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MOLECULAR CHARACTERIZATION OF *Bacillus subitlis* ISOLATED FROM SOME ENVIRONMENTAL SAMPLES IN UMUAHIA, ABIA STATE, NIGERIA

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ABSTRACT

Molecular Characterization of *Bacillus subitlis* from some Environmental Samples was studied with the aim of relating it to their antimicrobial production potentials .Water and soil samples were collected from top soil of a small stream Inem, in Eziama Ossah in Umuahia North Local Government area of Abia State. The samples were cultured and sub-cultured to isolate pure colonies of the desired bacteria on Nutrient Agar ,Brain heart infusion media and Trptic soy broth .Series of physiological, biochemical and molecular analysis were performed in order to identify the bacterium. The bacterium was gram positive, aerobic and able to reduce nitrate and gave positive results for biochemical tests like voges – prauskauer (VP), catalase, citrate utilization buts negative results for methyl red, and oxidase tests. The bacterium produced acid production from sugars like Glucose and thrived well at ambient temeratures. It's Pure genomic DNA was isolated from the cell and was amplified using polymerase chain reaction (PCR) technique with 16sr RNA gene forward and reverse primers. The sequencing conditions were 32 cycles of 90°C for 105, 55°C for 55 and 60°C for 40 minutes. The genetic sequence was subjected to BLAST search in gene bank with nr (Non-redundant) data base of NCBI. BLAST Results showed that the organism was similar to *Bacillus* with 99% similarity. Phylogenetic relationship of test organism was constructed using phylp 3.69 soft ware and tree visualiluation was done by Tree view X. Finally, the isolate was found to be under the group of *Bacillus sp* and closely related to *subtilis*. Further research is suggested to exploit its potentials.

Keywords: *Bacillus subtilis*, Antimicrobial, Soil, Water, Molecular typing

1.0 INTRODUCTION

Bacillus subtilis also known as hay *Bacillus* or grass *Bacillus* is gram-positive, aerobic, spore forming, rod shaped bacterium. (Perez, 2000). The bacterium has its natural habitat in the upper layers of soil, water and vegetation. It can also be found in the gastrointestinal tract of ruminants and humans as commensals (Hong *et al*., 2009). *B. subtilis* like all members of the genus *Bacillus* is a rod shaped bacterium that typically occurs in small clumps, short chains or single cells. It can form a tough protective endospore, allowing it to survive extreme environmental conditions of temperature and drought. Endospores of *B. subtilus* are resistant to heat, drying, radiation, acid, alkaline, osmotic freezing, disinfectant, oxidative conditions, ethanol and other conditions that would normally destroy other bacteria (Bandow, 2000). The endospores allows it to survive until conditions become favourable again.

This bacterium is therefore a model organism for studying endospore formation in bacteria. Endospores in *Bacillus subtilis*, are mostly formed in the tips of protuberances (Piggot *et al*., 2004). Many strains produce spores with brown pigments. Depletion of carbon, nitrogen, or phosphorous causes the process of sporulation to begin. Over the years, *B.subtilis* has proven highly amenable for genetic manipulations and has become widely used as a model organism for laboratory studies.

Identification of pure strains isolated from environmental samples based on morphological, physiological and biochemical characteristics for the identification and confirmation of *Bacillus subtilis* were studied by other researchers like Faruk *et al*., (2009). In their research work, 16 soil samples collected from different environmental sites were analysed for the presence of *Bacillus*. The identified bacteria species included mainly *Bacillus* after biochemical and morphological confirmation. A total of 29 *Bacillus* species were isolated from the soil s analysed by Ramachandran *et al*., (2004).

Molecular characterization of *Bacillus subtilis* surfactin producing strain and factor affecting production was studied by Abushandy *et al*., (2000). In their study, isolation of DNA of *B. subtilis* was carried out by extraction of pure Gene from genomic DNA of the studied isolates. PCR amplification of 16sr RNA was also carried out using forward and reverse primers. Result of PCR amplification and sequence suggested two isolates belong to the *Bacillus* group with highest identity to *B. subtilis*.

