The Molecular Characterization of Rhizobacteria Isolates from Saki, Nigeria

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ABSTRACT

Plant growth promoting Rhizobacteria (PGPR) are important in the agricultural sector. Although different microorganisms live in the soil but thrive in PGPR rhizosphere zones, improve the production and protection to them from diseases by production of metabolites, volatile compounds and phytohormones and Induced Systemic Resistance (ISR). This study was aimed at isolating and characterizing molecularly; the bacteria from the rhizosphere of pepper (Capsicum annuum), vegetable (Spinacia oleracea), rice (Oryza sativa) grown in Saki. The rhizosphere sample of pepper, rice and vegetable were collected between the hours of 1:40-2:00pm. The colonial characteristics, Gram staining techniques, biochemical tests were carried out on the isolates which were also tested against eight antibiotics (Ceftriaxone, Gentamicin, Cefazidime, Ofloxacin, Augmentin, Cefuroxime, Erythromycin, and Cloxacillin) by using disc diffusion method. PCR techniques and subsequent use of sanger method was used for sequencing. DNA extraction was obtained using the lysozyme-SDS-phenol chloroform method in the prepared kits by Jena Bioscience. Amplification of the 16S rRNA gene was performed with the use of T1-Thermocycler PCR machine with 1492R primers pA (5’-TAC GGYBTAC CGT GTT ACG ACT T3′) and 27F primers pH (5’-AGAGTTTGATCMTGGCTCAG3’). This Primer was used for PCR amplification of 16S rRNA gene. All bacterial isolates were catalase (+) and indole (-). Antibiotics screening showed that all isolates resist most of the antibiotics except Ofloxacin and ciprofloxacin while sample 1 isolated plant can be activated simultaneously [6].

Keywords: PGPR; ISR; Rhizobacteria; Biopesticides; Oryza sativa; 16S rRNA.

I. INTRODUCTION

Interactions happens between microorganisms from beneficial symbiotic relationships to detrimental pathogenic relationship in the rhizosphere [1]. The rhizobacteria that enhance beneficial effects on plant growth and development are known as plant growth promoting rhizobacteria (PGPR) [2]. PGPR’s enhance plant growth via their ability to produce growth regulators or solubilize mineral phosphates and varieties of nutrients or fix atmospheric nitrogen or antagonistic action against phytopathogenic microbes by the secretion of antibiotics, endospore, siderophores, and cyanide [3]-[5]. The experimental evidences suggest that plant growth stimulation is the net result of multiple mechanisms that may be activated simultaneously [6]. Rice consumption has been increasing over the years due to the increasing population. Unfortunately, as a result of decrease in soil fertility, poor management of soil resources, build-up of pathogens and accumulation of phytotoxic substances rice productivity has decrease as expected and demanded [7]-[9]. Farmers relies a lot on agrochemicals to maintain the crop productivity [10], [11]. Nevertheless, extravagant use of agrochemicals in crop fields increase nitrate, nitrite, ammonium and phosphate and other reactive chemical species in groundwater and surface water bodies, which leads to serious environmental and health hazards [12]-[14]. Vegetable environment is strictly threatened by non-organic and organic factors. Among which crop loss due to organic factors especially due diseases is huge and estimated as 50-80 % from the heavily infected fields [15]-[17]. Farmers are greatly concerned for their crop, and for a quick remedy they use excessive amount of chemicals to control the diseases which in turn leads to detrimental effects on environment and microbiota organisms including animals and human beings [18]. The rhizosphere of vegetable crops has widely been studied which has been examine to a minor [19]-[22]. According to [23] the inoculation of B. cereus L90 interferes with the suppression of stress conditions to the biological characteristics of plant rhizosphere. Bacillus species are aerobic or facultative anaerobic bacteria closely related species B. cereus, B. thuringiensis are Gram-positive spore forming rod-shaped characterized by multiple morphological state, the vegetative cell (from 1.0 to 1.2 µm in width and from 3.0 to 5.0 µm in length), motile or non-motile, and the endospore (non-swelling the sporangium). The genus is characterized by the presence of endospores, which help in resistant to many stressful environment conditions (heat, cold, radiation, dehydration and disinfectants) and secreted either in the
presence or in the absence of air. According to [24], the presence of B. thuringiensis strain is capable to produce a capsule. The Bacillus species have unique functions and features in plant rhizospheres such as phytostimulation, biofertilization and bio-protection.

