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Isolation of the *Azospirillum* Species from the Rhizosphere of the Leguminous *Bauhinia petersiana in* North Eastern Namibia

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Abstract

Bacteria associated with the plant roots of leguminous plants have the potential to alleviate the burden of using costly and harmful agrochemicals. Discovery of novel and suitable bacteria for enhancing plant growth is amongst the primary challenges involved in realizing the agronomic application of plant beneficial bacteria. A bacterium isolated from the rhizospheric soil of the legume *Bauhinia petersiana* was described based on several physiological and molecular analyses. The gram-negative bacterium designated as LMAB10 is motile, oxidase positive, catalase negative and displayed lipolytic activity. The bacterium's ability to utilize fourteen compounds as sole carbon sources was examined. A 340 kb fragment of the structural *nifH* gene that encodes for the Fe protein polypeptide of the nitrogenase was amplified from the genomic DNA of isolate LMAB10. Identification of the isolate via 16s rDNA via the BLASTn revealed that the isolate is an *Azospirillum* species with a 98 % similarity to *Azospirillum oryzae* JCM 21588^{T}. Phylogenetic analyses with MEGA5 software showed that the isolate shares an ancestor with *Azospirillum oryzae* JCM 21588^{T} and *Azospirillum zeae* N7^{T}, eventually branching off into a separate taxon. The partially-described isolate from the rhizosphere of *B. petersiana* shows promise for use as a crop inoculant.

Keywords: *Azospirillum* species , *Bauhinia petersiana,* Rhizosphere bacteria , Kavango-Namibia

1. Introduction

The association between plants and various microorganisms within the rhizosphere is a well-documented occurrence that is frequently reported as a beneficial and mutualistic relationship (Kiersi and Denison, 2008). Bacteria are able to make use of the abundant substrates and a low oxygen concentration that is available in the area surrounding the plant roots (rhizosphere), whilst the plant benefits from plant growth-promoting substances and fixed nitrogen provided by the bacteria (Madigan *et al.*, 2014). Associations between the plants and the rhizobacteria suggests that a long term and pronounced extent of adaptation has developed between the two types of organisms in evolutionary time (Nehl and Knox, 2006).

The genus *Azospirillum* is metabolically diverse, and belongs to the *α-Proteobacteria* class of bacteria. This group of bacteria are highly competitive and highly adaptable enabling their survival in various environments. *Azospirillum* spp. are free-living nitrogen fixing microaerophilic bacteria that are well-known to be in association with grasses and cereals (Boddey and Döbereiner, 1988; Baldani *et al.*, 1997; Peng *et al.*, 2006) These bacteria have also been isolated from the soil and from the roots of numerous plants (Ghai and Thomas, 1989; Bilal *et al.*, 1990; Eckert *et al.*, 2001 ; Reis *et al.*, 2001; Lin *et al.*, 2012) *Azospirillum* spp. are cited as biofertilizers (Steenhoudt and Vanderleyden, 2000 ;Gholami *et al.*, 2009) because of their ability to promote plant growth (Vessey, 2003), and are able to moderate osmotic stress in plants (Cassan *et al.*, 2009). To date, there are seventeen *Azospirillum* species reported, though only fifteen are presently valid after *A. irakense* and *A. amazonense* were recently reclassified (Young *et al.*, 2015): *A. lipoferum* (Tarrand *et al.*, 1978) , *A. amazonense* (Falk *et al.*, 1985) , *A. brasilense* (Tarrand *et al.*, 1978) , *A. canadense* (Mehnaz *et al.*, 2007a) , *A. doebereinerae* (Eckert *et al.*, 2001) , *A. fermentarium* (Lin *et al.*, 2013), *A. formosense* (Lin *et al.*, 2012), *A. halopraeferens* (Reinhold *et al.*, 1987), *A. humicireducens* (Zhou *et al.*, 2013), *A. irakense* (Khammas *et al.*, 1989) , *A. largimobile* (Ben Dekhil *et al.*, 1997) , *A. melinis* (Peng *et al.*, 2006), *A. oryzae* (Xie and Yokota, 2005) , *A. picis* (Lin *et al.*, 2009), *A. rugosum* (Young *et al.*, 2008) , *A. thiophilum* (Lavrinenko *et al.*, 2010) and *A. zeae* (Mehnaz *et al.*, 2007b).

