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RESEARCH ARTICLE OPEN ACCESS

Isolation and Molecular Characterization of Antibiotic Producing *Bacillus licheniformis* Strains Isolated from Soil

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Abstract

Currently, there is an increase prevalence of antibiotic-resistant bacteria worldwide. Therefore, the need for characterization of naturally occuring antibiotics with less antibiotic resistance is required. Soil resources contains valuable antibiotic producing microorganisms that increasingly being utilized for the production of suitable antibiotics. Therefore, this study aimed at identifying an antibiotic bacteria with ability of producing antibiotic that is isolated from soil samples collected from Al Zarqa provenance, an arid area in Jordan. Morphological and biochemical characterization of the isolates were carried out and found that all of the isolates belong to *Bacillus* genus. Further confirmation of the characterization of the bacteria was done by ribosomal RNA and PCR. The results reveal that the isolates represent *Basilluslicheniformis*. These bacilli were further investigated for antimicrobial activities against 6 ATCC human pathogens viz., *S. aureus*, *S. pneumonia*, *Salmonella typhi*., *E. coli, P. mirabels* and *E. cloacae*. Additionally, the results of Gas Chromatography Mass Spectrometry (GCMS) of ethyl acetate extracts for *B. licheniformis* secondary metabolites showed that they contain two main antimicrobial compounds namely Pyrrolo [1, 2-a] pyrazine-1, 4-dione,hexahydro and Trans-13-octadecenoic acid. The present work maybe suggests that soil isolates from the studied arid area include antibiotic producing strains that can be utilized commercially.

Keywords: *Bacillus*, Antibacterial activity, 16S rRNA, GCMS

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INTRODUCTION

The field of medicine and pharmacology are currently revolutionized thanks to the invention of antibiotics that successfully that has been fruitful in combating many infectious diseases. However, many infectious microbes have become increasingly resistant to most available antibiotic due to mainly the extensive these antimicrobial medicine[1]. Varied genetic mechanisms, such as mutation, genetic transfer, and epigenetic, have been adopted by resistant pathogens to make them successful adapted to antibiotics[2]. Antibiotics and antimicrobial metabolites extracted from microorganisms that inhabit environmental habitats, such soil, are providing an important role in fighting microbial disease and shown to be a promising source of new novel antibiotics[3]. Natural soil in general has a high biodiversity of bacteria and provide an important resource for these microbial diversity for possible novel antibiotics[4]. In fact, several medicinally new antibiotics have been characterized and identified from bacterial that inhabit varied natural soil, isolated from different soil samples[5,6,7]. The *Bacillus* is a heterogenous genera of bacteria with species that contain enormous antimicrobial compounds that act as agent of fighting several microbial diseases[8]. Among the most important species of bacteria that produce medically importan antibiotics are beloging to the genus *Bacillus*[9]. These species and other of *Bacillus* are among the most abundant bacterial strains found in soil with the ability of endospore-forming and Gram positive bacteria. Many investigations have been carried out to isolate different strains of terrestrial *bacillus* and identify their antimicrobial activities. This study attempts using biochemical and 16S rRNA to identify the microbial isolates from an arid are, Al Zarqa, of Jordan and to determine their antimicrobial activities.

MATERIAL AND METHODS

Microorganisms

Based on the study of Massadeh and Mahmoud[10], new isolates of antagonistic strains were gathered from soil that was collected from the Hashemite University Campus area and identified as *Bacillus* species. Three isolates were chosen for further screening of antimicrobial activities and possible production of secondary metabolites. Each isolate was maintained on nutrient agar slants, and preserved at 4°C.

Isolates identification

The isolates were characterized to the genus level by based on their morphology and biochemical characteristics[11]. The culture characteristics include colony morphology (size, opacity, form, elevation and margins), Gram reaction, spore formation, motility test, and Biochemical reactions. Further identification to the species level was achieved by using Microgen *Bacillus*-IDkits (Microgen bioproducts, UK) provided with Microgen identification software (MID-60).