Bacilli rhizobacteria are phytostimulators by the production of phytohormones which are gibberellic acid (GA) and indole-3-acetic acid (IAA) the direct PGPR mechanisms characterized by Bacilli rhizobacteria [25]. Although Bacilli rhizobacteria produce IAA but few have been known on their ability to secrete abscisic acid (ABA). The Phytohormones biosynthesis by Bacilli rhizobacteria directly connects to nutrient availability and subsequent growth promotion in different plants [26].

B. cereus was stated to be having the potential of controlling many rice phyogenic fungi. Bacillus cereus has also been revealed to be efficient in bio-protection against several fungal pathogens in the rhizosphere [27]. Evidence revealed that Bacilli cereus and B. thuringiensis as rhizobacteria have the ability of stimulating plant growth either directly or indirectly via multifarious [28] including induced systematic resistance (ISR), antibiotics, hydrolytic enzymes, siderophores and nitrogen (N₂) fixation, nutrient solubilization and biosynthesis of phytohormones in plants to their pathogens [29].

An English biochemist Frederick Sanger and his colleagues in 1977 who developed a chain termination method for sequencing which is Sanger sequencing. This was developed by Sanger and his colleagues in 1977. The method for determining the sequence of nucleotide bases in a piece of DNA (commonly less than 1,000 bp in length). Sanger sequencing with 99.99% base accuracy is regarded as the "gold standard" for validating DNA sequences, including those already sequenced by next-generation sequencing (NGS). Sanger sequencing was used in the Human Genome Project to determine the sequences of relatively small fragments of human DNA (900 bp or less).

II. MATERIALS AND METHODS

Collection of soil samples from the rhizosphere of vegetable, rice, and pepper (10-15 cm depth) was done aseptically, each sample container was labeled accordingly. The rhizosphere soil samples were collected from the root depth, aseptically collected into a petri dish for the isolation of rhizosphere bacteria. For isolation of bacteria, 1 g of rhizospheric soil sample from each site was serially diluted in 9 ml of distilled water to 10⁻³. The diluted suspensions (1 ml) were spread on pre-poured nutrient agar medium and incubated at 28±2 °C for twenty four hours. The isolated colonies that grew on nutrient agar were subcultured into differential and selective media. The isolates were tested against eight antibiotics (Ceftriaxone, Gentamicin, Cefazidime, Ofloxacin, Augmentin, Cefuroxime, Erythromycin, and Cloxacillin) by using disc diffusion method.

A. Biochemical Characterization

The isolates were characterized by some specific morphological and biochemical features. The morphological characterization was carried out by viewing the isolated colonies under a compound microscope for colony color, forms, elevation, and margin. Also, cell shape, size, endospore presence and Gram's reaction was noted. The difference biochemical characterizations are indole production, citrate utilization test, oxidase test, catalase production and starch hydrolysis were carried out as per Bergey’s Manual of Determinative Bacteriology [31].

B. DNA Isolation

Bacteria DNA Preparation Kit designed by JENA bioscience for easy and fast isolation of genomic DNA from both Gram-positive and Gram-negative bacteria samples. The solution based system reduces DNA fragmentation that may be problematic in the spin-column or filtration based techniques, because phenol or chloroform cannot not be used. Solution based genomic DNA purification kits guarantee minimal DNA fragmentation and yield DNA sized up to 150 kb. Expected yield of genomic DNA varies from sample to sample dependent on the amount, quality and type of material involves. At least an amount of approximately 40 μg purified DNA per preparation can be expected.

C. Cell Lysis for Gram-Positive Bacteria

1 ml of cultured cells was transferred into a 1.5 ml micro tube, to get the cells it was centrifuge at 15,000 g for 1 min and the supernatant was discarded. The cell pellet was re-suspended in 300 μl of cell resuspension solution. Add 2 μl of Lysozyme solution then mixed well by inverting the tubes and was incubated at 37 °C for 60 minutes by occasionally inverting. It was centrifuged at 15,000 g for 1 min and the supernatant was discarded then the pellet was re-suspended in 300 μl of Cell Lysis Solution.

D. Cell Lysis for Gram-Negative Bacteria

For Gram negative bacteria 1 ml of cultured cells was transferred into a 1.5 ml microtubes in order to harvest the cells and was centrifuged at 15,000 g for 1 minutes before the supernatant was discarded. The pellet was re-suspended in 300 μl of cell lysis solution.

