academicJournals

Vol. 9(14), pp. 1037-1043, 8 April, 2015
DOI: 10.5897/AJMR2015.7441
Article Number: FF2AA0F52318
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Isolation and characterization of antibiotic producing *Bacillus* species in Lake Bogoria, Kenya

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Received 20 February, 2015; Accepted 25 March, 2015

Bacilli are a large homogeneous group of bacteria that survive in a wide range of environmental conditions. Formation of resistant spores allows them to survive in high temperature zones where other organisms cannot. Eighty samples were collected and inoculated directly into nutrient broth. Of the eighty samples collected, thirty three exhibited growth. Nine of these were Gram positive rods, twenty were Gram negative rods, and four were Gram positive cocci. Only cultures that yielded Gram positive rods were processed further. Antimicrobial profiling was performed using standard organisms: *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *E. coli* 35218 and *Pseudomonas aeruginosa* ATCC 27853. No inhibition was noted against *S. aureus* ATCC 29213 and *E. coli* 35218. Five of the nine Gram positive isolates revealed inhibitory properties against the standard organisms. Phylogenetic analysis of amplified 16S rDNA gene confirmed that all the six antagonistic isolates formed close phylogenetic clusters with known members of *Bacillus* species with a 88-99% sequence identity. The current study shows the presence of thermophilic *Bacillus* species, which are potential biomolecule producers within the hot springs of Lake Bogoria, Kenya. However, further investigation will be useful for the discovery of novel bioactive substances effective against wide range of pathogens.

Key words: *Bacillus*, Lake Bogoria, secondary metabolites, antibiotics.

INTRODUCTION

The major impetus behind extremophile research is the biotechnological potential associated with extremophilic organism. Indeed, applying extremophiles in industries has opened a new era in biotechnology. In the last decade, the number of antibiotic multi-resistant bacterial strains as etiological agents of infectious diseases has increased at an alarming rate; challenging physicians to find an anti-infective therapy that ensures an effective result (Bansidhar et al., 2013). Extermophile micro-organisms produce molecules adapted to unusual life conditions and have been recognized as an important source of new biological products (Sanchez et al., 2009). Until recently, majority of antimicrobial compounds were isolated from terrestrial microorganisms. In the last two

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decades, however, the rate of discovery of novel compounds from this source has significantly declined. This phenolmenon has been exemplified by the fact that extracts from soil-derived actinomycetes have yielded high numbers of clinically unacceptable metabolites (Mincer et al., 2002). While these microorganisms continue to be studied extensively, the rate of discovery of novel metabolites from terrestrial microorganisms is decreasing (Sarker et al., 2010). Discovery and identification of new sources of natural products, therefore, plays an important role in the uncovering of novel drug candidates and drug development (Jorgensen and Turnidge, 2003). Saline and hot water lakes are now becoming increasingly appreciated as rich and untapped reservoirs of useful novel natural products.

Antibiotics have been recognized as the most significant means of effective microbial growth control (Bertrand, 2004) after the discovery of penicillin and other antimicrobial agents by Alexander Fleming in 1928. Since then, there has been ongoing search for effective antibiotics that can withstand emergence of drug resistance among microorganisms (Song, 2008). In Sub Saharan Africa for example, resistance to most available antibiotics has resulted in morbidity and mortality from treatment failures and the ever-increasing costs of antibiotics (Bertrand, 2004).The main approach in which new antibiotics have been discovered has been by screening groups of microorganisms such as *Bacillus, Penicillium, Streptomyces* and other microorganisms (Demain and Fang, 2000; Oluoch et al., 2010). Currently, standard reference strains such as the America Type Culture Collection (ATCC) organisms are used to standardize screening tests. Isolates that demonstrate evidence of antibiotic production are then subjected to further studies to determine if the antibiotic they produce is new. When an organism producing a new antibiotic is discovered, it is produced in large quantities, purified and tested for cytotoxicity and therapeutic activity in infected animals (Talaro and Talaro, 2006). Most of new antibiotics will fail the *in vivo* testing, but a few of the new antibiotics that prove medically useful are then produced commercially (Yarborough et al., 2009).