Molecular identification of the isolates

DNA extraction

The DNA of each bacterial strains was isolatedfrom a 24 hours old pure culture based on methodology of bacterial DNA extraction kit (Wizard®, Promega, USA). The DNA was kept frozen until sequencing.

16S rRNA gene amplification and sequencing

Two universal PCR primers were manufactured to amplify approximately 1,300 bp a region of 16S ribosomal RNA gene: the forward primer 63f (5`-CAG GCC TAA CAC ATG CAA GTC-3`) and the reverse primer 1387r (5`-GGG CGG WGT GTA CAA GGC-3`).The PCR mixtures contained approximately 2 µl of DNA and 1 µl of each primer and 12.5 µl of DNA master mix (Promega, USA) and about 8 µl of double distelled water. The reactions of PCRwere performed with a cycler (biorad thermal cycler). The cycler was programmed to perform 94°C for 30 min, followed by 25 cycles consisting of 94°C for 45 s, 55°C for 45 s, and 72°C for 90 s. followed by a final extension step of 10 min. at 72°C. PCR products were visualized by 1% agarose gel electrophoresis. The PCR product was properly labeled and sent to Princess Haya Biotechnology Center (Irbid-Jordan) for being identified by sequencing of the PCR products.

Antibacterial activity

Cell-free extract preparation

Each isolate was cultivated in a flask containing a 100ml of nutrient broth. The flasks were incubated at 30°C in a shaking incubator running at 150 rpm for 3 days and then subjected to centrifugation and then filltered with standard memebrane.

Ethyl acetate extract preparation

To concentrate the isolates metabolites, each extract was mixed with ethyl acetate at a ratio of 4:1. The mixture was agitated at 100 rpm for 2 hrs in an incubator shaker (Human Lab, Korea). The organic phase was concentrated by evaporating the ethyl acetate at 45°Cand the resulting extract was stored at 4°C until use[10].

Antimicrobial test

The inhibitory effect of each bacterial extract was tested against different pathogens using agar-well diffusion method. The tested pathogenic microorganisms include 2 Gram-positive bacteria (*Streptococcuspneumonia* ATCC 6303 and *Staphylococc*us *aureus* ATCC 11632) and 4 Gram-negative bacteria (*Proteus mirabilis* ATCC, *Enterobactercloacae* ATCC 13182., *Escherichia coli* ATCC 10145, and *Salmonella typhi*. ATCC 13076). A suspension of each test pathogens (0.1 ml) of an $O.D_{600}$ of 0.4 was spread on NA plates, and wells of 6mm. A 40 µl of extract was placed into the wells, and the plates were placed under 37°C for about 24 hours. The diameter of affected zone was estimated with calipers as mm. The antibiotic Ciprofloxacin (5 mcg) was used as a control.

Characterization of antibacterial metabolites

Proteolytic digestion

For testing proteolysis of metabolites was employed with a slight modification accordingto the procedure describe eleswhere[12]. One milliliter of eythl acetate extract showing antibacterial activity was subjected to proteolytic digestion using a 100 mg/ml proteinase K. The samples then were incubated overnight at 56°C. After incubation, samples were tested for antibacterial activity by the agar well diffusion method and a sample of ethyl acetate extract without proteinase K was used as a negative control.

Thermostability test

Each extract was incubated at 80°C for 1 hr. and the tested for antibacterial activity by the agar welldiffusion method. A sample of ethyl acetate extract without heat treatment was used as a negative control.

Detection of antibacterial compounds by Gas chromatography/Mass spectrometry (GC-MS)

Antibacterial metabolites in ethyl acetate extract were characterising using GC-MS by inserting a 1 µl solution into an Agilent 6890 GC detector GC-MS. The temperature level procedure was set at 80°C for about 3 min and then was increased to 80 to 500°C. Chemistation system was used to compare the percntage of mass to charge using standared spectrum library[13].

RESULT AND DISCUSSION

Morphological characteristics and biochemical activities

The major aim of current research was to identify and characterize bacterial strains isolated from soil Al Zarqa arid area that has the ability of producing natural antibiotic. The soil in this area was chosen as it contains large bacterial community with potential of antibiotic production[10].