RNase Treatment was done by the addition of 1.5 μl of RNase A and mixed by inverting the tubes. It was incubated at 37 °C for 15-30 min and cool on ice for 1 min.

Protein Precipitation: 100 μl of Protein Precipitation Solution was added and vortex vigorously for 20-30 sec then centrifuge at 15,000 g for 5 min.

DNA Precipitation: The supernatant was transferred to a clean 1.5 ml micro tube containing 300 μl 100% Isopropanol >99% then mixed by inverting gently for 1 minute. It was centrifuged at 15,000 g for the same duration (DNA was visible as a small white pellet). The supernatant was discarded and the tube was drained briefly on clean absorbent paper. About 500 μl of washing buffer was added with the tube inverted for several times to wash the DNA pellet. Then this was centrifuged at 15,000 g for 1 minute and the ethanol was discarded carefully. It was air dried at room temperature for 10-15 minutes.

5 DNA Hydration: Addition of 50-100 μl of DNA hydration solution into the dried DNA pellet and hydrated by incubating at 65 °C for 60 minutes. Then the DNA Stored at 4 °C. For long time storage, it was stored at -20 °C or -80 °C. Stored at -20 °C and used as template DNA in PCR to amplify the 16S rRNA for phylogenetic analysis.

DOI: http://dx.doi.org/10.24018/ejbio.2021.2.2.159
E. PCR Amplification and Sequencing for Total Genomic DNA

Isolates was carried out on the basis of 16S rRNA sequencing. For this, the isolates were sent to Humanizing Genomics MACROGEN Laboratory, United States of America. As per the details shared the 16S rRNA sequence was obtained using Sanger sequencing method. Amplification of the 16S rRNA gene was performed using a T1-Thermocycler PCR machine with 1492R primer spA (5′-TAC GGYTAC CTT GTT ACG ACT T-3′) and 27F primers pH (5′-AGA GTT TGC TTA CCG CGG TCA G-3′).

According to Sanger sequencing method, a DNA primer complementary to the template DNA (the DNA to be sequenced) is used to be a starting point for complementary to the template DNA (the DNA to be sequenced). The primer is extended from the 5′ end until it comes in contact with a complementary nucleotide from the template DNA. The enzyme responsible for this synthesis reaction is DNA polymerase. Following the synthesis reaction, nucleotides labeled with a distinct fluorescent dye are used to terminate the reaction. Compared to dNTPs, ddNTPs have an oxygen atom to the chain of nucleotides, four ddNTPs: ddATP, ddGTP, ddCTP, and ddTTP labeled with a fluorescent dye are used to terminate the synthesis reaction. Compared to dNTPs, ddNTP has an oxygen atom dismissed from the ribonucleotide, hence cannot form a link with the next nucleotide. Following synthesis, the reaction products are loaded into four lanes of a single gel based on the diverse chain-terminating nucleotide and subjected to gel electrophoresis. According to their sizes, the sequences of the DNA is then determined [32]. The nucleotide sequences of 16S rRNA gene were analyzed using BLAST online at http://blast.ncbi.nlm.nih.gov/Blast.cgi. The phylogenetic tree was constructed on the aligned data set using the neighbor-joining method (Saitou and Nei 1987) implemented in the program using MEGA4.0 software [33].

III. RESULTS AND DISCUSSION

The molecular characterization of Rhizobacteria isolates was carried out on the basis of 16S rRNA sequencing, the results of BLAST showed 100% similarity between the nucleotide sequences of 16S rRNA gene of the obtained isolates which included: B. cereus, B. thuringensis and B. wiedmannii with the nucleotide sequences of the 16SrRNA gene in GenBank. Online alignment was done at http://expasy.org/tools/ on each isolate which had a kinship with several bacteria which had 99% similarity of 16S rRNA gene.

Isolate 1: Query ID MW362295.1
Description Bacillus thuringiensis strain CBC 123 16S ribosomal RNA gene, partial sequence...
Molecule type nucleic acid Query Length 1483
Bacillus thuringiensis strain BT11 16S ribosomal RNA gene, partial sequence
Subject Sequence ID: MT29210J1 Length: 1539 Number of Matches: 1