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Kalahari bauhinia/ White bauhinia is a leguminous dicotyledonous plant that is able to grow in the dry agro-ecologies of Southern Africa including the Kalahari Desert. *B. petersiana* depends on soil nitrogen as it does not have any root nodules which are the biological apparatus used to fix atmospheric nitrogen by some legumes (Bosch, 2006). Plants are incapable of fixing nitrogen for their own use and therefore are dependent on nitrogen fixing microbes in the rhizosphere and soil to supply nitrogen for plant growth (Reddy, 2014). The plant's components have a variety of uses including medicinal (leaves and roots), animal feed (leaves and seeds), food source (pods, seeds, flowers, bark and roots) and as a dye (roots) (Curtis and Mannheimer, 2005; Bosch, 2006; Chelle, 2011). *B. petersiana* colonises disturbed areas, and has been considered as an arid plant with the potential of becoming a crop in semi-arid areas through domestication efforts and the possibility of reversing desertification effects (Curtis and Mannheimer, 2005; SASSCAL Integrated Science Plan, 2013).

In this study, a bacterium from the rhizosphere of *B. petersiana*, a leguminous shrub found in the wooded grasslands and in the Kalahari of Namibia, was isolated and characterized. The aim was to describe the bacterium associated with the rhizosphere of *B. petersiana*, by using physiological and molecular techniques in order to understand how the plant is able to survive in the soils that are low in nitrogen content.

2. Materials and Methods

2.1. Bacterial Isolation and Physiological Characterization

Rhizospheric soil was collected form *B. petersiana* plants in the East and West Kavango regions of North Eastern Namibia. Soils near the roots of the plants were dug with a trowel sterilized with 80 % ethanol and flaming. The samples were sealed in a sterile plastic container, and stored at ambient temperature until processing. Modified rhizobium LMG 201 medium (Atlas, 2005) was used as the enrichment medium, where the medium was supplemented with vitamin solution (Reinhold *et al.*, 1986), and ammonium chloride was used instead of glutamic acid. Rhizospheric soil (1gram) was aseptically transferred into test tubes containing 9ml enrichment broth medium. The test tubes were kept in a 30±2°C incubator for ten days, with occasional shaking. Spread plates were prepared from serial dilutions on yeast extract mannitol agar (YEMA) and incubated at 30±2°C for seven days. Bacterial isolates were subcultured onto fresh YEMA and Congo red agar (Caceres, 1982) media and incubated at 30±2°C for up to five days. Pure cultures were maintained on fresh agar plates for further observations by incubating at 33±2°C for three days. The gram staining procedure was performed. Bacteria were inoculated on S.I.M. medium (Oxoid Ltd; Hampshire, UK) according to the manufacturer's instructions to determine the motility. The oxidase test was performed by placing a loop full of bacterial colony onto Microbact Oxidase strips (Oxoid Ltd; Hampshire, UK). A colour change from white to blue-violet on the inoculated strip indicated a positive reaction, *Staphylococcus aureus* ATCC® 25923™ was used as a positive control. Three-day old isolates grown on agar plates were submerged in a solution of 3 % hydrogen peroxide (SMM Instruments (Pty) Ltd; Gauteng, SA) to test for catalase activity. The formation of air bubbles ascending from the bacterial colonies was indicative of a positive reaction, *Proteus vulgaris* ATCC® 6380™ was used as a positive control. Bacteria were streaked onto tributyrin agar supplemented with Difco lipase reagent (Difco; Becton, Dickinson and Company Sparks, MD), and incubated at 30±2°C for seven days to test for lipolytic activity. A transparent zone around the edges of the bacterial colony was indicative of lipase activity, *B. subtilis* ATCC® 11774™ and *Staphylococcus aureus* ATCC® 25923™ were used as the positive controls. Two-day old bacterial cultures were inoculated into phenol red carbohydrate broth (MacFaddin, 2000) to determine a carbohydrate utilization summary of the isolate. In this study utilization of the following carbohydrates were tested for: D-fructose (Merck KGaA; Darmstadt, Germany), D-glucose (Saarchem; Gauteng, SA), D-(+)-mannose (Sigma-Aldrich Co; St Louis, MO), D-mannitol (Sigma-Aldrich Co; St Louis, MO), D-(+)-melezitose monohydrate (Calbiochem corp; La Jolla, CA), glycerol (B&M Scientific cc; Capetown, SA), lactose monohydrate (Merck KGaA; Darmstadt, Germany), D+maltose monohydrate (Sigma-Aldrich Co; St Louis, MO), myo-inositol (Sigma-Aldrich Co; St Louis, MO), D-(+)-raffinose pentahydrate (Merck KGaA; Darmstadt, Germany), D-sorbitol (Sigma-Aldrich Co; St Louis, MO), sucrose (Sigma-Aldrich Co; St Louis, MO), D-(+)-xylose (Sigma-Aldrich Co; St Louis, MO), D-(+)-trehalose dehydrate (Sigma-Aldrich Co; St Louis, MO).

