grown in three regions in the Sudan, El-Obied, El-Gezira and El-Gadarif, DNA was extracted, *nifH* genes were amplified and sequenced and the sequences were compared with reference strain. The analysis of the sequences of *nifH* genes revealed that all the isolates and the reference strain are *Klebsiella* with identity of 99% and ensured the presence of *nifH* gene in all isolates and the reference strain and there is no difference in the sequences except the sequence of El-Gezira strain was found shorter than the others which led to difference of the *nifH* gene translated amino acids of this strain compared to the others.**

***Keywords*: endophytes, isolates, nitrogenase, nitrogen fixation, sequencing, strains.**

I. INTRODUCTION

For many years, a limited number of bacterial species were believed to be nitrogen fixers (Postgate, 1981)[1], but in the last 30 years nitrogen fixation has been shown to be a property with representatives in most of the phyla of bacteria and also in methanogenic *Archaea* (Young, 1992)[2].

Molecular nitrogen is converted to ammonia by the nitrogenase system in the biological process of nitrogen fixation. Synthesis of a functional nitrogenase requires the products of *nif* genes. The structural gene *nifH*, as an important *nif* gene, is involved in the formation of the Fe-protein complex (Cocking, 2003)[3]. Nitrogen-fixing bacteria can now be identified on the basis of the presence in their genome of *nif* DNA (Franche, 2009 and Reinhardt *et al*., 2008)[4-5]. The genetics of nitrogen fixation was initially elucidated in *Klebsiella oxytoca* strain M5a1 (first identified as *K. pneumoniae*). In that strain, *nif* genes necessary for synthesis of a functional nitrogenase are clustered in a 24 kb region (Arnold *et al*., 1988)[6]. Nitrogenase is the enzyme responsible for nitrogen fixation, and *nifH* is the gene that encodes for the iron protein subunit of nitrogenase, which is highly conserved among all nitrogen-fixing groups and serves as an ideal molecular marker for these microorganisms (Deslippe and Egger, 2006)[7]. The iron (Fe)-protein of the nitrogenase enzyme known as dinitrogenase reductase. It transfers electrons to the second part of the enzyme, the molybdenum (Mo)Fe-protein, which further reduces N_2 to NH^+_4(Howard and Rees, 1996)[8].

In recent years, many studies have addressed the importance and contribution of biological nitrogen fixation in ecologically unique terrestrial and aquatic habitats by focusing on the diversity of *nifH* sequences (Zehr *et al*., 2003)[9]. Such studies have provided a rapidly expanding database of *nifH* sequences and revealed a wide diversity of uncultured diazotrophs (Tan *et al*., 2003)[10]. The means of identifying nitrogen fixers became popular with the development of *nif*

gene cloning and sequencing and of DNA amplification by polymerase chain reaction (PCR). This led to the demonstration of the presence of *nif* DNA in putative nitrogen-fixing isolates by PCR amplification, followed by nucleotide sequencing of the amplicon (Franche *et al*., 2009)[4]. The cloning and sequencing of the *nifH* gene have provided a large and rapidly expanding database of *nifH* sequences from a number of diverse environments (Zehr *et al*., 2003)[9].

During the last decades the *nifH* gene has been widely used to study the presence and diversity of N_2-fixers. The *nifH* gene contains relatively conserved regions and can be amplified using degenerate primers (Zehr and McReynolds, 1989; Kirshtein *et al*., 1991)[11-12]. However, the diversity of nitrogen-fixing bacteria is still poorly described, and many of these microorganisms are yet to be discovered (Gaby and Buckley, 2011)[13]. Therefore this study was conducted to search and sequence the *nifH* genes in three locally Sudanese isolates of fast growing bacteria isolated from groundnut root nodules.

II. MATERIALS AND METHODS

Bacterial strains:

The strains used in this study were isolated from groundnut root nodules from different regions in the Sudan, one strain was isolated from El-Obied which located in the west part of Sudan and characterized by sandy soil (Arenosols). The other two strains were isolated from El-Gezira in the central Sudan and the third strain was isolated from nodules of groundnut grown in El-Gadarif which located in the eastern Sudan, the last two regions characterized by heavy clay cracking soils (Vertisols). The reference strain was obtained kindly from department of biofertilization - ministry of sciences and technology – Sudan. After surface sterilization, nodules were crushed and then the resulting suspension was streaked onto yeast extract mannitol agar (YEMA) at pH 6.8, the medium contains (g / l): mannitol, 10; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.2; NaCl, 0.4; yeast extract, 1; agar, 20 with Congo Red or Bromothymole Blue. Purity was assured by routine plating on YEMA supplemented with Congo red and the uniform colonies were selected. The basal growth medium is that described by Vincent (1970)[14].