Bacilli, which consists of a group of Gram-positive, facultative anaerobic, sporulating rods, is known to produce more than 45 antimicrobial molecules some of which are of clinical significance (Stein, 2005). *Bacillus* species produce antibiotics in a soluble protein form which they synthesize and secret into the growing medium. Therefore, the antibiotics they produce have been found to be cheaper and effective, hence preferred in commercial production. The potent antimicrobial activities of *Bacillus* lantibiotics against pathogenic microorganisms such as *Bacillus cereus*, *L. monocytogenes* and *Staphylococcus* spp. make them good candidates for application in the food and medical industry (Stein, 2005).

Bacillus has for a longtime been regarded as a phylogenetic heterogenous group (Ash et al., 1991). Conventionally, *Bacillus* has been identified in the laboratory through biochemical tests and fatty acid methyl ester profiling (Bobbie and White, 1980; Vaerewijck et al., 2001). Since 1990 however, 16S rDNA has been successfully applied in determining phylogenetic relationships of the aerobic, endospore forming bacteria which played an important role in the creation of several families and genera Bacillales (Garrity et al., 2007). To date, 16S rDNA forms a vital standard of taxonomy not only for *Bacilli* species but bacteria in general.

In the present research study, we investigated the presence of thermophilic Bacilli in the various hot springs of Lake Bogoria and evaluated their antibiotic producing capabilities by performing antibacterial studies.

MATERIALS AND METHODS

Collection of water samples

Samples were collected randomly from each of the four hot springs of Lake Bogoria. Ten sediment and ten surface water samples were collected from four sites each from Chemurkei, Loburu, Koiobei and Losaramat hot spring to make a total of 80. The samples were obtained within a temperature range of 45-60°C. Samples were randomly collected and inoculated directly into nutrient broth in universal bottles. In every hot spring, each of ten universal bottles containing 15 ml nutrient broth was directly inoculated with 5 ml sediment sample. Equally, 5 ml of surface water sample was inoculated into ten 15 ml of nutrient broth in universal bottles from each of the four hot springs. The inoculated bottles were put into insulated boxes and immediately transported to the laboratory.

Culture and isolation of *Bacillus* species from samples

Samples in nutrient broth were incubated without shaking for two days (48 h) at 45°C. Growth was determined visually by checking for turbidity. All tubes that showed growth were sub-cultured in nutrient agar plates using streak plate method. The plates were then incubated at 40°C for 24 h. Different colonies obtained after incubation were used to make smears on clean slides which were subsequently Gram-stained. Colonies with Gram-positive rods (nine) were sub-cultured in fresh nutrient agar as pure cultures. Since *Bacilli* are Gram-positive rods, all Gram-positive cocci and Gram-negative rods were not processed further. Stock cultures of each selected strain were prepared and preserved in nutrient agar slants at 4°C.

Biochemical identification

Bacilli isolates were characterized using glucose and lactose fermentation, gas production, catalase production, hydrogen sulfide production, indole production and motility tests according to the methods described by Sneath (1984).

Sensitivity testing

The selected isolates were inoculated into nutrient broth in conical flasks enriched with 1% glucose and incubated in a shaker incubator (100 rpm) at 37°C for 4 days. Five ml of the broth from each of the flasks was taken and put in labeled sterile tubes and

Table 1. Morphological characterization of Gram positive rods isolated from Lake Bogoria.

Isolate	Colony characterization				Cell characterization	
	Colour	Form	Elevation	Margin	Cell arrangement	Gram reaction
S7	White	Irregular	Flat	Undulate	Large rods	Positive
D8	Cream	Irregular	Flat	Undulate	Small rods	Positive
S17	White	Irregular	Flat	Undulate	Large rods	Positive
D22	White	Irregular	Flat	Undulate	Large rods	Positive
D5	White	Irregular	Flat	Undulate	Large rods	Positive
D18	Cream	Irregular	Raised	Even	Large rods	Positive
D10	Cream	Spreading	Flat	Undulate	Small rods	Positive
S13	Cream	Spreading	Flat	Undulate	Small rods	Positive
D1	White	Irregular	Raised	Undulate	Large rods	Positive

centrifuged at 10,000 rpm for 15 min. The cell free supernatant was sterilized using 0.2 µl filter paper and put into sterile Eppendorf tubes. Six millimeter sterile filter paper disks were dipped in each of the sterile supernatant in the Eppendorf tubes and dried in a vacuum for 10 min.

