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Isolation and Molecular Characterization of Phosphate-Solubilizing Bacteria from Root Nodules of Cowpea (*Vigna unguiculata*) Seeds Planted at Ota, Ogun State, Nigeria

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ABSTRACT: In line with the race to increase worldwide food production to cater to the growing world population, there is a growing interest in exploring the plant growth-promoting attributes of rhizobia. This is in a bid to curb environmental and health damage arising from the use of chemical fertilizers. Hence, the objective of this work was to isolate and characterize phosphate-solubilizing bacteria from root nodules of cowpea (Vigna unguiculata) seeds planted at Ota, Ogun State, Nigeria using standard techniques. The 16S rRNA gene amplification and sequencing of the organisms identified the 2 isolates which were positive for phosphate solubilization as *Pseudomonas straminea* and *Ralstonia mannitolilytica.* Their sequences were deposited to GenBank with the assession numbers MK590690 and MK590695 respectively. Isolate *Pseudomonas straminea* was more efficient and produced a halo zone (16mm) with a phosphate solubility index (PSI) of 2.0 while isolate *Ralstonia mannitolilytica* produced a halo zone (14 mm) with PSI of 1.6 after 7 days of incubation. The results obtained from this study indicate that these organisms could be potential candidates to be used as biofertilizers to improve crop yield.

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The rise in both environmental damage and human population pressure worldwide indicates that global food production may soon become insufficient to feed the entire world population. It is therefore crucial that agricultural productivity be drastically increased in the next few decades (Glick, 2012). In order to meet this demand, farmers have resorted to the use of chemical fertilizers. The excessive use of chemical fertilizers on the long run however, has presented with adverse effects both to human health and the environment including soil salinity, radionuclide and heavy metal accumulation in soil and plant systems, water eutrophication and the accumulation of nitrates and phosphorus in drinking water and rivers (Savci, 2012). As a result, agricultural practice is inclining towards more sustainable and environmentally friendly techniques. One of the approaches to reduce the negative impacts arising from the use of chemical fertilizers is the use of plant growth-promoting bacteria (PGPR) (Pérez-Montaño *et al*., 2014; Glick, 2012; Naziya *et al.*, 2019; Malgioglio *et al.*, 2022). Rhizobia, which are bacteria that form root nodules on leguminous plants and convert atmospheric nitrogen (N_2) into usable nitrogen (ammonia), are considered a vital part of plant growth-promoting rhizobacteria (PGPR) due to their ability to solubilize insoluble phosphate, secrete siderophores and phytohormones as well as plant defense reactions (Lindström and Martinez-Romero, 2005; Denison and Kiers, 2011; Vargas *et al*., 2017). Phosphate solubilization which

makes the phosphorus in soil available for plant growth is considered as one of the important attributes of PGPR (Chen *et al*., 2006). Khan *et al.* (2007) reported that the fundamental work on phosphate solubilization by nodule bacteria has been significantly less even though it is known that phosphorus is the most limiting factor for nitrogen fixation by Rhizobium-legume symbiosis. Organisms with phosphate-solubilizing potential can enhance plant growth by increasing the availability of other micronutrients such as iron, zinc, *etc*., and by production of plant growth-promoting regulators (Ponmurugan and Gopi, 2006).

As a sustainable strategy, the application of phosphate-solubilizing bacteria (PSBs) holds great potential in aiding the achievement of the Sustainable Development Goals (SDGs) 1 and 2; No poverty and zero hunger respectively by improving agricultural produce of farmers leading to more income and increased availability of food; 6, 14 and 15: Clean water and sanitation, Life below water and Life on land respectively may benefit from the use of PSBs as they will lead to the use of less of chemical fertilizers which contaminate surface and underground water sources. Therefore, the objective of this work is to isolate and characterize phosphate-solubilizing bacteria from root nodules of cowpea (Vigna unguiculata) seeds planted at Ota, Ogun State, Nigeria.