2.2. Amplification of nifH Gene Fragment

The genomic DNA of bacteria cultured in Luria-Bertani Broth (AMRESCO; Solon, OH) for three days was isolated using Zymo Research Fungal/Bacterial DNA MiniPrep™ according to the manufacturer's instructions. The nitrogenase *nifH* fragment was amplified by nested polymerase chain reaction (PCR) with primers FGPH19 (5′-TACGGCAARGGTGGNATHG-3′) and PolR (5′-ATSGCCATCATYNTCRCCGGA-3′) for the first step and PolF (5′-TGCGAYCCSAARGCBGACTC-3′) and AQER (5′-GACGATGTAGATYTCCTG-3′) for the second step (Sato *et al.*, *2009*). In the first step a 25µl PCR mixture (12.5µl DreamTaq Green PCR Master Mix 2× (Thermo Scientific; South Africa), 2µl Genomic DNA, 0.5µM of each primer and nuclease-free water) was used for the reaction in a PCR cycle with the following steps: initial denaturation at 94°C for four min, thirty cycles at 94°C for one minute, 55 °C for one minute and 72 °C for two minutes, and final extension at 72 °C for five minutes. For the second step a 25µL PCR mixture (12.5µL DreamTaq Green PCR Master Mix 2× (ThermoScientific), 2µl template from the first step, 0.5µM of each primer and nuclease-free water) was used for the reaction in a PCR cycle with the following steps: initial denaturation at 94°C for four minutes, thirty cycles at 94°C for one minute, 56 °C for one minute and 72 °C for two minutes, final extension at 72 °C for five minutes and hold at 4°C. All amplifications were done in a Bio-Rad MyCycler™ thermal cycler. The PCR products were viewed under UV light after being separated by electrophoresis in a 1.2 % agarose gel stained with ethidium bromide (Sigma-Aldrich Co.; St. Louis, MO).