Four standard ATCC organisms were used to test for production of antibiotics: *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, and *Pseudomonas aeruginosa* ATCC 27853. Agar disc diffusion assay was performed on 90 mm Petri-plates containing 20 ml of Mueller Hinton agar. A lawn of the test organism standardized using Mac Farland 0.5 turbidity standard was prepared and the extract impregnated disks placed on top. The Petri-dishes were incubated overnight at 37°C for 24 h. Zones of inhibition were noted as clear areas around the disks.

Phylogenetic analysis of 16S rDNA of the isolates

ZR Fungal/Bacterial DNA Miniprep™(Zymo Research, USA) was used to extract genomic DNA from the bacterial cells according to the manufacturer's specification based on the method of James et al. (2003). The DNA product was semi-quantified on 1% agarose gel at 80 V for one hour and visualized by ethidium bromide staining (Sambrook et al., 1989). Target 16S rDNA sequences were amplified using universal bacterial primers 27f AGAGTTTGATCCTGGCTCAG and 1525r AAGGAGGTGATCCAGCCGCA (Bioneer, USA) in relation to *E. coli* gene sequence (Lane, 1991; Embley and Stackebrandt, 1994). Amplification was performed using an advanced Eppendorf 96 AG, model 22331 thermal cycler (Hamburg, Germany) according to the procedure described by Roux (1995). The PCR products were separated on agarose gel electrophoresis (FisherBiotech Electrophoresis system Australia) at 80 V for one hour and visualized by ethidium bromide staining (Sambrook et al., 1989).

Sequencing of purified product was done in both directions without cloning using a commercial service provider (BECA, ILRI). Sequencing reactions were carried out with ABI PRISM Dye terminator Cycle Sequence Ready Reaction Kit (Applied Biosystems Inc., USA) on an ABI 310 DNA Sequencer according to manufacturer's instructions. Sequence data was analyzed with CLC Main Workbench version 7.0.3 software package (http://www.clcbio.com). Alignments were checked and corrected manually where necessary based on conserved regions. The 16S rDNA gene sequences obtained were compared with sequences in the public database using basic local alignment search tool (BLAST) on the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nih.gov) in order to determine similarity with sequences in the GeneBank database (Altschul et al., 1990; Shyne et al., 2003). The 16S rDNA sequence identity was confirmed using the RDP Naive Bayesian rRNA Classifier Version 2.6 (Wang et al., 2007), Michigan State University, (https://rdp.cme.msu.edu). The 16S rDNA gene sequences with high similarities to those determined in the study were retrieved and used in the construction of a phylogenetic tree. Sequences were aligned using Molecular Evolutionary Genetics Analysis version six (MEGA6) (Tamura et al., 2013) software and the aligned sequences used to construct a maximum likelihood phylogenetic tree.

RESULTS

Isolation

There was high diversity of bacteria in hot waters of Lake Bogoria. Isolates grew well producing different colonies on nutrient agar. Colonial morphology description and Gram stain reaction (Table 1) of the isolates were the initial identification criteria used. Micro-morphological observation of the isolates revealed the organisms had typical characteristics of the group Bacilli including formation of spores and presence of branched rods. Of the 33 isolates that grew on nutrient media, 27.3% were Gram positive rods.

Characterization of isolates

All the nine isolates were subjected to baseline biochemical tests as suggested by Brock (2006). Physiological tests showed that all the isolates were catalase positive, all except D22, D5, D18 and D1 were indole positive. Most of the organisms utilized glucose as an energy source whereas none produced gas from sugar fermentation. S13 and D10 were lactose and/or sucrose fermenters as indicated by the butt and slant turning yellow. D22, S7 and D8 fermented glucose and produced enough acid to turn the butt yellow. D5, S17 and D18 did not change the color of the medium. Isolates S13, D10 and D8 produced hydrogen sulfide which was shown by blackening of the butt. The details of physiological and biochemical characteristics of the isolates are given in Table 2.

Table 2. Biochemical results for all nine Gram positive isolates.

Isolate	Biochemical test		Indole	Lactose fermentation	Glucose fermentation	H_2S production	Gas production
	Catalase	Motility					
S13	+	+	+	+	+	+	-
D10	+	+	+	+	+	+	-
S7	+	+	+	+	+	-	-
D22	+	+	+	+	+	-	-
D8	+	+	+	+	+	+	-
S17	+	-	-	-	-	-	-
D5	+	-	-	-	-	-	-
D18	+	-	-	-	-	-	-
D1	+	-	-	-	-	-	-

(+) Positive, (-) Negative.