DNA extraction:

Genomic DNA was isolated similarly as described by Dhaese *et al*. (1979)[15]. Strains were grown in AG broth medium in incubator shaker (150 rev/minutes) at 28°C for 2 days. About 20 ml of the bacterial culture were collected by centrifugation. After washing the bacterial biomass once with TE buffer (10 mM tris, 1 mM EDTA, pH 8), bacteria were resuspended in 300 µl TE buffer. 100 µl of 5% SDS (Sodium dodecyl sulfate**)** and 100 µl pronase E (2.5 mg/ml in TE buffer pre-incubated for 90 minutes at 37℃) were added. After mixing, the solution was incubated for overnight. Then the DNA was thoroughly sheared using a syringe. The DNA was purified by two extractions with 300 µl of Tris-buffered phenol and one extraction with methylene chloride. DNA was precipitated with 2.5 volumes of ethanol. The quality and quantity of the DNA were assessed using a NanoDrop ND-1000 device (Spectrophotometer, USA) and agarose gel electrophoresis stained with ethidium bromide, using a marker ladder as reference. All experiments were done in Melecular Genetics lab - Institute of Genetics – Technical University of Dresden – Germany.

Amplification and sequencing of nitrogen fixing (*nifH*) gene:

For amplification of *nifH* region for the different strains two primers, (nifHKleF50ATCCTGCACGCTAAAGCACA30 and nifHKleR: 50TGTAGATCTCCTGGGCTTTGT30), were used (Ibanez *et al*., 2009)[16]. A 50 µl reaction mixture was prepared as follows: 5 µl of 10x Dream Taq buffer, 1 µl dNTPs, 1 µl forward primer, 1 µl reverse primer, 1 µl template DNA, 1 µl Dream Taq DNA polymerase, 1.5 µl DMSO and 38.5 µl double distilled water. The thermal cycler condition for *nifH* amplification were: Initial denaturation for 3 minutes at 95℃ which was followed by 32 cycles at 94℃ for 1 minute, 56℃ for 1 minute and 72℃ for 45seconds with a final extension of 72℃ for 5 minutes and after that PCR product was visualized using 1% agarose gel and stained with ethidium bromide, using a marker ladder as reference. PCR products were purified by MEGAquick-spin Total fragment DNA purification kit according to manufacture instructions. Sequencing was done by GATC Biotech (Konstanz, Germany).

Data analysis:

The sequence analyses were performed by using the algorithm BLASTN (Altschul *et al*., 1997)[17] to identify similarities and the software programs APE (A Plasmid Editor) and EMBOSS (European Molecular Biology Open Software Siute) were used for sequences alignments, merging and translation.

Nucleotide sequences accession numbers:

The *nifH* gene sequences obtained in this study were deposited in the Gene Bank data base under the following numbers: KJ940122, KJ940123 and KJ940124 for El-Gadarif, El-Gezira and El-Obied strains, respectively.