The results revealed 3 isolates with mainly yellow-white opaque morphological characteristic for studied colonies in accordance with similar studies that suggested soil as an important natural resource of medically important bacteria[14]. In agreement with similar the resultsthe biochemical tests of the four isolates revealed that all isolated were positive for catalase production[15]. Additionally, Microgen *Bacillus*-ID identified 3 possible isolates with varied percentage of probability: *B. subtilis*, 82.7%, *B. licheniformis* with both 37.9% and 80.2% (Table 1). Moreover, the results showed that are positive to citrate utilization and have significant capability of fermentation of the sugars cellobiose, mannitole, mannose, salicin, sucrose, trehalose and xylose (Tables 2).

Table 1. Identification of species of the four isolates by Microgen Identification System Software (MID-60)

Isolate	Identification	Percent probability	Identification comments
1	*B. subtilis*	82.69%	Acceptable Identification, additional tests may improve the identification
2	*B. licheniformis*	37.94%	Identification to the species level will require additional tests
3	*B. licheniformis*	80.18%	Acceptable Identification

Genetic characterization of bacterial strains by using ribosomal RNA

To confirm the identification and the classification of the studied strains all isolates were subjected to advanced identification using 16S rRNA sequencing[16]. DNA of each isolate was extracted, amplified in PCR, checked in electrophoresis and finally sequenced. Fig. 1 and Table 3 shows the main products of gel electrophoresis and identification according to 16S RNA sequencing. Moreover, a phylogenetic tree, constructed by Neighbor-joining, showed a four major group of *Bacillus* strains that clustered based on their sequence similarity of the 16S rRNA gene from all strains[17] (Fig. 2). This clustering method is preferable to the conventional biochemical tests as it reveals the identification of isolates within 2-3 days compared to several weeks[18]. In agreement with several previous reports our results revealed that ribosomal RNA analysis is an efficient method for classification of bacteria and provide support for earlier studies which have shown that *Bacillus* species as common bacteria occur in soil[19].

Table 2. Biochemical reactions of using the test of Microgen Biochemical ID Software Microgen GN-A(after 24 h)

Reaction name	1	2	3
ARA	+	+	+
CEL	+	+	+
INO	-	+	-
MAN	+	+	+
MNS	+	+	+
RAF	-	-	-
RHF	-	-	-
SAL	+	+	+
BOR	+	+	+
SUC	+	+	+
TRE	+	+	+
XYL	+	+	+
ADO	-	-	+
GAL	+	-	+
MDM	-	-	-
MDG	+	+	+
INV	-	-	+
MLZ	-	-	-
INO	----	----	----
ONPG	+	+	+
ARG	-	-	-
CIT	+	+	+
VP	----	----	----
NIT	----	----	----

Antimicrobial activity

The antimicrobial activity of the 3 isolates was studied against tested human pathogens. The results revealed that the ethyl acetate extract of the 3 isolates of *B. licheniformis* DSM 13 have an inhibitory effect against all test pathogens except for *E. coli* ATCC 10145 and *Salmonella typhi*. ATCC 13076 (Table 4; Fig. 3). It was found that the highest antimicrobial activitywas achieved by the extract *B. licheniformis* DSM 13 (isolate 1) against *S.* Aureus ATCC 11632 where the inhibition zone was 13.33 ± 0.58 mm, while the lowest antimicrobial activity was exhibited by *B. licheniformis* DSM 13 (isolate 2) extract against *E. cloacae* ATCC 13182, *P. mirabilis* ATCC 12453 and *S. pneumonia* ATCC 6303 with inhibition zones of 10.33 ± 0.58 mm (Table 4). Previous reportesindicated that *B. licheniformis* is widely distributed in nature and can readily be isolated from soils[3]. Moreover, *B. licheniformis* has been applied widely for producing extracellular enzymes, antibiotics, and special chemicals with a low risk of adverse effects to human health or the environment[20].

