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SHORT COMMUNICATION

Phylogenetic Analysis Based on 16S rRNA Gene of a Thermophilic Protease-Secreting *Bacillus gelatini*-TPNK-3 Isolate from Kiteezi Landfill, Uganda

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Abstract

A thermophilic protease-secreting bacterial isolate, TPNK-3, from Kiteezi landfill, is an aerobic Gram-positive spore-forming bacterium with rod-shaped cells (3.28 µm long and 0.45 µm wide). Optimal growth was observed at 55°C and pH of 7.0, and the isolate tolerates up to 5% (w/v) NaCl, exhibits extracellular amylolytic, cellulolytic and caseinolytic activities, utilizes a range of carbon and nitrogen sources and has a GC content of 45 mol%. The 16S rRNA gene sequence analysis showed that the studied bacterium belongs to the genus *Bacillus*, and closest to *Bacillus gelatini* (> 99.9%). Consequently, isolate TPNK3 is tentatively described as *Bacillus gelatini* strain TPNK3.

Key words: 16S rRNA-gene, phylogenetic relationship, thermophilic

Phylogenetic methods based on the sequencing and cataloguing of rRNAs (16S and 23S), have played a major role in modern taxonomic and phylogenetic studies of newly isolated microorganisms. Typically, the analyses of 16S rRNA gene sequences are generally used as a framework for bacterial classification (De Clerck *et al*., 2004) and both archaeal and bacterial domains have been phylogenetically described using rRNA-based methods. For instance, analysis based on 16S rRNA has been used in multiple studies such as (i) characterization of the *Ehrlichia canis* strains involved in naturally occurring canine monocytic ehrlichiosis (CME) and to investigate the differences between the 16S rRNA genes of *Ehrlichia canis* responsive to mild non-myelosuppressive and severe myelosuppressive forms of CME (Siarkou *et al*., 2007); (ii) establishing the genotypic diversity among marine *Bacillus* species (Ki *et al*., 2009); (iii) establishing phylogenetic relationships between hydantoinase-producing bacteria (Mei *et al.,* 2009); and (iv) differentiation the species in the genus *Anaplasma* in China (Liu *et al.,* 2005). Furthermore, the strength of the rRNA sequencing approach has been illustrated in its application to the phylogenetic placement of unculturable microorganisms (Nogales *et al.*, 2001). Thermophilic organisms are promising sources of thermostable enzymes. Since these enzymes tend to remain stable under harsh industrial conditions, they are used in a number of biotechnological bioprocesses (Jaenicke *et al.*, 1996). For instance, as components of laundry detergents, in the bioconversion of waste into biomass, leather tanning, dairy and food processing, aerobic digestion of sewerage sludge and pharmaceutical industries (Genckal and Tari, 2006; Liu *et al*., 2011). The wide use of thermostable enzymes in the industry prompts multiple screens of environments that can contain bacteria producing such enzymes.

In our study, we screened Kiteezi landfill site in search for bacterial isolates that can produce thermostable enzymes. The sampling site encompasses an area of four acres of which three contained freshly damped municipal solid waste. Thirty (30) soil samples of approximately 200 g each were collected from the depth of at least two feet where we noted temperature above 50°C. Samples were collected from freshly damped decomposing garbage and suspended in two volumes of sterile distilled water. After agitation for 30 min to achieve thorough mixing, the samples were serially diluted and plated onto casein agar (pH 7.5) then incubated at 55°C for 48 h.

The standard medium for routine growth of the studied isolate was prepared as describe previously (Hawumba *et al*., 2001). Casein medium, which was

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used in the production of crude enzyme solution, was prepared by modifying the standard medium by replacement of the peptone with casein at 2% (w/v). To investigate the utilization various individual carbon and nitrogen substrates as sole carbon and nitrogen sources, respectively, the standard medium was modified by supplying: (a) the carbon substrates (*i.e.* L-sorbose, sorbitol, L-arabinose, D-fructose, D-arabinose, D-xylose, inulin, D (+) maltose, mannitol, D-galactose, dextrin, inositol, cellobiose, starch, cellulose, sodium acetate, sodium malonate, sodium oxalate, sodium succinate, and sodium malate) and (b) nitrogen substrates (D and L-serine, L-asparagine, D and L-phenylalanine, D and L-valine, L-glutamate, D and L-tyrosine, L-cysteine, glycine, L-aspartic acid, L-tryptophan, L-arginine, D and L-histidine, D-alanine, D-methionine, D and L-leucine, L-tyrosine, urea, and D and L-ornithine). To solidify the media, 1.5 % (w/v) of agar was added to the broth. The pH was adjusted to pH 7.5 using 2 N NaOH or 1N HCl before autoclaving at 121°C for 25 minutes.

