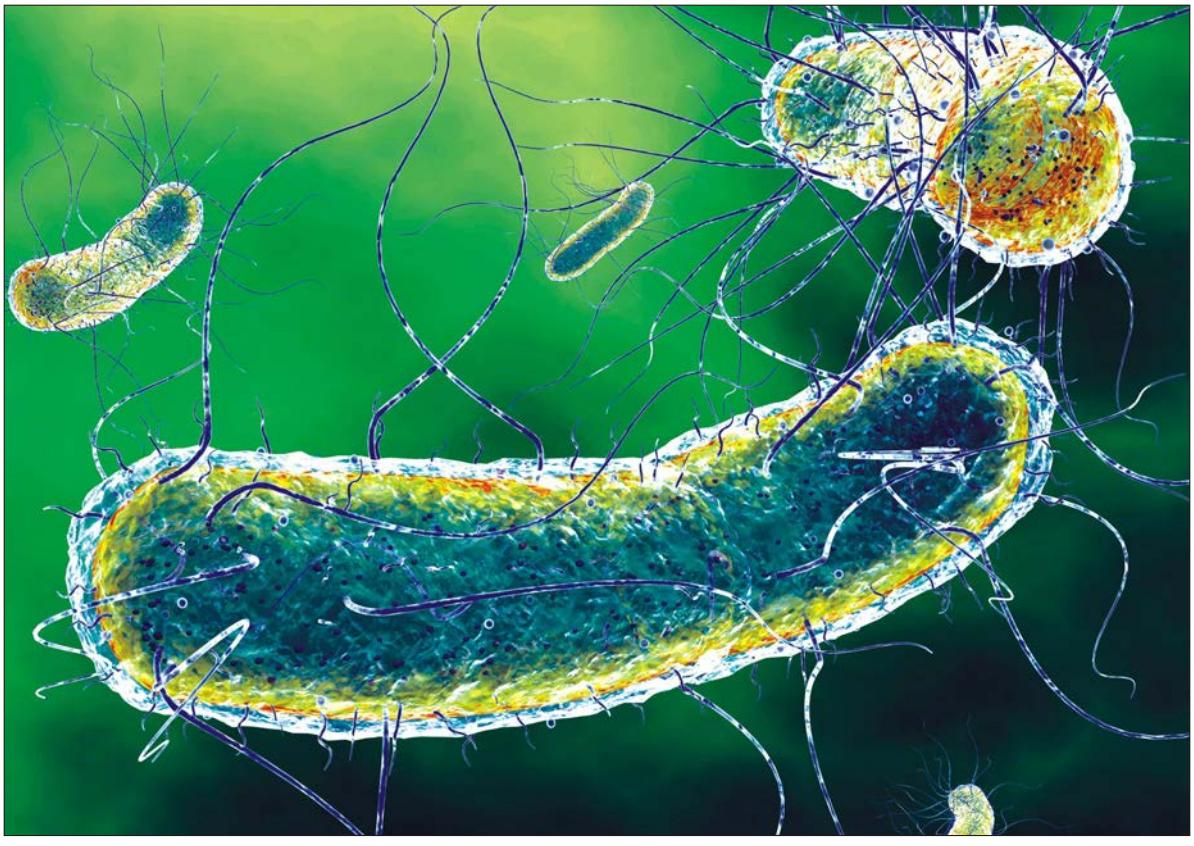

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Bacillus sp. 1A1 as a Producer of Antibacterial Crude Extract: Taxonomy, Cultivation and Partial Purification

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ABSTRACT

Despite the availability of modern medicine, number of new infections and resistant bacterial strains is escalating. As a large number of soil bacteria are currently unknown, they represent a promising source for new bioactive natural products. *Bacillus* sp. 1A1 was isolated from a soil sample, identified, cultivated and its crude extract and purified fractions were evaluated for their potent antibacterial activities. It was identified based on morphological, biochemical and physiological characteristics as well as on molecular level. The 16S rRNA revealed that *Bacillus* sp. 1A1 is closely related to *B. amyloliquefaciens*. Its crude extract exhibited inhibiting activity against Gram-positive bacteria. Partial purification of the produced crude extract by size exclusion chromatography yielded three antibacterial fractions that caused inhibition zones of 10-20 mm in agar diffusion assay. Fraction S_8 was the most promising fraction that inhibited the growth of tested Gram-positive bacteria with Minimum Inhibitory Concentration (MIC) 31-125 μg mL^{-1}.