Table 3. Antimicrobial assay of extracts obtained from the Gram positive isolates against *P. aeroginosa, S. aureus* and two strains of *E. coli* using disc diffusion method.

Organism	Zones of inhibition			
	***P. aeruginosa* ATCC 27853**	***S. aureus* ATCC 29213**	***E. coli* ATCC 35218**	***E. coli* ATCC 25922**
D10	R	R	R	R
S17	R	R	R	R
D18	R	R	R	R
S7	R	R	R	R
D22	+++	R	R	R
D1	+	R	R	R
D8	+	R	R	R
S13	+	R	R	+
D5	++	R	R	++
Control	+++	R	R	++

Inhibition was regarded as clear zones around the discs; (R) resistance, (+) weak inhibition, (++) strong inhibition, (+++) very strong inhibition. Control (Trimethoprim/sulfamethoxazole).

Bioassay against test bacteria

Antimicrobial screening in the present study was carried out at 37°C against four standard ATCC organisms. Five of the nine Gram positive isolates screened showed antagonistic activity against one or more of the standard test organisms. The four isolates that did not show any antagonistic activity against the test organisms were not investigated further. The isolates which were active against the standard organisms were identified with the following codes: D1, D22, D8 and D5 from sediment samples; S13 for surface sample. Antimicrobial activity of the five isolates ranged from weak to strong antagonism using the paper disc assay (Table 3).

16S rDNA gene sequences of the isolates

BLAST analysis of partial 16S rDNA gene sequences showed that the five isolates were closely affiliated with members of the genus *Bacillus.* A phylogenetic tree was constructed using nearest neighbor obtained from the GeneBank to show the phylogenetic position of each of the isolates studied (Figure 1). The isolates shared identities of between 88 and 99% with known *Bacillus* species. D1 and D5 clustered together with a bootstrap value of 63% and supported by a sequence identity of 96%. These isolates also clustered very closely with *Bacillus halodurans* (Acc. NR_025446.1) with isolate D5 having a sequence identity of 99% while isolate D1 had a sequence identity of 95% with the organism. Isolate D8 clustered with isolate S15 with a high bootsrap value of 99% and supported by a sequence identity of 88%. They also clustered with *Paenibacillus thiaminolyticus* (Acc. NR_113266 and NR_040887) and *Paenibacillus dentritiformis* (Acc. NR_042861) supported by a bootstrap value of 81% and sequence identities of 89, 89 and 90%, respectively for D8 and 88, 92 and 94% for S8,