The present study was designed to examine the molecular characteristics of different strains of the genus *Bacillus* and confirming their identity through PCR after morphological, physiological and Biochemical characterization. Molecular techniques (PCR) have been widely used in recent times and they have the advantage that they are accurate, sensitive and rapid detection of single bacteria or genes. It also shorten detection time, and detect and quantify bacteria in real time and highly sensitive, specific and reproduceable.

In the course of this research work, molecular characterization was based on

16s rRNA primers since Ribosomal RNA's are now relatively easy to analyse, because they can be directly sequenced from crude cell extracts using reverse transcriptase (petti, 2007). Therefore the use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic maker used for a number of reasons which include: it's presence in almost all bacteria existing as multigene family, and the function of 16S rRNA gene over time has not changed suggesting that Random sequence changes are a more accurate measure of time (Kohji, 2009).

2.0 MATERIALS AND METHODS

Sources of Reagents, Chemicals and Culture Media.

Reagents and chemicals used in the morphological and Biochemical identification, culture media (nutrient agar and Tryptic soy broth) etc used in this present study were commercially purchased. All chemicals and kit for biochemical analysis were also purchased commercially. Nutrient agar used for the major culturing and sub culturing work of the isolates, was 1S0 13485 model with lot M3EIH POI Prepared by TITAN BIOTECH LTD, Rajasthan, India, while the Brain heart infusion Agar (BHI) was sigma – Aldrich St. Louis, MO prepared by Bio Rad Laboratories Inc. Hercules. Indicator *Bacillus subtilis* was Purchased Commercially from NAFDAC Office, Abuja Nigeria. The PCR mix which included: X2 Dream Taq Master Mix was supplied by Inqaba, South Africa. The forward and the reverse primers were also supplied by Inqaba South Africa.

Collection of Samples

Soil samples from different locations in the upper layers of the soil near the stream, and soil from the stream were aseptically collected in sterilized bottles and transported to the department of Medical laboratory Science Laboratory in Imo State University for analysis.

Cultivation of Samples and Isolation of Bacillus

The soil and water samples were analysed in this present study using standard bacteriological methods/procedures. Pour plate, soil sprinkling and streaking methods were used to culture the samples on Nutrient Agar medium. Both were incluabted at 37oC for 24-48hrs. The isolates were further sub cultured on fresh nutrient agar for 24-48hrs to get pure strains. The pure isolates were grown aerobically in Brain Heart infusion broth and muellar Hinton agar at 37oC with shaking (225rpm). All strains were maintained/preserved in BH1 medium Containing 20% glycerol.

Identification of Isolates

Isolates were identified morphologically based on shape, Gram staining reaction, spore staining for spore formation, motility, Acid fast stain, slime production, presence of capsule. And culture characterization on Agar plates: (Colonies, growth temperature, colour, margins, elevation and density). Biochemical test like; oxidase and urease, catalase enzymes production, Nitrate reduction, methyl red, vogues proskaeur citrate utilization, indole production, starch hydrolysis were applied

according to Bergey's Manual of determinative Bacteriology (Bergy and Holt, 1994).

Molecular Identification of Isolates on the basis of 16s rRNA Profile

Molecular identification of isolates using PCR involved the following: DNA Extraction (Boiling method), DNA quantification, 16s rRNA Amplification, sequencing, phylogenetic analysis. DNA Extraction was carried out using 5ml of an overnight broth culture of the bacterium in Luria Bertani (LB). It was spun at 1400rpm for 3 minutes. The cells were then re-suspended in 500µl of Normal saline and heated at 95°C for 20 minutes. The heated bacterial suspension was then cooled on ice and spun for 3 mins at 1400rpm. The supernatant containing the DNA was transferred to a 1.5ml microcentrifuge tube and stored at 20°C for other reactions.

DNA Quantification

The extracted genomic DNA was quantified using the NANODROP 1000 spectrophotometer. The equipment was blanked using normal saline and initialized with 2 µl of sterile distilled water. 2 microlitres of the extracted DNA was loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The concentration of the DNA was measured by clicking on the measure button.