MATERIALS AND METHODS

Isolation of Root Nodule Bacteria: Cowpea (*Vigna unguiculata*) seeds were bought from a market at Lagos and planted in pots at Bells University of Technology, Ota, Ogun State, Nigeria. Nodule samples were taken from the cowpea plants 35 days after planting and surface-sterilized according to the method described by Leite *et al.* (2017). Individual nodules were crushed with sterile glass rods in test tubes containing 1 ml distilled water to make a suspension. Aliquots of 0.1 ml taken from the suspension were spread on nitrogen-free yeast extract mannitol (YEM) agar medium (Küçük *et al*., 2006) containing 1.0 g L^{-1} yeast extract; 10 g L^{-1} mannitol; 0.5 g L^{-1} K_2HPO_4; 0.2 g L^{-1} $MgSO_4.7H_2O$; 0.1 g L^{-1} NaCl; 1 g L^{-1} $CaCO_3$ and 15 g L^{-1} agar and sterilized by autoclaving at 121 ^oC for 15 min. Following incubation at 32 ^oC for 24 hours, single morphologically different colonies were selected and sub-cultured on nutrient agar to obtain pure cultures for screening (Hamza and Alebejo, 2017).

Morphological and biochemical characterization of the Isolates: All 5 rhizobia isolated from legume root noodles were characterized by carrying out Gram reactions and other biochemical tests (Oxidase, catalase, citrate utilization and indole production, sulphide production and sugar fermentation) which were done using standard biochemical methods.

Screening for phosphate solubilizing bacteria: All isolates were tested for phosphate-solubization ability by an agar assay using Pikovskaya's agar medium containing 10 g L^{-1} glucose; 5 g L^{-1} tricalcium phosphate (TCP); 0.5 g L^{-1} yeast extract; 0.5 g L^{-1} $(NH_4)SO_4$; 0.2 g L^{-1} KCl; 0.1 g L^{-1} $MgSO_4.7H_2O$; trace quantities of $FeSO_4.7H_2O$ and $MnSO_4$ as well as 1.5 g L^{-1} agar agar. The isolates were pre-incubated in Nutrient Broth then 0.1 ml of the broth culture was on the Pikovskaya's agar medium in petri plates using a micropipette. The colony and halo diameters were measured after incubating for 7 days at 25 ^oC. The ability of the bacteria to solubilize insoluble phosphate was described by the phosphate solubility index (PSI) as stated below (Islam *et al.*, 2007).

$$SI = \frac{[diameter\ of\ halo\ zone\ (including\ colony\ diameter) in\ mm]}{colony\ diameter\ (in\ mm)}$$

Molecular Identification of phosphate-solubilizing rhizobia: Two phosphate-solubilizing bacteria isolated from the root nodules were identified by sequencing based their 16S rRNA genes sequencing. Total genomic DNA was extracted from isolates grown in Luria-Bertani (LB) broth for 24 hours using a commercial kit: illustra bacteria genomicPrep Mini Spin Kit (General Electric Company, UK) and quantified using a Nanodrop spectrophotometer (GenovaNano^{TM}, Jenway, UK). The 16S rRNA gene region of extracted DNA was amplified by Polymerase Chain Reaction (PCR) using an automated thermocycler and the universal primers: 27F and 1492R (Inqaba Biotech Ltd, Pretoria, South Africa). The PCR thermal cycling programme used was as follows: initial denaturation at 95 ^oC for 5min; 30 cycles of denaturation, annealing and extension at 94 ^oC, 52 ^oC and 72 ^oC for 30 s, 30 s and 1min 25s respectively, followed by a final extension at 72 ^oC for 10 min and kept at a hold temperature of 4 ^oC. The size of the amplified fragments was verified by electrophoresing the products on 2 % Agarose gel stained with ethidium bromide and viewed using a UV transilluminator. Sequences of the PCR products were determined by Sanger Sequencing technique (Inqaba Biotech Ltd, Pretoria, South Africa). The 16S rRNA sequences of the isolates were aligned with reference sequences from the GenBank database on NCBI

(National Centre for Biotechnology Information). Evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al.,* 2004). Phylogenetic trees were constructed using MEGA 6 software (Kumar *et al.,* 2018).

Phylogenetic Analysis: Reference sequences representing nearest neighbors were obtained from the National Centre for Biotechnology Information (NCBI) database and were included in phylogenetic analysis. Evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al.,* 2004). A phylogenetic tree was constructed using MEGA X software (Kumar *et al.,* 2018).

RESULTS AND DISCUSSION

Nowadays, it is urgent to maintain consistently high agricultural productivity by the use of chemical fertilizers, but it is equally becoming urgent to alter as little as possible our natural environment. Clearly we must then head for a more environmentally sustainable agriculture while maintaining ecosystems and biodiversity. One potential way to decrease negative environmental impact resulting from continued use of chemical fertilizers, herbicides and pesticides is the use of plant growth-promoting rhizobacteria (PGPR) (Pérez-Montaño *et al.*, 2014). Application of PGPR in legumes has been mainly restricted to rhizobia manipulation for studies on increase in legume growth and development, specifically by means of nodulation and nitrogen fixation. Obviously, the main reason for that is because a broad range of soil-borne rhizobia species can establish symbiosis with legumes (Cooper, 2008). Rhizobia therefore, can be considered the best known beneficial plant associated bacteria and the most important biofertilizers (Pérez-Montaño *et al.*, 2014).