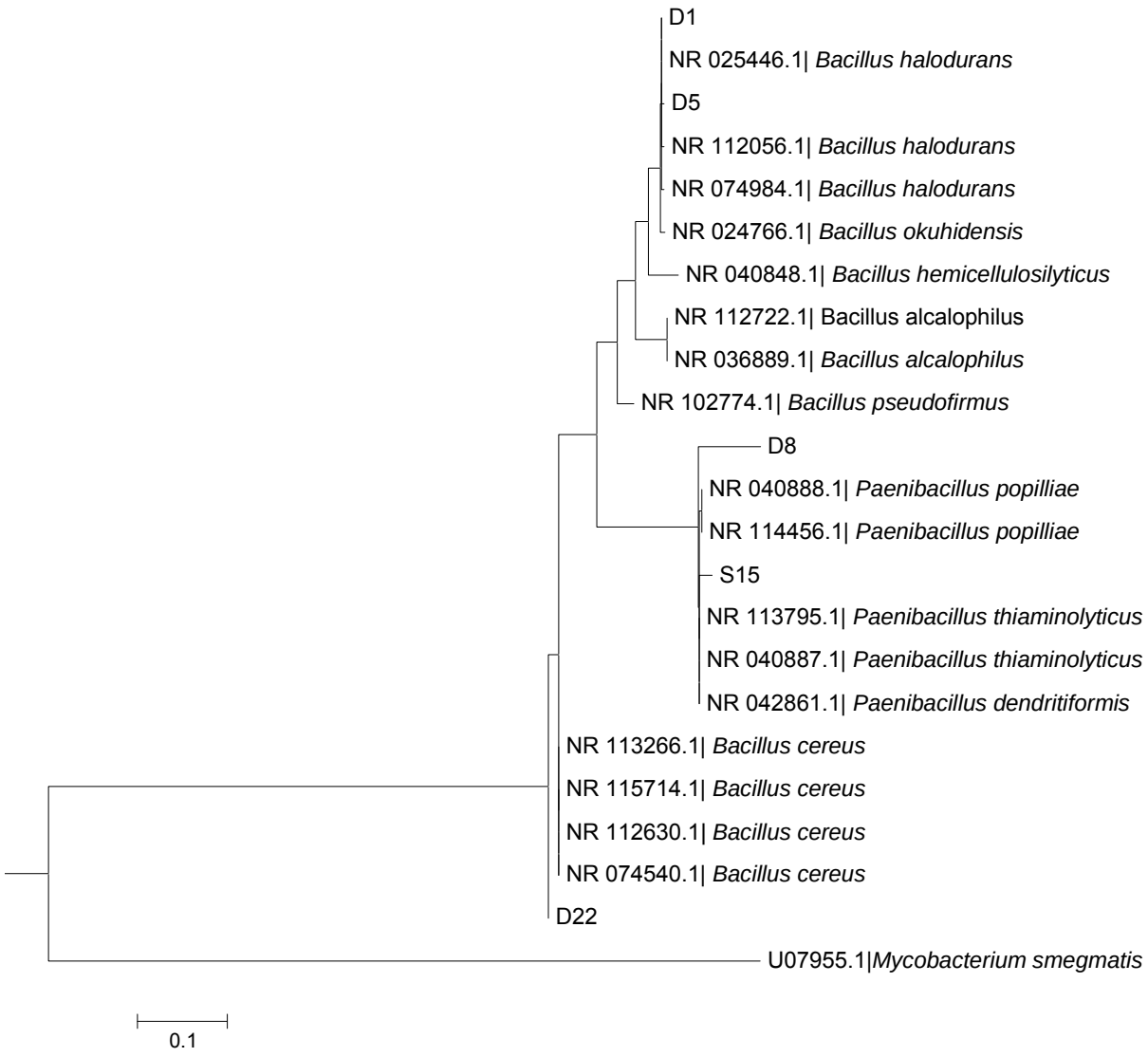

Figure 1. A maximum likelihood tree based on partial 16S rDNA gene sequences showing the phylogenetic relationships between the isolates with antibacterial activity against standard ATCC bacteria and related to *Bacillus* species. The tree is drawn to scale, with the branch lengths measured in the number of substitutions per site. The 16S rDNA sequence of *Mycobacterium smegmatis* was used as an outgroup.

respectively. D22 clustered with *B. cereus* group with a bootstrap value of 11% and sequence identities of 93%.

DISCUSSION

The main goal in this research was to bioprospect for thermophilic Bacilli from selected hotsprings in Lake Bogoria and to characterize those with antibacterial activity using morphological, biochemical and molecular methods. The results obtained showed the presence of a wide variety of bacteria in these environments predominantly Gram-negative rods. However, our main interest was focused on Gram-positive rods as they represent the group of interest, the Bacilli. Sampling was done on both surface (denoted as S) and sediment (denoted as D) samples. We found that more growth was obtained from samples obtained from below the surface. This region was warmer than the surface. However, we could only speculate that Bacilli preferred living in the warmer waters below the surface, since extensive literature search did not reveal any theory to support this.

Different scientists have reported inhibition of various organisms by microbial products produced by *Bacillus* species. Marahiel et al. (1997) isolated a strain of *B. subtilis* C126 from sugarcane fermentation that produced a polypeptide antibiotic, bacitracin, which inhibited the growth of *Micrococcus flavus.* A *Bacillus licheniformis* strain, 189,

isolated from a hot spring environment in the Azores, Portugal, was found to inhibit strongly growth of Gram-positive bacteria by producing peptide antibiotic (Mendo et al., 2004), further confirming the significance of extreme environments as sources of antimicrobials.

Initial screening in the present study was carried out at 37°C against four standard ATCC organisms. Inhibition was achieved against *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922. No inhibition was noted against *S. aureus* ATCC 29213 and *E. coli* ATCC 35218 using the disk diffusion method. Diameters of zones of growth inhibition were approximated and taken to be related to susceptibility of isolates to the extracts (Wayne, 2009).