16Sr RNA Amplification

The 16S rRNA region of the genes of the isolates were amplified using forward and reverse primers. The 27f:5[1]-AGAGTTTGATCMTGGCTCAG-3[1] and 1492R:5[1] – CGGTTACCTTGTTAGACTT-3[1]. An ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. The PCR conditions were as follows: initial denaturation, 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 52°C for 30 seconds. Extension to 72°C for 30 seconds for 35 cycles and final extension at 72°C for 5 minutes. The product was resolved on a 1% agarose gel at 130v for 25 minutes and visualized on a blue light transiluminator.

Sequencing

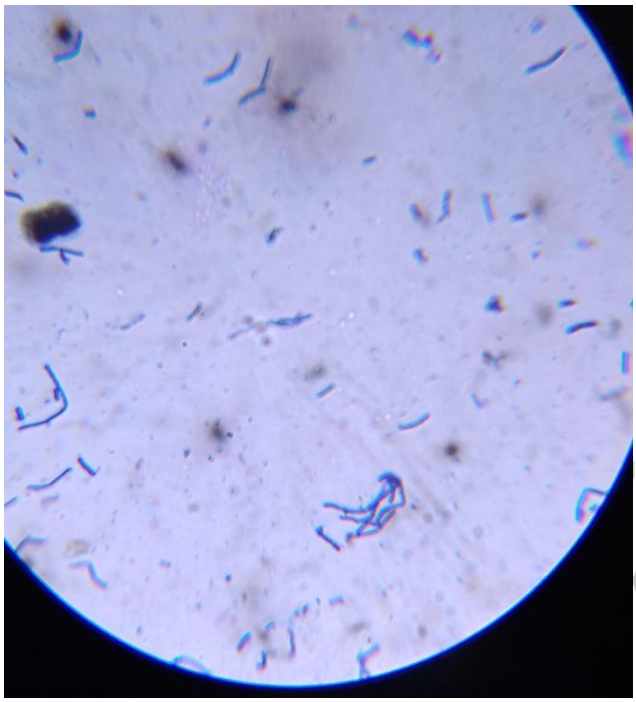
Sequencing was done using Big Dye Terminator kit on a 3510 ABI sequencer at a final volume of 10µl, the components included 0.25µl Big Dye. The sequencing conditions were as follows: 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4 minutes.

Phylogenetic analysis

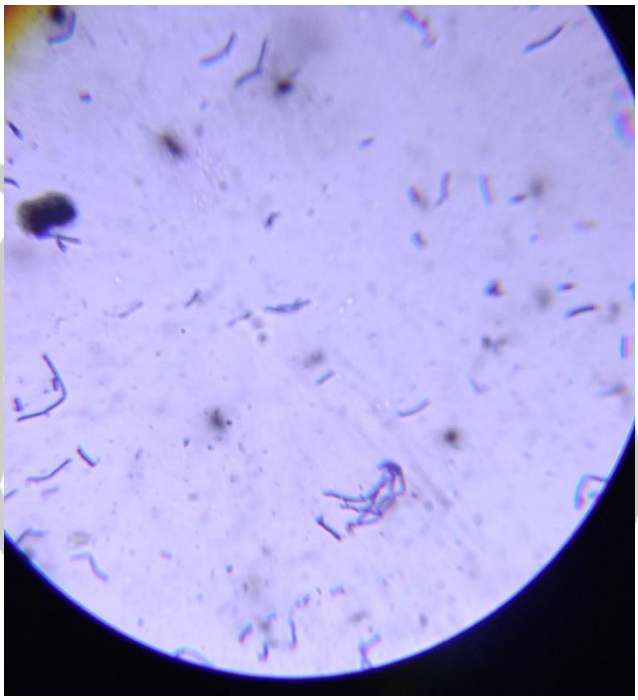
The sequences obtained, were edited using bioinformatics algorithm Trace edit just similar sequences from the NCBI data base using BLASTIN. The evolutionary distances were computed using Juke – Cantors method (Jukes and Cantor, 1969).