To isolate pure cultures, individual colonies which produced clear zones of hydrolysis were purified by repeated streaking on the standard medium and finally on the casein agar medium. Four isolates exhibiting extracellular caseinolytic activity were identified and designated as: TPNK1, TPNK2, TPNK3 and TPNK4. Isolate TPNK3, producing the largest hydrolysis zone around its colonies, was selected for further characterization. The optimal pH for growth of each isolate was determined in 50 ml of the medium buffered with 10 mM benzoic acid at pH 4, 10 mM pyridine at pH 5, 10 mM phosphate buffer at pH 6 and 10 mM Tris-HCl at pH 7 to 9. The optimal growth temperature was determined by culturing the isolate in 100 ml of standard medium at temperatures: 40, 45, 50, 55, 60, and 65°C, respectively, at optimal pH, bacterial growth was monitored by measuring optical density at 600 nm every 30 min for 10 h. At each investigated temperature, the growth rate (μ) was calculated for the period of exponential increase. The ability of isolate TPNK3 to utilize various substrates as sole carbon and nitrogen sources, respectively, was investigated. The medium was individually supplied with appropriate substrates and cultured at optimal conditions of temperature and pH. Catalase activity was examined upon addition of 3% H_2O_2, appearance of gas bubbles was considered a positive test for catalase activity.

TPNK3 isolate grew at pH from 5.8 to 8.2, with an optimum pH of 7.0. The growth rate was determined at optimal pH within the temperature range of 40 to 65°C. Maximum growth rate was observed at 55°C and no growth occurred above 65 and below 40°C, respectively. At the optimal growth temperature and optimal pH, the generation time was 35 min. Isolate TPNK3 is also halotolerant and could grow in broth containing 5% (w/v) NaCl. However, no growth occurred above this value. The catalase test revealed that the isolate is either aerobic or facultatively anaerobic.

Genomic DNA was isolated as described previously (Hawumba *et al.*, 2001). The mol % G+C was determined by method of Owen and Hill (1979). The gene encoding 16S rRNA was amplified using the universal primers fD1 and rP2 (Weisburg *et al.*, 1991) and its nucleotide sequence was determined by automated sequencing with an ABI PRISM™ BigDye™ Terminator Cycle Sequencing Ready Reaction mixture (Perkin-Elmer Applied Biosystems, Foster City, CA USA), in a Hitachi 3100 capillary array automated DNA sequencer. The resulting sequence was edited to a total length of 1350 nucleotides. A BLAST search of the GenBank database of the National Center for Biotechnology Information, (www.ncbi.nlm.nih.gov/GenBank) retrieved several sequences that were compared with that of TPNK3. These sequences were edited using the BioEdit program and the phylogenetic relationship of isolates TPNK3 was determined by performing a multiple alignment of the sequencing data with sequences of related *Bacillus* spp. (GenBank database of the National Center for Biotechnology Information, www.ncbi.nlm.nih.gov/GenBank/) using ClustalW (Thompson *et al.*, 1994). The tree was constructed using the neighbor-joining method of Saitou and Nei (1987) in combination with the bootstrap method (Felsenstein, 1985). The 16S rRNA gene sequence obtained was deposited in GenBank under accession number: FJ748503

The sequencing of the 16S rRNA gene of TPNK3 strain allowed us to determine the sequence of approximately 90% of the complete gene. The 16S rDNA phylogenetic tree (Fig. 1) indicated that isolate TPNK3 clusters together with the strains that are classified to genus *Bacillus,* sharing >99.9% sequence similarity with 16S rDNA sequences of of *Bacillus gelatini* strain TMW 2.552 (GenBank Accession No. AJ809500), *Bacillus gelatini* strain R-13822 (GenBank Accession No. AJ586347), and 98.30% sequence similarity with *Bacillus gelatini* strain 506 (GenBank accession No. DQ350818). Based on this high sequence similarity to *Bacillus gelatini* members, isolate TPNK3 may be tentatively described as *Bacillus gelatini* strain TPNK3. However, it is assumed that organisms showing greater than 97% 16S rDNA sequence similarity are likely to have 70% or more DNA-DNA relatedness and thus can be considered to be the same species (Nazina *et al.*, 2001). Nevertheless, in accordance to the rules of taxonomy, the proper classification of bacteria to the species requires to determine the degree of DNA-DNA hybridization. So far described *Bacillus gelatini* strains are mesophilic and therefore the isolation of a thermophilic strain points to the heterogencity of member of the genus *Bacillus.* Therefore it is