2.3. 16S rRNA Gene Amplification and Phylogenetic Analysis

Amplification of the 16S rRNA gene fragment was carried out with universal primers 27F (5′-GAGTTTGATCM TGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) using bacterial genomic DNA as a template (Lane, 1991). The 25µL PCR mixture consisted of 12.5µl DreamTaq Green PCR Master Mix 2× (Thermo Scientific; South Africa), 2µL Genomic DNA, 1µM of each primer and 9.5µL Nuclease-free Water. The reaction was carried out according to (Grönemeyer *et al.*, 2012) in a Bio-Rad MyCycler™ thermal cycler. The PCR cycle steps were as follows: initial denaturation at 95°C for four minutes, thirty-five cycles of denaturation at 95°C for one minute, annealing at 50 °C for thirty seconds, extension at 72 °C for one minute, final extension at 72 °C for ten minutes and hold at 4°C. The products were separated by electrophoresis in a 1 % agarose gel and viewed under UV light. Purification and sequencing of the PCR fragments were carried out by Inqaba Biotec (Pretoria, South Africa). The sequence was analysed in the nucleotide databases using the NCBI's Basic Local Alignment Search Tool program to identify the bacteria and submitted to GenBank (KT944355). Related sequences of *Azospirillum* spp. and *Lacibacterium aquitile* strain LTC-2 were obtained from the NCBI's nucleotide database (www.ncbi.nlm.gov/nucleotide) and included in the multiple alignment using the MEGA5 program (Tamura *et al*., 2011). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) via MEGA5 with 1000 bootstrap replicates (Felsenstein, 1985). The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969).

3. Results

3.1. Cultural and Physiological Characteristics of Isolate

Fifteen distinctive colonies of bacteria were initially isolated from the rhizosphere soil of *B. petersiana.* However, only isolate LMAB10 showed the characteristics of a potential *Azospirillum* isolate when grown on Congo red agar (Caceres, 1982). Thus, isolate LMAB10 was selected for further study. The bacterium exhibited moderate growth on both YEMA and Congo red agar. The colonies of the isolate are convex, generally round with wavy margins, raised edges, and are light pink in colour on YEMA. The appearance on Congo red agar is similar with exception to the colour which is crimson/scarlet in this case (Figure 1). The surface of the colonies are smooth to begin with, but after approximately one week at room temperature, the surface of the colonies seemed dry and scorched while maintaining their colour. Results for the remaining tests and carbon source utilization tests are indicated in Table 1.

Table 1 Physiological and carbohydrate utilization characteristics of isolate LMAB10 and three of its closest relatives.

Test	LMAB10	*A. melinis*	*A. oryzae*	*A. zeae*
Gram	-	-	-	-
Motility	+	+	+	+
Oxidase	+	nd	+	+
Catalase	-	nd	+	+
Lipase	+	nd	nd	nd
Fructose	+	+	+	+
Glucose	+	+	+	variable
Glycerol	+	+	nd	+
Lactose	-	+	-	-
Maltose	+	+	-	-
Mannitol	+	+	-	+
Mannose	-	+	nd	-
Melezitose	+	nd	nd	nd
myo-Inositol	+	+	-	-
Raffinose	-	nd	nd	nd
Sorbitol	-	+	-	+
Sucrose	+	+	-	-
Trehalose	+	+	nd	-
Xylose	+	nd	nd	nd

A. oryzae, *A. melinis* and *A. zeae* data sourced from Xie and Yokota (2005), Peng *et al*. (2006), Mehnaz *et al.* (2007b) and Reis *et al.* (2015).

+ positive; − negative; nd = not determined

3.2. Presence of the nifH Gene and Phylogenetic Relationships with 18 Documented Azospirillum Species

The attempt to amplify the *nifH* gene fragment from the genomics DNA of isolate LMAB10 yielded a PCR product of approximately 340kb in size (image not shown). A BLASTn search of the 16S rRNA gene sequence (1 059 kb) revealed that isolate LMAB10 is most similar to *Azospirillum oryzae* JCM 21588^T (98%) (Figure 2). Based on the 16S rRNA gene sequences, the phylogenetic tree (Figure 3) shows that the isolated bacterium is closely related to the strains *A. oryzae* JCM 21588^T and *A. zeae* N7^T. Nevertheless, isolate LMAB10 is attached to a branch on a different taxon than that of its most similar strains.