3.0 RESULTS

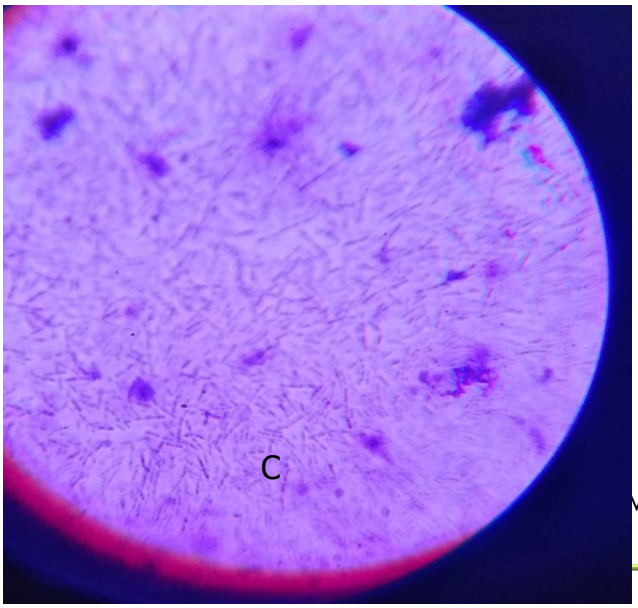
3.1 Gram – staining reaction of isolates



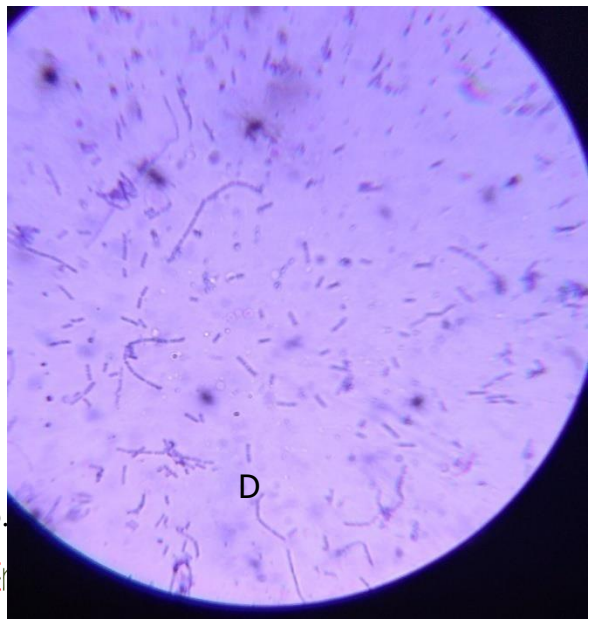
A



B



C



D

Figure 1 (a – d) shows *B. subtilis* from soil and water.

The isolates following gram stain were gram – positive bacteria, rod-shaped which is typical of *B. subtilis*

Morphological criteria for the identification of *B. subtilis*

The table below shows the morphological tests carried out to identify *B. subtilis*. These shape and arrangement of bacteria were short chains, the colony shape on the nutrient agar was irregular. Capsule, gram, slime spore, motility test were positive, while anerobic acid fast tests were negative. Morphological test carried out showed that the most probable identify of the isolate was *Bacillus subtilis* (Table 1).

Table 1: Morphological tests for the identification of *B. subtilis*

Test	Morphological results
Shape and arrangement	Short chains
Colony shape on nutrient agar	Irregular
Presence of capsule	Positive
Slime Production	Positive
Anaerobic growth	Negative
Gram staining	Positive
Spore formation	Positive
Motility	Motile
Acid fast staining	Negative

Result of culture characterization on Agar plates that was used in identification of *B. subtilis* is given in table 2. The isolates when cultured on Agar medium presented results typical of *B. subtilis*. The colonies appeared whitse and growth at optimum temperature was positive with creamy white colours with serrated margins. They helped in identifying *B. subtilis*.

Table 2: Cultural characterization on Agar Plates used in Identification of *Bacillus subtilis*

Test	Result
Colonies	White round abundant multiage
Temperature growth	
20°C	Positive
30°C	Positive
39°C	Positive
42°C	Positive
50°C	Negative
60°C	Negative
Colour	White and Creamy
Growth	Aboundant
Form	Irregular
Margin	Serrated
Elevation	Flat
Density	Translucent
Temperature	Optimum

Culture characterization on Broth media is presented in table 3. On the broth media, the surface growth clouding and sediment, appeared pellicle, slight and flaky confirming *B. subtilis*.