Morphological and Biochemical Characteristics of the Isolates: Five distinct isolates were identified and code-named T001, T002, T003, T005 and T006. Observed morphological and biochemical properties of the isolates are summarized in Table 1. All the 5 isolates were gram-negative rods and catalase positive. Two of them (T002 and T003) fermented glucose but none fermented either lactose or sucrose. Isolates T005 and T006 produced hydrogen sulphide gas while only T004 produced indole. Only T002 and T006 were able to utilize citrate. In addition, T001, T002, T003 and T006 were all oxidase positive.

Table 1: Morphological and biochemical properties of isolates

Test	Isolates				
	T001	T002	T003	T004	T005
Gram reaction	-	-	-	-	-
Glucose fermentation	-	+	+	-	+
Lactose fermentation	-	-	-	-	-
Sucrose fermentation	-	-	-	-	-
Sulphide production	-	-	-	+	+
Indole	-	-	-	+	-
Citrate utilization	-	+	-	-	+
Oxidase	+	+	+	-	+
Colony morphology	Rods	Rods	Rods	Rods	Rods
Identity of isolate	*Pseudomonas straminea*	*Delftia tsuruhatensis*	*Variovorax guangxiensis*	*Burkholderia cepacia*	*Ralstonia mannitolilytica*

- = Negative; + = Positive

Screening for phosphate solubilisation: To test for their ability to solubilize phosphate, the isolates were incubated on Pikovskaya's agar medium. Only two of the isolates showed results in solubilizing phosphate in the agar assay. The isolate identified as *Pseudomonas straminea* strain ETEOC01 solubilized calcium phosphate in the medium with a halo zone diameter of 16 mm and PSI of 2.0 while *Ralstonia mannitolilytica* strain ETEOC06 had a halo zone diameter of 14 mm and PSI of 1.6 (Table 2). *P. straminea* showed better efficiency in phosphate solubilization with a solubilization index (SI) of 2.0 which was greater than that of the *Ralstonia mannitolilytica* (1.6). It is noteworthy that several species of plant-associated *Pseudomonas* promote plant growth by suppressing pathogenic micro-organisms (Naziya *et al.,* 2019; Attia *et al.,* 2022; Adedayo and Babalola, 2023), synthesizing growth-stimulating plant hormones (Naziya *et al.,* 2019) and promoting increased plant disease resistance (Preston, 2003). However, *Pseudomonas straminea* identified in this research showed the ability to solubilize phosphate which is one of the limiting nutrients in soil crucial for plant growth. Islam and co-worker in 2007 reported that *Pseudomonas* sp. BRS-2 isolated from the Rhizoplane of *Oryza sativa* L. cv. BR29 of Bangladesh had an SI values ranging from 1.2 to 6.7 which lies within the SI values obtained from this present study (Islam *et al.,* 2007). Vyas and Gulati (2009) reported the discovery of efficient phosphate-solubilizing strains of

Pseudomonas sp. which led to increase in plant height, shoot and root dry weight in maize as a result of organic acid production. Similarly, Midekssa *et al*. (2016) also documented plant growth promotion in chick pea by phosphate solubilizing *Pseudomonas* and *Ralstonia* strains. *Ralstonia mannitolilytica* was recently reported to possess wide range characteristics as plant growth-promoting bacteria due to its ability to solubilize phosphate and fix atmospheric nitrogen that support plant growth (Cortes *et al.*, 2020; Paul and Datta, 2016).

Table 2: Phosphate solubilization index for the isolates

Organism	Solubilization zone diameter (mm)	Phosphate solubilzation index (PSI)
Pseudomonas straminea	16±0.56	2.0
Ralstonia mannitolilytica	14±0.83	1.6

Cortes and co-workers reported that *Ralstonia mannitolytica* isolated from Cacao (Theobroma cacao L.) rhizosphere treated with bamboo biochar and arbuscular mycorrhizal fungi was able to solubilize phosphate with an SI value of 1.41. However, *R. mannitolilytica* isolated from this study showed a better phosphate solubilisation potential which could be attributed to variation in geographical location of the two *Ralstonia* species.