Key words: *Bacillus*, antibacterial, crude extract, Jordan

INTRODUCTION

Interestingly, a large number of drugs approved nowadays are based on natural products. Thirteen natural product related drugs were approved from 2005-2007 (Harvey, 2008) and a total of twenty five natural products and natural products derived drugs were approved for marketing from 2008-2013 (Butler *et al*., 2014), some of which are of microbial origin. These compounds are derived from secondary metabolism of these microorganisms (Bérdy, 2005). Moreover, several studies pointed out to the microbial origin of some pharmaceutical active agents that were previously ascribed to macroorganisms (Arai *et al*., 1979; Piel, 2004). Due to their chemical diversity, the activity of these compounds extends beyond inhibition of microbial growth (i.e., antibiotics) to metabolites that act as effectors of ecological competition and symbiosis, enzyme inhibitors, antitumor agents and growth promoters of animals and plants (Demain, 1998). Nevertheless, new infections and resistant pathogens are still emerged. Therefore, the need for novel therapeutically active compounds is urgent.

As less than 1% of soil bacterial species are currently known, soil represent a large untrapped pool for discovering new natural products (MacNeil *et al*., 2001). Among the interesting genera is *Bacillus*. Various reports confirmed the ability of *Bacillus* members to produce antibacterial, antifungal, antiviral and compounds with potential biotechnological and pharmaceutical

applications (Awais *et al*., 2007; Hassan *et al*., 2014). Herein, isolation and cultivation of *Bacillus* sp. 1A1 is described as well as the activity of its crude extract and the partially purified fractions against selected bacterial test strains.

MATERIALS AND METHODS

Isolation of producing organism: *Bacillus* sp. 1A1 was isolated from soil sample collected from Al-Mazar (south Al-Karak/Jordan) by serial dilution technique. It has been deposited in the collections of the Department of Biology, Mutah University.

Identification of *Bacillus* sp. 1A1

Morphological, biochemical and physiological characterization: Morphological studies were carried out on culture grown for 48-72 h on nutrient agar plates (Oxoid, U.K) using a stereomicroscope (Nikon/Japan). Gram-staining was examined under a light compound microscope (Olympus CX2, China). Physiological and biochemical characteristics were determined using standard procedures (Baumann *et al*., 1972; Bowman *et al*., 1997; Corstvet *et al*., 1982; Schaeffer and Fulton, 1933; Steel, 1961). Production of acid from different carbohydrates was carried out as described by Helmke and Weyland (1984).

Sequencing and analysis of 16S rRNA: Genomic DNA was prepared using the EZ-10 Spin Column Genomic DNA kit according to the manufacturer's instructions (Bio Basic Inc. Canada). The 16S rRNA was amplified using Polymerase Chain Reaction (PCR) as mentioned previously (Al-Zereini *et al*., 2007). The primers were synthesized by MWG-biotech (Ebersberg, Germany). The PCR product (~1500 bp) was detected by electrophoresis (Sambrook and Russell, 2001) and purified with E.Z.N.A Gel Extraction Kit (Omega Bio-Tek Inc, USA) following the manufacturer's instructions. The 16S rRNA gene sequence was compared with those available in the GenBank and Ribosomal database project II (RDP).