Table 3: Culture characterization on Broth

Test	Result
Surface growth	Pellicle
Clouding	Slight
Sediment	Flaky

Biochemical tests for confirmation were carried out to confirm identity of *B. subtilis*. Like oxidase, catalase, nitrate reduction, litmus milk, urease test, H_2S production. Oxidase test was negative, catalase positive. The ability to reduce nitrate positive and urease test negative.

Table 4: Biochemical tests result given in table 4, confirming identity of the isolate to be *B. subtilis*

Biochemical Tests	**Results**
Oxidase	-
Catalase	+
Nitrate reduction	+
Litmus milk	Alkaline
Urease	-
H_2S production	-
Methyl red	-
Vogues Proskaeur	+
Citrate utilization	+
Indole Production	-
Carbohydrate fermentation	
Lactose	-
Surcrose	+
Glucose	+
Maltose	+
Sorbitol	-
Starch hydrolysis	+ rapid

Gelatin hydrolysis	+
Casein hydrolysis	+
Lipid hydrolysis	+

Key: + Positive - Negative

Result of Molecular Analysis

The obtained 16s rRNA sequence from the isolate, produced an exact match during the mega blast search for highly similar sequences from NCBI database. The 16sr RNA of the isolates B1, B2, B3 and B4 showed a percentage similarity to other species at 99 –100$^0/_0$. The evolutionary distances computed using the Juke-cantor method were in agreement with the phylogenetic placement of the 16s rRNA of the isolates with the *Bacillus Sp* and revealed a closely relatedness to *Bacillus subtilis* than other *Bacillus* Species. Fig. 1 showed the result of electrophoresis analysis of the 16s rRNA gene of the isolates. The lane 1, 2, 3, etc represents the lane for the isolates while m lane represents the standard DNA marker 100bp ladder. (Patel, 2001).

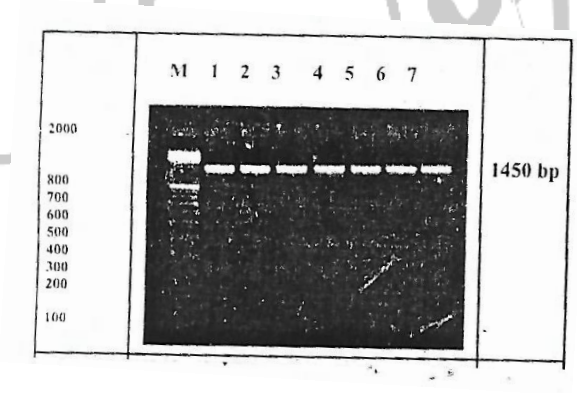


Fig. 2: Electrophoresis analysis of 16srRNA gene of the selected isolates M. DNA Marker.

4.0. DISCUSSION

The present study was carried out to isolate, identify and molecularly characterize *Bacillus subtilis* isolated from environmental samples. *Bacillus subtilis* isolated from the soil by soil was identified as *B. subtilis.* This supports the reports of table 1. experiments carried out by Bushra *et al*., (2007) and Awis *et al*., (2008). For the isolation of *Bacillus subtilis*. Morphological

characteristics of *B. subtilis* were studied through growth on solid media and presented in table 1. The organism was short and in chains, colony shape on nutrient agar was irregular with the presence of capsule, gram stain positive etc. These morphological characteristics were purely confirmatory for *Bacillus subtilis*. Table 2 presents the culture characterization on Agar plates used in identification of *Bacillus subtilis.* The isolates were identified as white round aboundant colonies typical of *Bacillus subtilis,* their forms were irregular, margins serrated and elevation flat. Growth was observed to be between PH 5-9 and growth temperature of isolate was observed in between 22-42^{o}C. Table 3 presents characteristics on Broth media. The characteristics on broth is pellicle, slight and sediment flacky this further confirms *Bacillus subtilis.* These results were in line with the results of work of Prasenjit *et al*., (2011) in the isolation, identification and molecular characterization of potential bacteria, *Bacillus subtilis.* Biochemical characteristics of the isolates (table 4) shows that the isolates were able to produce acid from surcrose, glucose and maltose but unable to ferment sorbitol and lactose. It was catalase positive. oxidase negative, confirming typical *Bacillus subtilis*. The isolate has the ability to hydrolyze starch, but unable to hydrolyse urea. This is also in line with the findings by Prasenjit *et al*., (2011).