III. RESULTS AND DISCUSSION

Amplification of about 300bp of *nifH* gene for the three isolates and the reference strain was achieved (Fig. 1). The concentrations of PCR product used for *nifH* gene sequencing were 68.5, 26.7, 84.2 and 22.4 ng / µl for El-Gadarif, El-Gezira, El-Obied and the reference strains, respectively. The sequencing resulted in (for forward and reverse primers) (266 and 262 bp), (263 and 251 bp), (262 and 211 bp) and (267 and 253 bp) for the reference, El-Gadarif, El-Gezira and El-Obied strains, respectively. The alignments of *nifH* gene sequences revealed that there were no differences in nucleotide sequences between the different isolates and the reference strain, this result was completely in agreement with Ruvkin *et al*. (1980)[18], they reported that the structural *nif* genes from taxonomically diverse microbes are nearly identical and function in a similar manner to encode nitrogenase. The number of the aligned nucleotides was 263bp except for El-Gezira strain the *nifH* gene sequence was found shorter than the other strains by 17 nucleotides it was 246bp. The reduction of the number of the nucleotides of El-Gezira *nifH* gene changed the translated amino acids number and order of this strain which was differed totally from the amino acids number and order of the other strains, the numbers of the amino acids were 85 and 87 for El-Gezira and the other strains, respectively. The identity of the *nifH* gene sequences of the different isolates and the reference strain may show that the different environment and soil types have no effect on this gene. The *nifH* genes analyses revealed that the three isolates and the reference strain are *Klebsiella spp*. like with identity of 99%. However Zehr *et al*. (2003)[9] reported that *nifH* gene may have different nucleotide sequences between species and even within the same species. The *nifH* gene product serves as a component of the nitrogenase system and has a role in the formation of the Fe-protein complex. It is therefore safe to assume that the presence of the *nifH* gene is indicative of the existence of the nitrogenase system and the ability to fix molecular nitrogen (Franche *et al*., 2009)[4]. The sequence analysis of *nifH* gene is a strategy that has been reported to be a useful tool for checking the presence of the *nifH* gene in microorganisms (Zakhia *et al*., 2006)[19]. The presence of *nifH* gene in *Klebsiella spp*. isolated from groundnut was confirmed before by Ibanez *et al*. (2009)[16] who reported that analysis of symbiotic genes showed that the *nifH* gene was only detected for the *Klebsiella*-like isolates. They also found that the phylogenetic analyses of these *nifH* sequences revealed that they were closely related to *Klebsiella spp.* Also Pandya *et al*. (2013)[20] reported that PCR amplification confirmed the presence of *nifH* genes in *E. adhaerens* and *Klebsiella pneumoniae subsp. ozaenae*. Homology was observed between cloned *nif* sequences of *K. pneumoniae*, *Anabaena* and *Rhizobium phaseoli* and *Rhizobium ORS571* total DNA (Norel *et al*., 1985)[21]. Seventeen genes involved in nitrogen fixation are clustered on the chromosome of *K. pneumoniae*. In addition to the structural genes for the nitrogenase *(nifHDK)*, seven genes *(nifQBMVSNE)* are thought to code for proteins that are necessary for a functional enzyme. The other genes are involved in regulation (*nifLA*) and electron transport *(nifFJ).* It was first shown by hybridization with *K. pneumoniae nif* probes (Ruvkun and Ausubel, 1980)[18] that *nifHDK* were highly conserved among different genera of diazotrophs. It can be assumed that genes coding for proteins with functions similar to those required for a functional nitrogenase in *K. pneumoniae*, could be present in other diazotrophs, and that consequently some homology of DNA sequences might exist between these genes (Norel *et al*., 1985)[21]. Ji Hong *et al*. (2008)[22] discovered that there is a similarity between *nifH* of endophytic bacteria other than *Klebsiella* and *Bradyrhizobium* and stated that the 99% similarities found in the *nifH* genes of *Bradyrhizobium japonicum* and of the endophytic *Bacillus* strains strongly indicated that horizontal transfer of symbiotic genes happened between the symbiotic bacteria and the endophytes. In addition, the horizontal transfer of symbiotic genes from *B. japonicum* to other symbiotic bacteria has been reported (Barcellos *et al*., 2007)[23], indicating that the symbiotic genes of *B. japonicum* could be transferred into companying bacteria in nodules. The most important result in this study is that the isolates and the reference strain share morphological and biochemical characteristics with fast growing rhizobia. This will lead them to be classified as Rhizobia. The inoculation with these strains may give results better than inoculation with rhizobia in the field as they were described as endophytic and opportunistic bacteria which means they use rhizobial

infection to enter in the plant tissue i.e they enter plant tissues in couple with any rhizobia which will resolve the competitiveness problems between the native and introduced rhizobia in the field. Fouts *et al*. (2008)[24] reported that the study of endophytes is increasing in intensity due to the roles they may play in multiple biotechnological applications,