Alignment statistics for match #1
Query 1 GTAGAACCCTGCGGGCCGGTCTCAATTACATCGCAAGTCCAGCGAATGGATTGAGTCTTCG
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SBjct 33 GTGGAACCGTGTGCCGGCGGTCAATACATCGCAAGTCCAGCGAATGGATTGAGTCTTCG 92
Query 61 CTCAGAAGATGCTGGCCGACGGTGTAACAGGCTGGCTCAATAGGCTGG 120
SBjct 93 CTCAGAAGATGCTGGCCGACGGTGTAACAGGCTGGCTCAATAGGCTGG 152
Query 121 ATACATCGCAAGTCCAGCGAATGGATTGAGTCTTCG 180
SBjct 153 ATACATCGCAAGTCCAGCGAATGGATTGAGTCTTCG 212
Query 181 GAAAGGGCCGGTCTGGGCTCAATAGGCTGGCTCAATAGGCTGG 240
SBjct 213 GAAAGGGCCGGTCTGGGCTCAATAGGCTGGCTCAATAGGCTGG 272
Query 241 GTAACCGCTCACAAGCGAAGATGCTGGCCGACTGAGGCTGATCGGCAACTGG 300
SBjct 273 GTAACCGCTCACAAGCGAAGATGCTGGCCGACTGAGGCTGATCGGCAACTGG 332
Query 301 GACTGAGACGGCCCGAGTCCGAGGCTGGCCGACTGAGGCTGATCGGCAACTGG 360
SBjct 333 GACTGAGACGGCCCGAGTCCGAGGCTGGCCGACTGAGGCTGATCGGCAACTGG 392
Query 361 GAAAGGTCTCAGCGAAGTCCGAGGCTGGCTCAGGCGGACTGAGGCTGATCGGCAACTGG 420
SBjct 393 GAAAGGTCTCAGCGAAGTCCGAGGCTGGCTCAGGCGGACTGAGGCTGATCGGCAACTGG 452
Query 421 TGTTTGGAGAAGAATGGCTATGGTAAATAGGCTGGACCTTGGCAGGCTGACTTAAAGCAAGAGG 480
SBjct 453 TGTTTGGAGAAGAATGGCTATGGTAAATAGGCTGGACCTTGGCAGGCTGACTTAAAGCAAGAGG 512
Query 481 AGCCACAGCGTTAATCAGGTGACCGCCGACTGAGGCTGGCGAGGCTGG 540
SBjct 513 AGCCACAGCGTTAATCAGGTGACCGCCGACTGAGGCTGGCGAGGCTGG 572
Query 541 AATATTTGGCCTGAAGCCCGCCGAGGCTGGACCTTGGCAGGCTGACTTAAAGCAAGAGG 600
SBjct 573 AATATTTGGCCTGAAGCCCGCCGAGGCTGGACCTTGGCAGGCTGACTTAAAGCAAGAGG 632
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Description
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Query Length 1478

Subject Sequence ID: CP053954.1

Number of Matches: 14
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CACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCA
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**Isolate 4 Query ID MW362292.1**

**Description**  
Bacillus wiedmannii strain ABC 123 16S ribosomal RNA gene, partial sequence...

**Query Length**  
1493

Bacillus wiedmannii strain SX13.1LB 16S ribosomal RNA gene, partial sequence

**Sequence ID:** MT052668.1 Length: 1540 Number of Matches: 1

**Query**  
60

**Sbjct**  
1100

**DOI:** http://dx.doi.org/10.24018/efjbio.2021.2.2.159
Bacillus cereus strain HFBP18 16S ribosomal RNA gene, partial sequence

**Isolate 5**

**Query ID** MW362291.1

**Description** Bacillus cereus strain ABC 12 16S ribosomal RNA gene, partial sequence

**Query Length** 1489

**Bacillus cereus strain HFBP18 16S ribosomal RNA gene, partial sequence**

**Sequence ID:** MT538265.1

**Length:** 159 Number of Matches: 1

**Query**

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**Sbjct**

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Bacillus cereus strain ABC 12 16S ribosomal RNA gene, partial sequence

Length: 1535 Number of Matches: 1
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**Query**

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1080 ACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGGTTC
1140 CGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTC
1200 CAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACG
1260 AGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACT
1320 TACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTT
1380 GACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGA
1440 GGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGC
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**Sbjct**

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Bacillus cereus strain ABC 12 16S ribosomal RNA gene, partial sequence

Length: 1535 Number of Matches: 1
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**Query ID** www.ejbio.org European Journal of Biology and Biotechnology

**DOI:** http://dx.doi.org/10.24018/ejbio.2021.2.2.159

**Vol 2 | Issue 2 | March 2021**
ORIGINAL ARTICLE

European Journal of Biology and Biotechnology
www.ejbio.org

DOI: http://dx.doi.org/10.24018/ejbio.2021.2.2.159

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GGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGC

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1261
**Isolate 6 Query ID MW362901**

**Description**

*Bacillus cereus* strain ABC 11 16S ribosomal RNA gene, partial sequence ...