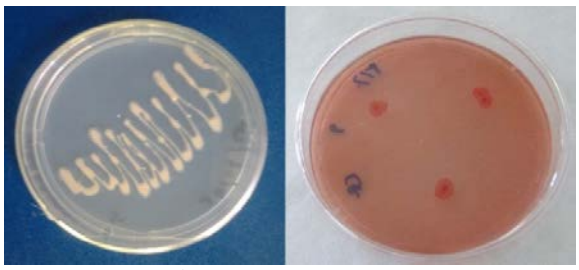

Figure 1. Growth of light pink coloured isolate (LMAB10) on YEMA (left) and crimson coloured spot inoculated colonies on Congo-red agar (right).

Description	Max score	Total score	Query cover	E value	Ident	Accession
Azospirillum oryzae strain JCM 21588 16S ribosomal RNA gene, partial sequence	1820	1820	98%	0.0	98%	NR_117482.1
Azospirillum oryzae strain NBRC 102291 16S ribosomal RNA gene, partial sequence	1816	1816	98%	0.0	98%	NR_114059.1
Azospirillum oryzae strain COC8 16S ribosomal RNA gene, partial sequence	1808	1808	98%	0.0	98%	NR_041233.1
Azospirillum picis strain IMMIB TAR-3 16S ribosomal RNA gene, partial sequence	1770	1770	98%	0.0	97%	NR_042682.1
Azospirillum zeae strain N7 16S ribosomal RNA, partial sequence	1766	1766	98%	0.0	97%	NR_043934.1
Azospirillum melinis strain TMCY 0552 16S ribosomal RNA gene, partial sequence	1759	1759	98%	0.0	97%	NR_043483.1

Figure 2. Highest six BLASTn search results based on the sequence of a 16s rRNA fragment amplified from genomic DNA of isolate LMAB10.

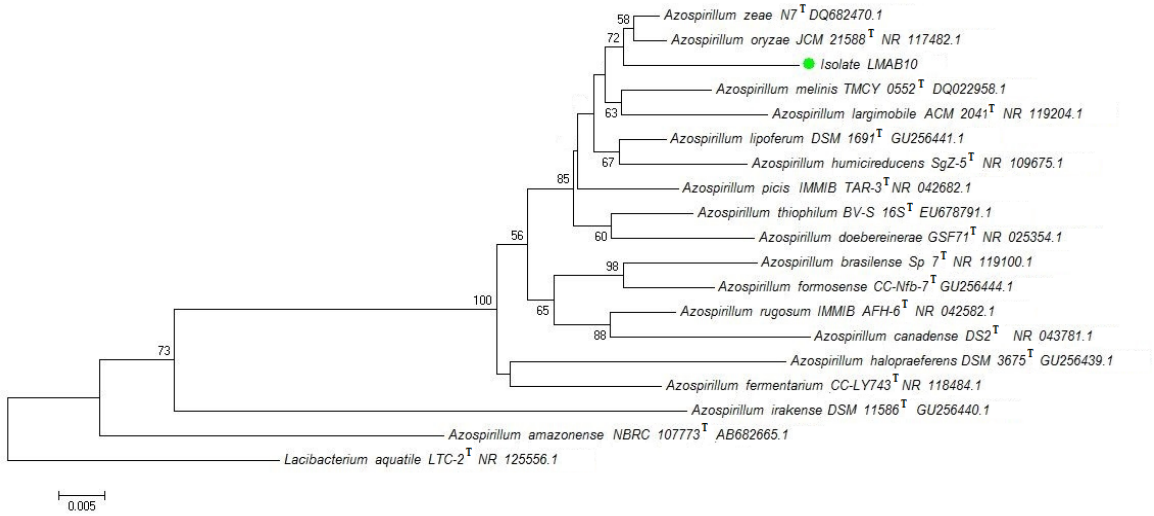

Figure 3. Evolutionary relationship of 17 selected *Azospirillum* strains, isolate LMAB10 and *L. aquatile* strain LTC-2 based on 16S rRNA sequences. *L. aquatile* strain LTC-2 was used as an out-group. The percentage (>50 %) of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Bar, 0.005 substitutions per nucleotide position were used. The evolutionary history was inferred via the Neighbor-Joining method using MEGA 5.