Characterization of ethyl acetate extract of *B. licheniformis* isolates

The extracts were subjected to heat treatment and proteolysis by enzymes. The results revealed that heat treatment of the etheyl acetate extracts of the 3 isolates did not reduce the antibacterial activity. The activity could still be demonstrated even after subjecting the extracts to 80°C for 1 hr. Some reportes indicated that the thermal stability of many antibacterial

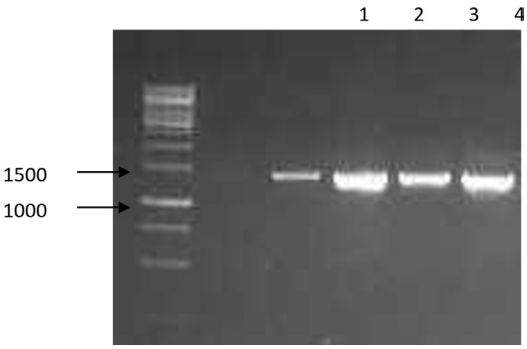

Fig. 1. PCR product with primers 63f and 1387r

compounds isolated from *B. licheniformis*[21,22]. Other, hovere, claimed that the most metabolites of *Bacillus* sp. were proved to be stable at different temperatures[23]. On the other hand, incubating the extracts supplemented with Proteinase K did not affect the antibacterial activity of the extracts of the 3 active isolates suggests that the active compounds are not proteinaceous in nature.

GCMS analysis

Chemical investigations for the 3 ethyl acetate extracts based on GCMS indicated the identification of 2 major compounds (Table 5): (1) Pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro with a retention time of 12.397 min. It was evident that this cyclic peptide is produced by some *Streptomyces* strains and sponge-associated