Cultivation of *Bacillus* sp. 1A1, extraction of culture fluid and partial purification of crude extract: *Bacillus* sp. 1A1 was inoculated in (10×2 L) Erlenmeyer flasks containing 1 L of nutrient broth (Oxoid, U.K) on orbital shaker (130 rpm, Forma Orbital Shaker, Thermo electron cooperation, USA) at 27°C. During cultivation process, 50 mL sample and thereafter daily samples were collected and used to measure bacterial growth parameters. Growth was followed by measuring the increase in Optical Density (OD_{600nm}) (UV. Spectrometer, Lambda 16, Perkin-Elmer, LANGEN) and changes in pH values (pH model 523, WTW, Germany). The cultivation was stopped directly after the OD_{600nm} value decreased. The cells were separated from the culture fluid by centrifugation (8000 rpm, 15 min, Sorvall® RC-5B Refrigerated super speed, Dupont company/USA). The supernatant was adjusted to pH 8 and extracted with an equal volume of ethyl acetate. The organic phase was dried over sodium sulfate (anhydrous) and concentrated in vacuum evaporator at 45°C (Büchi Rotavapor R-215, Switzerland). The resulting crude extract was dissolved in methanol to 50 mg mL^{-1} and stored at 4°C.

The crude extract was partially purified by size exclusion chromatography (Sephadex LH-20, column size 3×35 cm) and eluted with methanol and dichloromethane (9:1) under atmospheric pressure. The 10 mL fractions were collected and monitored by Thin Layer Chromatography (TLC). The fractions with similar bands migration pattern on TLC were combined together and concentrated in vacuum evaporator at 45°C.

The resulting combined fractions were re-spotted on TLC plates, detected using UV light at 254 and 366 nm. Moreover, developed spots were visualized with ninhydrin (2,2-dihydroxyin-1,3-dandion; 0,1% spray-reagent) with warming at 120°C for the detection of amino acids, bromocresol green (0.1 g bromocresol green in 500 mL ethanol and 5 mL 0.1 M NaOH) for the detection of organic acid and bromothymol blue (0.1% bromothymol blue in 10% aqueous ethanol) for detection of lipid and phospholipids (Touchstone, 1992).

***In vitro* antibacterial activity:** Antibacterial activity of the bacterial crude extract and partially purified fractions was determined by agar diffusion technique and serial dilution assay using standard methods as mentioned in National Committee for Clinical Laboratory Standards in 2004 with modifications described by Al-Zereini (2014).

The test microorganisms used in present work were the Gram-positive bacteria *Staphylococcus aureus* (ATCC 43300), *Bacillus subtilis* (ATCC 6633), *Micrococcus luteus* (ATCC 10240) and the Gram-negative bacteria *Proteus vulgaris* (ATCC 13048), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883) seeded on LB agar plates (0.5% tryptone, 0.5% yeast extract, 1% NaCl, 1.8% agar). Aliquots of 500 μg/disc of crude extract and 200 μg/disc of partially purified fractions were used in agar diffusion test, while concentrations starting from 200 μg mL^{-1} of silica gel fractions and 100 μg mL^{-1} of positive control (Penicillin G) were used in serial dilution assay to deduce the Minimum Inhibitory Concentration (MIC). All assays were performed in triplicate and mean values were presented.

Evolutionary analyses: Evolutionary analyses were conducted in MEGA6 free software. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura and Nei (1993).

RESULTS

Strain 1A1 formed white circular colonies with entire margin on nutrient agar. The colonies became transparent after 7 days of cultivation. It was found to be Gram positive, endospore-forming, capsulated bacilli. The cells are 1.7-3 μm long and 1-1.2 μm wide. It is oxidase negative and catalase positive. Reduce nitrate into nitrite. The biochemical and physiological characteristics are listed in Table 1.

16S rRNA sequence of this isolate revealed that strain 1A1 is closely related to *Bacillus* bacteria (97-98% similarity level). The depicted phylogenetic tree showed that strain 1A1 has highest homology with *Bacillus amyloliquefaciens* strain MPA 1034 (98% similarity level, access No. NR_117946) (Fig. 1).

Cultivation of *Bacillus* sp. 1A1 varied between 72-96 h. During which, the pH value was increased up to 8.7 at the end of cultivation period. The antibacterial activity against *M. luteus* could be detected early in the logarithmic growth phase, after 12 h, reaching the maximum at the beginning of the stationary phase with no significant decrease during prolonged incubation (Fig. 2). Extraction of culture fluid gave rise to 30 μg mL^{-1} of crude extract. Applying of 500 μg/disc of the resulting crude extract caused 17-21 mm inhibition zones in tested Gram-positive bacteria. Gram-negative bacteria were resistant to the tested extract.