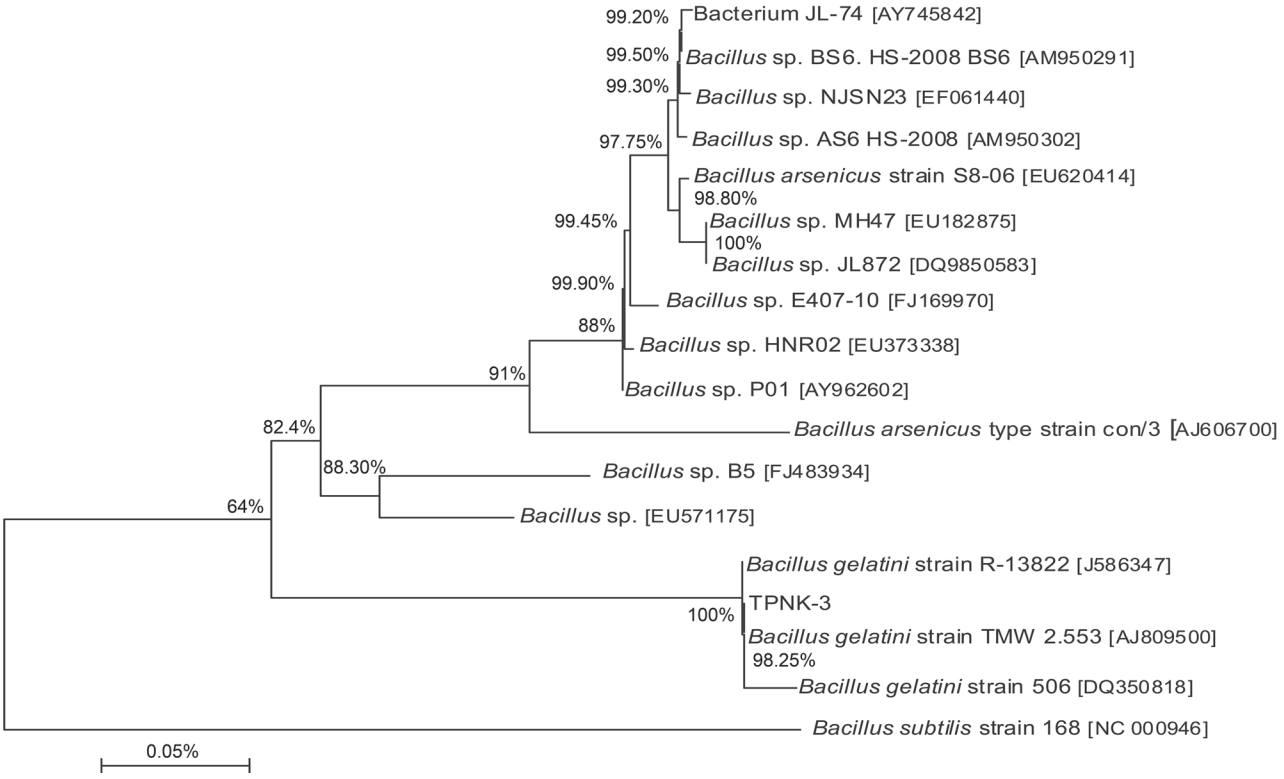

Fig. 1. Neighbor-joining tree showing the phylogenetic affiliation of isolate TPNK3 with selected members of Bacillus spp., with *Bacillus subtilis* strain 168 included as an out-group. Bootstrap values greater than 50% are indicated. The scale bar represents 0.05% of the estimated substitutions per nucleotide position

necessary to underline that TPNK3 strain is the first thermophilic *Bacillus gelatini* strain described.

In addition, the mol % G+C of isolate TPNK3 of 45%, was also in the range found for *Bacillus gelatini* strains (De Clerck *et al*., 2004; Yoon *et al*., 2005) and *Anoxybacillus contaminans* sp. (De Clerck *et al*., 2004).