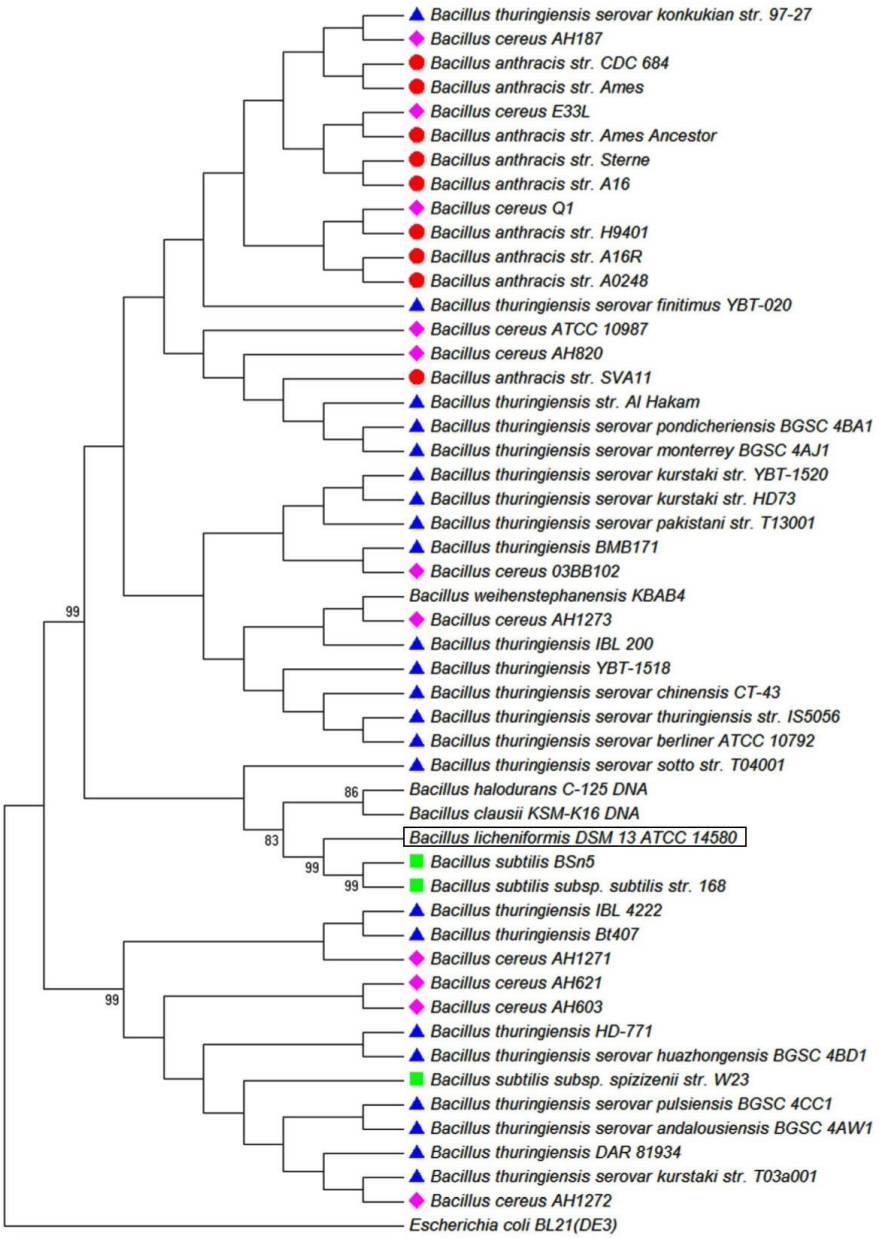

Fig. 2. Phylogenetic tree constructed by Neighbor-joining based on the sequences of the 16S rRNA gene from 50 *Bacillus* strains (Wang and Ash, 2015)

marine bacteria, in addition to some endophytes. Some researchers reported the isolation of *Streptomyces* strain MUSC149^T mangrove soil with a strong antioxidant activity[24]. The chemical analysis this strain's extract revealed the identification of Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro as the antioxidant agent. On the other hand, some researchersidentified the presence of this compound in the extract of an Antarctic endophytic fungus exhibiting a strong antibacterial activity against *E. coli, Pseudomonas aeruginosa* and *Enterococcus faecalis*[13]. Moreover, this compound was produced by sponge associated bacteria (SAB) that was capable of inhibiting *Vibrio alginolyticus*[25]. (2) Trans-13-octadecenoic acid with a retention time of 16.127 min. this Trans fatty acid was detected in tuber extracts of *Solena amplexicaulis* plant and has been reported as anti-inflammatory and cancer compound[26].However, other scientists identified this compound as an anti-inflammatory and antimicrobial compound[22].

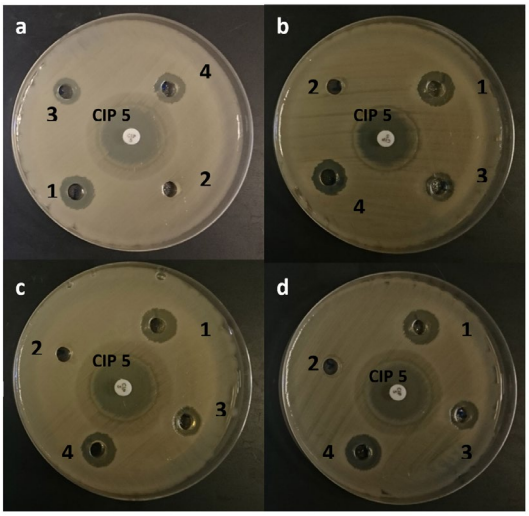

Fig. 3. Antimicrobial activity of the 3 isolates against the test pathogens: In this figure: a: *Enterobacter* sp., b: *P. mirables*, c: *Staphylococcus* sp., d: *Streptococcus* sp. No. 2 well and CIP 5 (Ciprofloxacin 5 µg/disc) were used as a negative and positive control.