Partial purification of the bacterial extract (0.3 g) by size exclusion chromatography yielded four fractions: S_1 (2 mg), S_2 (15 mg), S_3 (60.6 mg) and S_4 (5.7 mg). Fractions S_1-S_3 exhibited antibacterial activity against susceptible Gram-positive bacteria with S_3 being the most potent bioactive metabolite (MIC 31-125 μg mL^{-1}) as shown in Table 2 and 3.

Table 1: Biochemical characterization of *Bacillus* sp. 1A1

Test	Experimental result
Oxide/Ferm Oxidation/Fermentation	
D-Glucose	+/+
Sucrose	+/+
Sorbitol	+/+
Manitol	+/+
Maltose	+/+
D-Xylose	+/+
Organic compounds as carbon source	
Sodium acetate	+
Glycine	-
L-Tyrosine	-
Sodium pyruvate	+
Organic compounds as carbon and nitrogen source	
L-Leucine	-
L-lysine	-
L-Histidine	+
L-Asparagine	-
L-Tyrosine	-
L-Phenylalanine	-
Hydrolysis	
Starch	+
Gelatine	+
DNA	+
Reduction of nitrate to nitrite	+
Production of H_2S	-

Table 2: Inhibition zones caused by 200 µg/disc of bioactive sub-fractions from *Bacillus* sp. 1A1 crude extract against the susceptible bacterial strains in the agar diffusion test

	Inhibition zone (mm)		
Compounds	*M. luteus*	*S. aureus*	*B. subtilis*
S_1	10	9	8d
S_2	.	.	13d
S_3	20	8	15

d: Diffused inhibition zone

Table 3: Minimal Inhibitory Concentration (MIC) of the bioactive sub-fractions in the serial dilution assay against the susceptible bacterial strains

	MIC (µg mL^{-1})		
Compound	*M. luteus*	*S. aureus*	*B. subtilis*
S_1	1000c	1000c	1000s
S_2	>1000	>1000	500s
S_3	31c	1000s	125c
Penicillium	<8	62.5s	<8