The crude extract antimicrobial profile we obtained against the standard ATCC organisms agrees with the findings of another study by researchers Maria et al. (2006), who also found strain ATCC 35218 to be resistant and strains ATCC 25922 and ATCC 27853 to be susceptible to phenolic extracts obtained from apple fruits. *S. aureus* ATCC 29213 was resistant to all extracts. In a different study, the effect of a “non-antibiotic” trimebutine was investigated against reference ATCC organisms (Kountouras et al., 2012). It was noted that the MIC and MBC were similar for *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 whereas for *S. aureus* ATCC 29213 and *E. coli* ATCC 35218 the MBC was higher. Five of the nine isolates showed inhibition against the standard organisms used indicating that the remaining isolates were not able to produce antimicrobial substances effective against any of the tested organisms.

Phylogenetic analysis showed all isolated organisms lied within the Bacilli group. Isolate D1 and D5 were closely related with 96% sequence identity. The two organisms closely grouped with *B. halodurans* (Acc. NR_025446.1) with D1 having a sequence identity of 99% while D5 had a 96% sequence identity showing that the two organisms could be different strains with the organism. The two could morphologically be separated from each other by D1 having raised colonies whereas D5 had flat colonies. *B. halodurans* has been found to contain unique genes and sigma factors that have aided its adaptation to more alkaline conditions (Takami, 2000) and strains of it have been known to produce a two-peptide lantibiotics, haloduracin, with activity against a wide range of Gram-positive bacteria (Lawton et al., 2007). Trent and Wilfred (2009) reported that though Haloduracin was active against Gram-positive bacteria, it had no potency against Gram-negative bacteria. Extracts from isolate D1 and D5 were active against Gram-negative bacteria indicating that they are different from *B. halodurans.*

Isolate D22 clustered with a group of different strains of *B. cereus* with a sequence identity of 93%. Typical *B. cereus* colonies are large, raised and opaque with undulate margins and lack the ability to split indole from the amino acid tryptophan (Wong et al., 1988). The colonies of isolate D22 were flat and the organism degraded tryptophan showing it to be different from typical colonies of *B. cerus.* Strains of *B. cereus* have been known to produce a few antibiotics such as zwittermicin A (Laura et al., 1994) that has a wide spectrum of activity (Laura et al., 1998) including against diverse Gram-negative bacteria and certain Gram-positive bacteria.

Isolate S15 was the only isolate obtained from surface water that had any inhibitory effect against the standard organisms. The isolate clustered with D8 with a sequence identity of 88% showing that their relation is significant. Isolate D8 clustered with *P. thiaminolyticus* (Acc. NR_113266 and NR_040887 and *P. dentritiformis* (Acc. NR_042861) with sequence identities of 89, 89 and 90% respectively, whereas S15 clustered with the same organisms with sequence identities of 88, 92 and 94% respectively. Both D8 and S15 produced cream colored colonies with undulate margins and the Gram appearance presented small Gram-positive rods. D8 had irregular colonies whereas S15 had spreading colonies. D8 and S15 are able to produce the enzyme catalase, hydrogen sufide and indole indicating that they could be different trains of the same organism. *Paenibacillus*, a genus initially included in the genus *Bacillus* was reclassified as a separate genus in 1993 (Priest and Collins, 1993). Like D8 and S15, *Paenibacillus* are Gram-positive endorespore forming bacteria (Ash et al., 1991) that develop complex colonies with intricate architectures (Ingham and Jacob, 2008).

The five isolates that showed antimicrobial activity are likely to be potential candidates for discovery of novel bioactive molecules for bio-control and biotechnological applications. The isolates present novel strains that should be investigated further to determine their identity, biochemical and biotechnological properties. Indeed the isolates that have active antimicrobial properties could be developed as good biological control agents.

Conflict of interests

The author(s) did not declare any conflict of interest.

ACKNOWLEDGEMENTS

The authors are grateful to Kenya Wildlife Service for allowing them to collect samples in Lake Bogoria, which is a protected site in Kenya and for providing technical assistance. They are also indebted to the National Council of Science, Technology and Innovation for their technical and financial support. This work was supported by Kenyatta University Departments’ of Biochemistry and Biotechnology and Medical Laboratory Sciences.

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