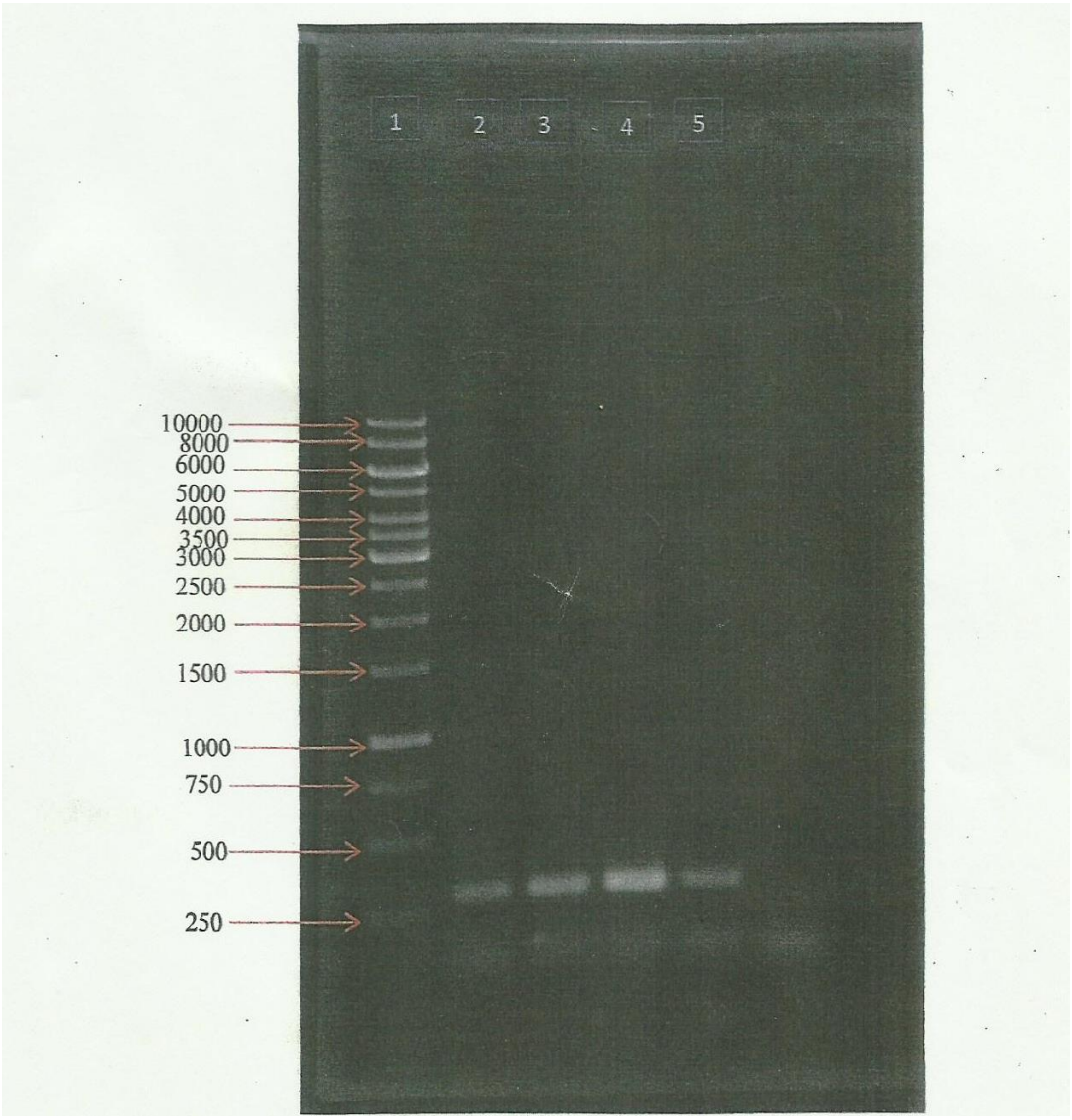

Fig. 1 Ampolification of nifH gene. Ladder (1), Reference (2), El-Obied (3), El-Gezira (4) and El-Gadarif (5)

including enhancing crop growth and nutrition, bioremediation, and development of plant-derived products and biofuel. In addition to that recently it was reported that associative bacteria as well as endophytic bacteria use the same mechanisms to influence plant growth (Jha *et al*., 2013)[25]. The same authors reported that, in terms of benefiting through nitrogen fixation, endophytic bacteria are considered to be better than that of rhizospheric one as they provide fixed nitrogen directly to their host plant and fix nitrogen more efficiently due to lower oxygen pressure in the interior of plants than that of soil. It was found that *Klebsiella pneumoniae* strain 342 colonized the interior of a wide variety of host plants with a very small inoculum dose (Dong *et al*., 2003)[26].

IV. CONCLUSIONS

The differences in the environment and soils have no effect on the *nifH* gene and these *Klebsiella* like isolates can be used as inoculates in different regions. The using of these isolates as inoculates may help to provide legumes and non legume plants by fixed nitrogen as they were described as endophytes. The presence of *nifH* gene in our isolates may resulted from horizontal transfer of this gene from other symbiotic bacteria to these isolates in the nodules. Finally the amplification and sequencing of *nifH* gene is the best tool for detection of nitrogen fixing bacteria (diazotrophs).

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