4. Discussion

Some cultural features belonging to the isolate named LMAB10 are similar to those described by (Young *et al.*, 2008; Lin *et al.*, 2012). A bacterium from the rhizosphere soil of *B. petersiana* was isolated, described and identified as belonging to the *Azospirillum* genus. Results show a 98 % percentage similarity of the 16S rRNA gene to its closest relative *Azospirillum oryzae* JCM 21588^{T}. Though a polyphasic taxonomic approach is necessary to properly distinguish genus members. Based on their polyphasic taxonomic characteristics, *A. irakense* and *A. amazonense* have been described as belonging to taxons outside the *Azospirillum* group and have appropriately been reclassified to *Niveispirillum irakense* and to *Nitrospirillum amazonense* (Prakash *et al.*, 2007; Lin *et al.*, 2014). Closely-related species to isolate LMAB10 are documented to associate with rice (*A. oryzae)*, maize (*A. zeae*) and a molasses grass (*A. melinis*) that grows well in poor nutrient soils of hot environments (Xie and Yokota, 2005; Peng *et al.*, 2006, Mehnaz *et al.*, 2007b). Benefits that the *Azosprillum* spp. confer to cereals include root growth, mitigated osmotic stress, salt stress and drought stress tolerance (Bacilio *et al.*, 2004; Cassan *et al.*, 2009, Bano *et al.*, 2013).

The utilization of numerous carbon sources is a good indicator of a high level of adaptability. The carbohydrate utilization profile also aids in the description of novel species and development of improved culturing and storage media (Reis *et al.*, 2015). As in this case, the differences in the physiological profile between isolate LMAB and its closest relatives support its claim as a novel isolate (Table 1). The isolate LMAB10 was designated as a potential diazotrophic bacterium based on the presence of the amplified *nifH* gene. The *nifHDK* nitrogenase structural genes are one of the most conserved genes within the prokaryotes (Masepohl and Forchhammer, 2007). Most of the *Azospirillum* spp. have demonstrated nitrogen fixation capacity when examined by means of the acetylene reduction assay (Mehnaz, 2015). Microorganism and plants in the nutrient poor arenosol soils of Northern-Eastern Namibia may have more developed relationships than organisms in other soils

(Sato *et al*., 2009, Jones *et al*., 2013). Much like *Tylosema esculentum* (a former member of the genus *Bauhinia*), a semi-arid adapted plant from the Ceasalpiniaceae family, it is highly likely that *B. petersiana* acquires most of its nitrogen from the association with diazotrophic rhizobacteria such as the isolate LMAB10.

Once again the non-specificity of *Azospirillum* spp. association to host plants comes to the fore. *Azospirillum* possess several chemotaxis signal transduction pathways and many receptors more than most soil bacteria, which allows the bacterium to detect an array of signals thus granting it an advantage by making the due adjustments for survival (Hauwaerts *et al*., 2002; Parales and Harwood, 2002; Bible *et al*., 2008; Kaneko *et al*., 2010; Sant'Anna *et al*., 2011; Wisniewski-Dye *et al*., 2012; Alexandre, 2015). The chemotaxis enables the bacterium to locate useful nutrients resources from the plant rhizosphere in order to establish a relationship with the plant in the root zone. The high adaptability, non-pathogenicity to both plants and humans and plant growth promoting properties validate the candidacy of *Azospirillum* spp. for use as biofertilizer constituents (Vacheron *et al*., 2013; Mehnaz, 2015).

5. Conclusion

With a total of 1 % arable land, the promotion of crop agricultural technologies which are sustainably effective and regionally native is vital for the largely semi-arid Namibia. Various plants take advantage of association with *Azospirillum* species in natural settings, therefore, advances can be made to convey and optimize their effectiveness in sustainable agricultural settings. The authors formally conclude that an *Azospirillium* species isolated from the rhizosphere of *B. petersiana* for the first time has been partially described. Consequently, the isolate shows good indications for development into a crop inoculant for an improved plant growth.

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