Molecular Identification of phosphate-solubilizing rhizobia: Out of the 5 bacterial strains isolated the only two that solubilized phosphate were identified based on their 16S rRNA gene sequences as *Pseudomonas straminea* strain ETEOC01, and *Ralstonia mannitolilytica* strain ETEOC06. The characterized sequences: ETEOC01 and ETEOC06 were deposited at the NCBI database under the accession numbers MK590690 and MK590695 respectively.

Table 3: Isolates with their GenBank Accession Numbers and suggested names

Isolate	Suggested Name	% Similarity to those deposited in Genbank	Accession Number
T001	*Pseudomonas straminea* strain ETEOC1	98.87	MK590690
T005	*Ralstonia mannitolilytica* strain ETEOC06	97.60	MK590695

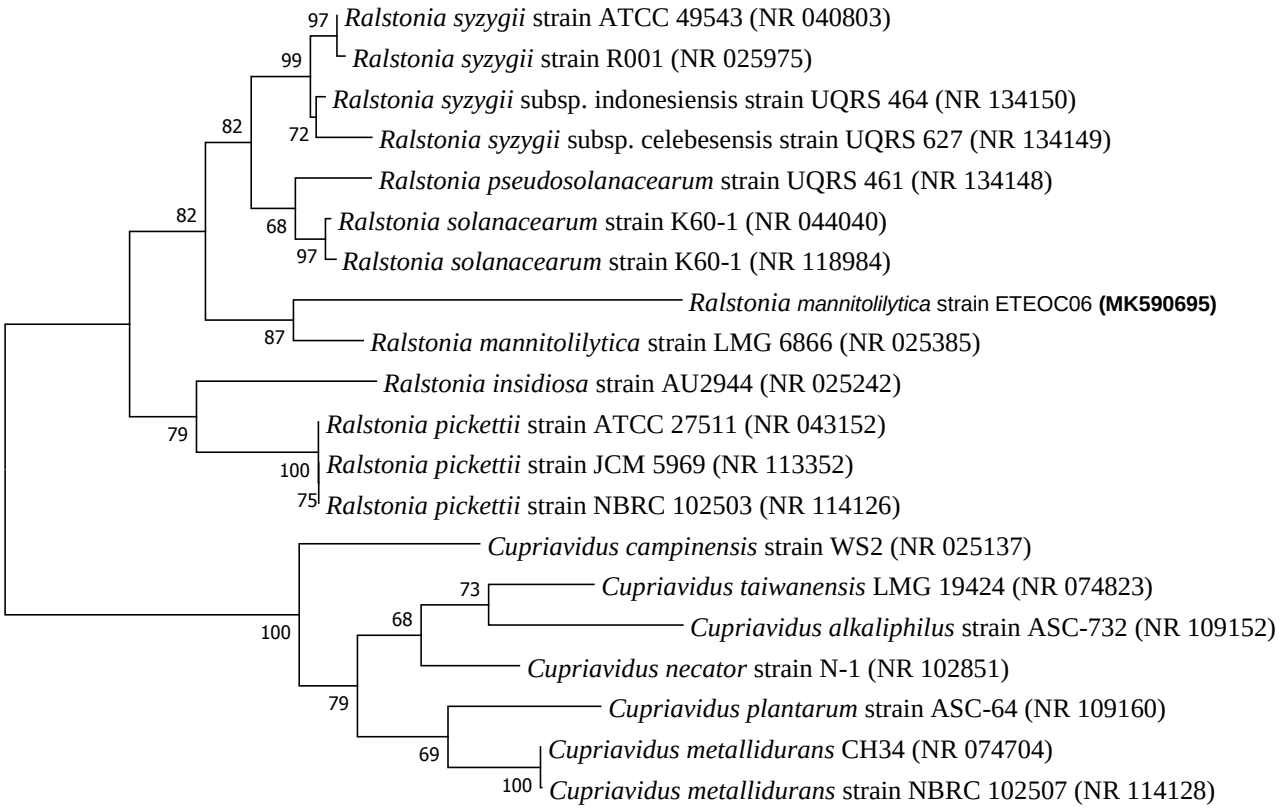

Fig 1: Phylogenetic tree indicating the phylogenetic relationship of the 16S rRNA gene sequences from *Ralstonia mannotolilytica strain* ETEOC06 isolated in this study to those in the GenBank database.