In order to establish the morphology of TPNK 3 strain, cells were stained with Gram stain and studied under the microscope (Olympus, LH50A, Tokyo, Japan) and image captured using a TTL Auto-exposure 35 mm SLR camera (Olympus SC35 Type 12, Tokyo, Japan) at a magnification of x 1000. Cell size was estimated using a calibrated eyepiece, and was an average of 10 measurements of distinct cells. Cells were Gram-positive, rod-shaped (3.28 μm long and 0.45 μm wide), and form spherical terminal spores (Fig. 2). The ability of the TPNK3 to utilize various polymeric substrates was studied by its capacity to secrete extracellular enzymes: protease (s), amylase (s) and cellulose (s) enzymes (Table I). Furthermore, the isolate could utilize several carbon and nitrogen sources (Table I) indicating its heterotrophic growth pattern (Stackebrandt and Geobel, 1994), characteristic of members of the genus *Bacillus* and related genera.

Endospore-forming aerobic bacterial species such as *Bacillus* sp., among others, are ubiquitous and have been isolated from a variety of habitats ranging from very cold to extremely hot. *Bacillus* sp. are very successful in colonizing various habitats, and are often tested in search of valuable bioproducts, such as enzymes,

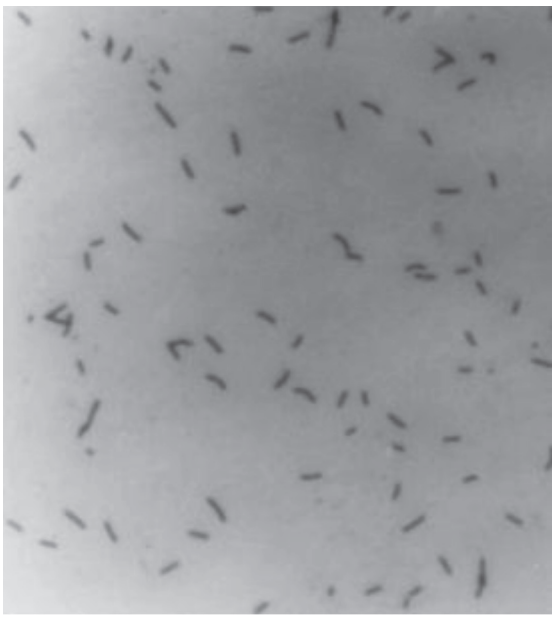

Fig. 2. Micrograph presents typical cell morphology of 6 hr culture of TPNK3 in standard broth medium. A camera (Olympus SC 35 typer 12 Tokyo, Japan) mounted on a phase contrast microscope (Olympus LH 50A, Tokyo, Japan) was used to take this photomicrograph. The magnification used was X 1000

Table I
Growth characteristics of isolate TPNK3

Test parameter	Result
Temperature range of growth (^{0}C)	40–60
pH growth range	5–8
Growth in NaCl (%)	0–7
Indole	+
Mo l % G+C	45
Catalase	+
Temperature range of growth (^{0}C)	40–60
pH growth range	5–8
Growth in NaCl (%)	0–7
Carbon utilization with acid production	
L (–) Sorbos	+
Sorbital	+
L (+) Arabinose	+
B-D (–) Fructose	+
D (–) Arabinose	+
D (+) Xylose	+
Inulin	+
D (+) Maltose	+
d-Mannitol	–
Cellulose	+
D (+) Galactose	+
Dextrin	+
I Inosital	–
Cellobiose	–
D (+) Glucose	+
Starch	+
Sodium acetate	–
Sodium malonate	–
Sodium oxalate	–
Sodium succinate	–
Sodium malate	+
Nitrogen utilization	
DL-Serine	+
L-Asparagine	–
DL-Phenylalanine	+
DL-Valine	+
L-Glutamate	+
L-Arganine	+
DL-Tyrosine	–
L-Cystein	+
Glycine	
L-Aspartic acid	–
L-Tryptophan	+
L-Arginine	–
DL-Histidine	–
D-Alanine	+
D-Methionine	+
DL-Leucine	+
L-Tyrosine	–
Urea	–
DL-Ornithine	+

Key: + = positive, – = negative, N = not determined

for application in industry and other biotechnological operations. Hot and other extreme environments are considered the most valuable sources of organisms that produce enzymes that can be used in industrial processes. In this study, protease-secreting thermophilic bacteria were isolated from compost heaps from Kiteezi landfill of Kampala City Council. Since there is a strong interest in the utilization of thermostable enzymes, TPNK3 strain can be a valuable source of novel proteolytic enzyme (s) that could be utilized in various biotechnological processes.

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