The isolate was identified by 16sr RNA gene sequence data analysis. The identification of the isolate was confirmed by the Nucleotide Division of National Centre for Biotecnology information (NCBI). A search by the BLAST, of the data base indicated a close relationship to other isolates of *Bacillus subtilis* phylogenetic tree construction was carried out using Phlp 3.69 software and visualization was done using Tree view x. 16sr RNA gene sequence data analysis of the isolates, showed high arrangements between traditional and molecular identification as set up and the isolates was finally identified as *Bacillus subtilis.*

Conclusion

Morphological, biochemical and Molecular Characterization of *Bacillus subtilis* isolated from environmental samples (water and soil) in this work. *Bacillus subtilis* can easily be isolated from it's natural habitat soil and water and can be identified Biochemically and molecularly using PCR amplification of 16s rRNA gene. Moreover, the genetic sequence result showed that the isolate was under the group of *Bacillus* sp and confirmed it to be closest to *Bacillus subtilis*.

REFERENCES

Abushady, H.M. Banshady, A.S., Aziz W.H. and Ibrahim, H.M. (2005) Molecular Characterization of Bacillus subtilis surfactin Producing strain and the factors affecting it's production. International Journal of Agriculture and Biology 7: 1560 – 8530.

Awis, M., Arshid, P, Sadia O. and Saleem, M. (2008). Effects of Glucose, incubation period and PH on the Production of Peptide antibiotics by Bacillus Pulmilus. African Journal of Microbiology 2:114 – 119.

Bandow, J.E, Brotz, H. and Hecker, M. (2002) Bacillus. Tolerance of Moderate Concentrations of Rifampin involves the B-

dependent general and Multiple Response. Journal of Bacteriology, 184(2): 459 – 467.

Bergey, D.H and Holt, J.G. (1994) Bergey's Manual of Determinative bacteriology. Methods of determining the Morphology of bacteria. 9th edition. Williams and Wilkins, Baltimore.

Bushra, J. Fariha, H., Abdul, H. and Safia, A. (2007). Isolation of Bacillus subtilis mh-4 from soil and it's potential of polypeptidic antibiotic Production, Pakistan. Journal of Pharmaceutical Sciences 20:26-31.

Faruk, A.K. Lohya, N and Priscillia, Y.O. (2009) Screening of Bacillus species with Potentials of Antibiotics Production: Journal of Applied Medical Informatics. 24(1):42-46.

Hong, H.A, Khaneju, R. Tam N.M., Cazzato, A., and Uvdaci, M. (2009). Bacillus subtilis isolated from human gastrointestinal tract. Journal of Research in Microbiology 160(2): 34-43.

Juke J.H. and Cantor, C.R. (1969). Evolution of Protein Molecules. Mammalian Protein Metabolism. PP. 21 – 132. Academic Press.

Kohji Hasunuma (2009). Genetics and Molecular Biology. Yokohoma City University Japan ESBN. Pg. 73.

Patel, J.B. (2001). 16s rRNA gene sequencing for Bacterial Pathogen Identification in the Clinical Laboratory. Journal of Molecular Diagnostic, 6:313 – 321.

Perez, A.R. and Dogliano, A.O. (2000). Spoll B localizes to active sites of Biogenesis and Partially regulates septal thining during engulfment in Bacillus subtilis. Journal of Bacteriology, 182 (4): 1096-1108.

Petti, C.A. (2007). 16sr RNA gene sequencing for Bacterial Pathogen Identification in the Clinical Laboratory. Journal of Molecular Diagnostics, 6: 313 – 321.

Piggot, P.J. and David, W.H. (2004). Sporulation of Bacillus subtilis. Current Opinion in Microbiology 7:579 – 586.

Prasenjit, B., Amrita, B., Partha, B., Keshab, C.M. and Pradeep, K. D.M. (2011). Isolation, Identification and molecular characterization of potential probiotic bacterium, Bacillus subtilis PPPB from Penaeus Monodon. Research article Biotechnology 1 (4): 473 – 482.

Ramachandran I and Ajah, G.C. (2014) A broad Spectrum antimicrobial activity of Bacillus subtilis RLD. The Scientific World Journal. 127-131.