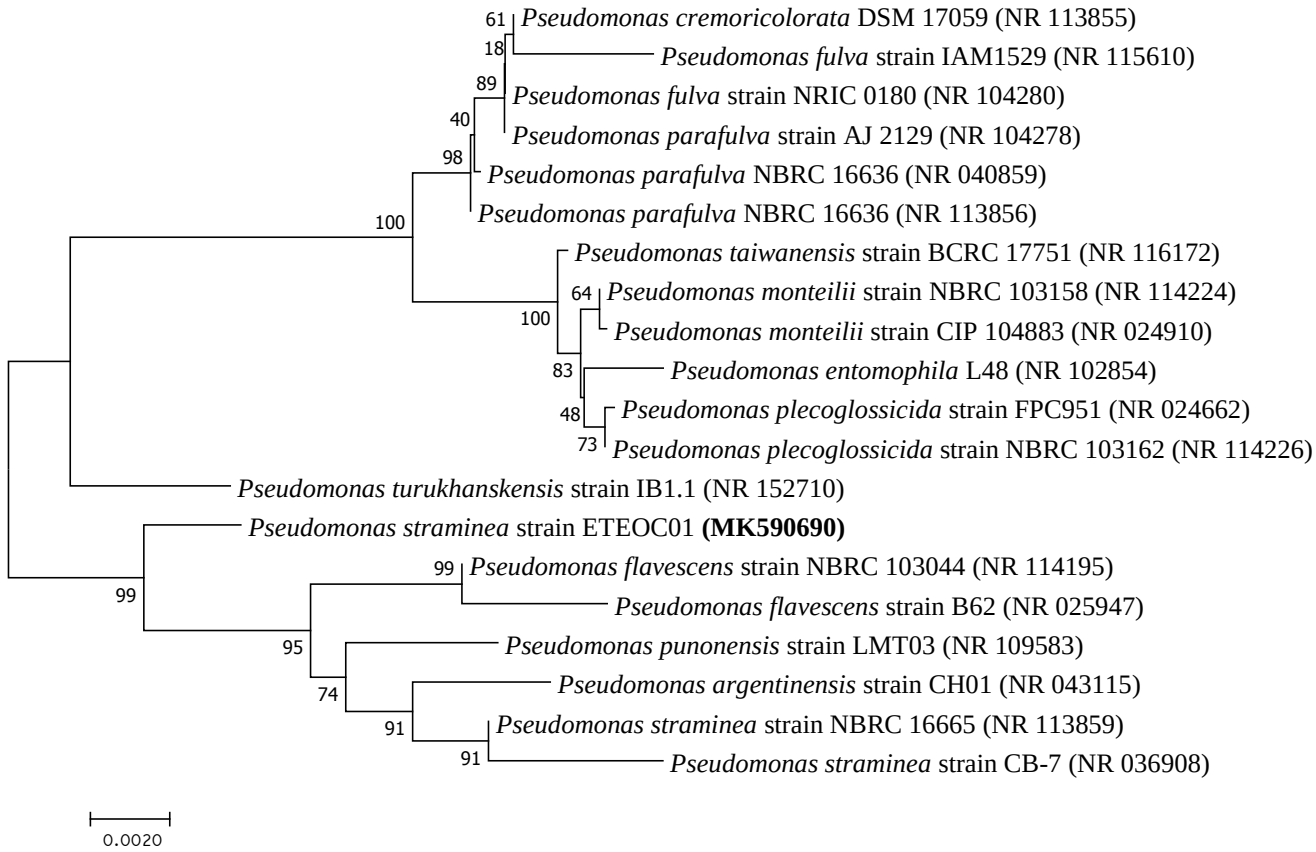

Fig 2: Phylogenetic tree indicating the phylogenetic relationship of the 16S rRNA gene sequences from *Pseudomonas straminea* strain ETE0C01 isolated in this study to those in the GenBank database.

Based on the nucleotide sequences obtained, the neighbor joining tree constructed showed that these organisms had strong relationships with other members of their genus as shown in figures 1 and 2. *P. stramenea* and R. *mannitolilytica* clustered very closely with other species that have previously been reported by other researchers.

Conclusion: The deployment of phosphate-solubilizing rhizobia holds promise as a viable alternative to chemical phosphate fertilizers. The identification of highly efficient phosphate-solubilizing rhizobia could lead to eventual replacement of the chemical fertilizers in agricultural practice by increasing the bioavailability of phosphorus already present in soils. This will lend support to food security and equally protect the environment as a natural practice and health of various life forms.

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