**Query Length**

1493

*Bacillus cereus* strain HFBP18 16S ribosomal RNA gene, partial sequence

**Sequence ID:** MT538265.1:Length: 1555

Number of Matches: 1

**Query**

Length: 1535

Number of Matches: 1

**Subject**

Length: 1535

Number of Matches: 1

**Query ID**

1493

**Sbjct ID**

1493

**Description**

Isolate 6

GCTCAGGATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAG

CAGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGT

AAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAA

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DOI: http://dx.doi.org/10.24018/ejbio.2021.2.2.159

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The Query Cover (QC) for six isolates of bacteria had a value in the range of 100% (Table I). The E-value of 0.0 indicated perfect number of alignments with a score equal to or higher than expected to occur in the database by chance. Therefore, the lower the E-value was, the more significant the score and the better quality of BLAST alignment search were. In this study, the 16S rRNA nuclear gene had a length of about less than 1535 base pairs (bp). Then the search for similarities with the limited query sequence was performed. According to Claverie and Notredame (2003), DNA sequences have a high similarity if the Query Cover value approaches 100% and the E-value approaches 0.0. Based on the Query Cover (QC), the E-value of 0.0, and similarity.

| Accession number | Lengths | No of matches | E-Value | Query cover |
|------------------|---------|---------------|---------|-------------|
| Query            |         |               |         |             |
| MW362290.1       | 1493    | 1             | 0.0     | 100%        |
| Subject          | MT538265.1 | 1507         | 1       | 100%        |
| Query            | MT362291.1 | 1489         | 1       | 0.0         |
| Subject          | MT538265.1 | 1503         | 1       | 0.0         |
| Query            | MT362292.1 | 1493         | 1       | 0.0         |
| Subject          | MT052668.1 | 1507         | 1       | 0.0         |
| Query            | MT362293.1 | 1483         | 1       | 0.0         |
| Subject          | MT538265.1 | 1503         | 1       | 0.0         |
| Query            | MW362294.1 | 1483         | 14      | 0.0         |
| Subject          | CP053954.1 | 1478         |         |             |
| Query            | MW362295.1 | 1483         | 1       | 0.0         |
| Subject          | MT292101.1 | 1515         | 1       | 0.0         |

16S rRNA gene has a characteristic size of about 500 bases until 1550 bp. For the 16S rRNA used for sequencing measuring 1535 bp. The use of 16S rRNA is often used in prokaryotic organisms rather than 23S rRNA because of its higher variation. In Eukaryote it uses 18S rRNA for identification. Therefore, uses 16S rRNA is of good identification in bacteria. A phylogenetic relationship based on 16S rRNA nuclear gene. The construction of phylogenetic tree (Fig. 1) described the phylogenetic relationship of the 6 species found, namely were three Bacillus cereus strain and Bacillus weidnani strain and B. thuringiensis.

Mahwish et al. reported that the genera Bacillus and Pseudomonas were the most dominant and most commonly found in various plant studies According to Cakmakci et al. [34], similar results were presented by [35] who reported an increase in nutrient absorption efficiency by PGPRs inoculation which resulted in increased root growth and hence efficient absorption of nutrients by plants. Plants will be more resistant to drought, salinity, and toxins derived from metals and metals. PGPR also maintain plants against pathogens and pests.

IV. CONCLUSION

The study showed a remarkable identity between all the isolates (query) and the subjects with very considerable number of matches and the number of nucleotides sequences. The prompt assignment of accession numbers to the isolates showed the validity and reliability of the protocols used ranging from the conventional identification procedures and the molecular analysis. MW362295 (Bt) is distantly related to others in the evolutionary trend.

ACKNOWLEDGMENTS

Our deepest appreciation goes to Mr. and Mrs. Olaniyi for their moral supports.
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Mr. Adeoti Olatunde is member of learned societies with some of his selected membership and distinguished academic laurels herein stated. Awards and Fellowship

- Editorial Board Member of Biomedical Sciences of Science Publishing Group, New York, NY 10018, United State of America 2018.
- The Founder, Face Out Malaria and AIDS Foundation (FOMA), Non-Governmental Organization which is a non-governmental organization from, 2005 to date.