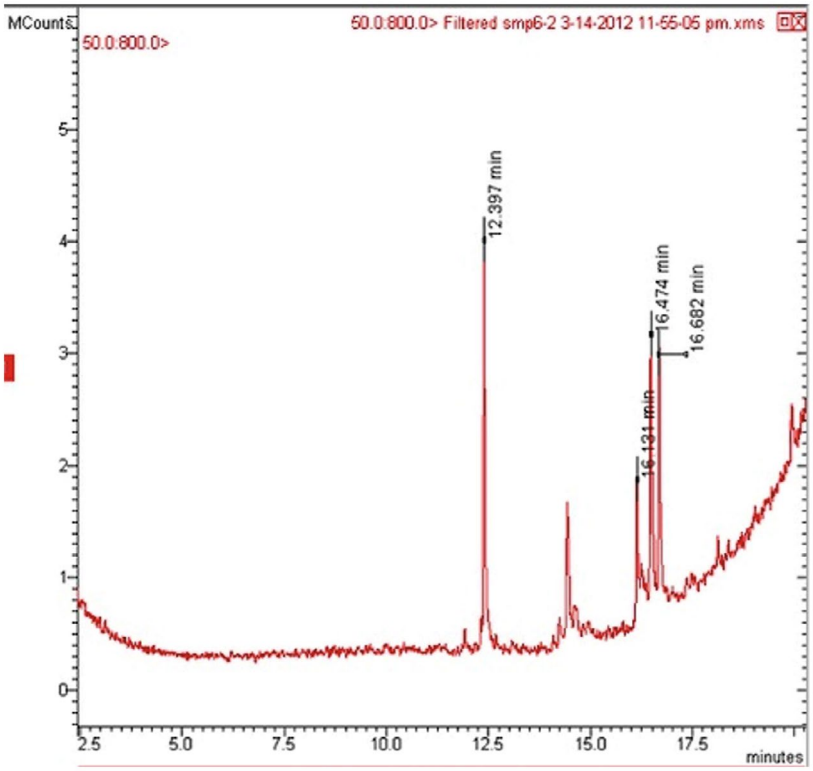

Fig. 4. Chromatogram showingresults of the major compounds from the active isolates.

Table 3. Classification of bacterial strains according to 16S rRNA (results obtained from BLAST/NCBI)

Isolate	Classification	Ident	Accession No. on BLAST
1	*B. licheniformis DSM 13*	87%	NR 118996.1
2	*B. licheniformis DSM 13*	90%	NR 118996.1
3	*B. licheniformis DSM 13*	97%	NR 118996.1

Table 4. Antimicrobial activity of the three isolates of *B. lichinifomis* against test pathogens

Pathogen	Diameters of inhibition zone (mm) by the four isolates			
	1	2	3	CIP 5
E. cloacae ATCC 13182	13.00 ± 1.00	10.33 ± 0.58	12.33 ± 0. 58	21.33 ± 0.58
P. mirabilis ATCC 12453	12.67 ± 0.58	10.33 ± 0.58	11.67 ± 0.58	22.00 ± 1.00
E. coli ATCC 10145	0	0	0	0
Salmonella typhi ATCC 13076	0	0	0	0
S. pneumonia ATCC 6303	12.67 ± 0.58	10.33 ± 0.58	11.67 ± 0.58	20.33 ± 0.58
S. aureus ATCC 11632	13.33 ± 0.58	11.67 ± 0.58	12.67 ± 0.58	22.00 ± 1.00

Table 5. Gas chromatogram results of the major compounds of the three active isolates

isolate	Compound	Retention time (min)
1	Pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro	12.397
	Trans-13-octadecenoic acid	16.127
2	Pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro	12.397
	Trans-13-octadecenoic acid	16.127
3	Pyrrolo [1, 2-a] pyrazine-1, 4-dione, hexahydro	12.397

CONCLUSION

The miss use of antibiotics has led to the evolution of pathogens with resistance to major available antibiotics. Accordingly, natural habitat such as soil are emerging procedure for the synthesis of new, safe and developed antibiotics. Three antagonistic bacterial strains were isolated from the soil of an arid area of Hashemite University and identified to be *B. licheniformis* based on biochemical tests and 16S rRNA gene sequencing with antibacterial activity against important human pathogens. Our finding promote that the bacterial strains obtained from the studied soil sables can be exploit commercially. Based on our study, we recommend further research that aim to use MIC to determine antibacterial activities, in addition to molecular docking and in *vivo* investigations to identify active compounds.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All Authors contributed equally to literature collection, designing and writing the manuscript.

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ETHICS STATEMENT

Not applicable.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

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