s: Bacteriostatic, c: Bactericidal

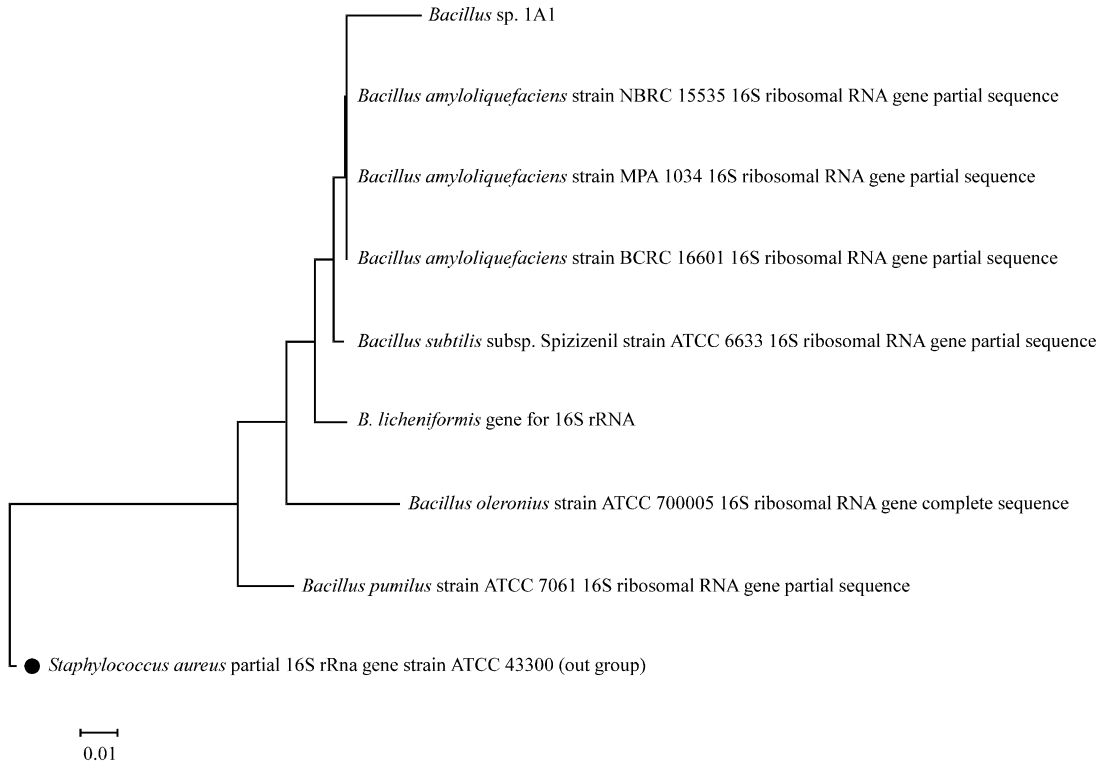

Fig. 1: Phylogenetic tree of strain 1A1, reconstructed by using the maximum likelihood algorithm based on the 16S rRNA gene sequence of strain 1A1 and related members of the *Firmicutes* phylum. The scale bar indicates 1% estimated base substitution per nucleotide position

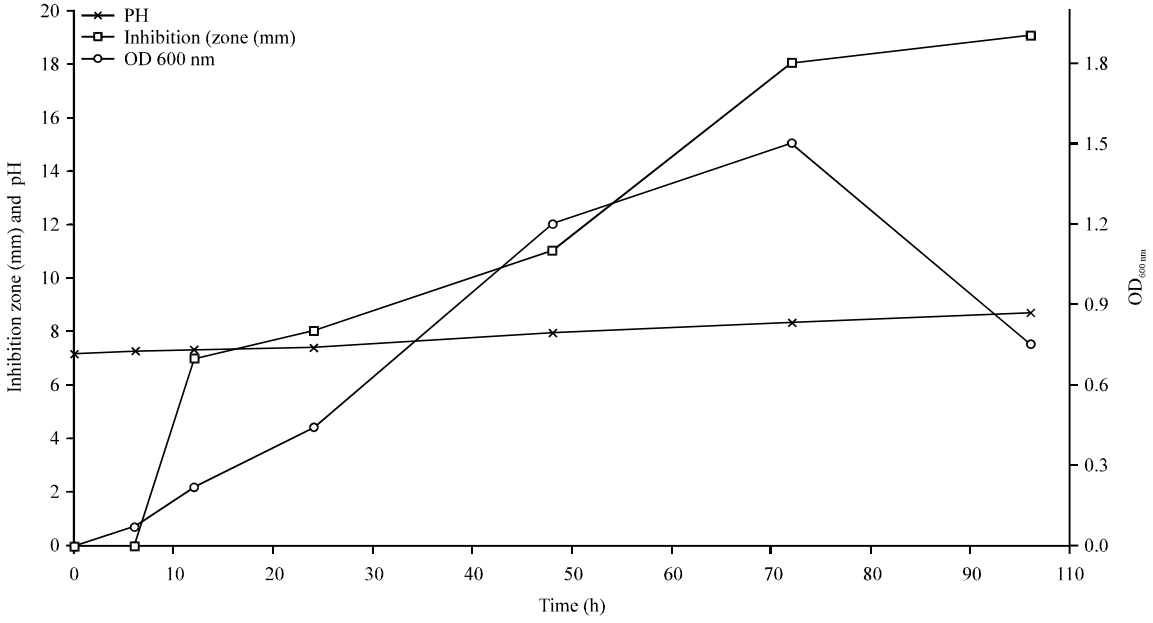

Fig. 2: Cultivation parameters of *Bacillus* sp. 1A1 in nutrient broth medium. Changing in pH values and increase in cell density ($OD_{600\ nm}$) are shown with the inhibition zone caused by 500 µg/disc of applied crude extract against *M. luteus*

DISCUSSION

In this study the authors were interested in obtaining bacterial isolates able to produce bioactive crude extracts. Isolated soil bacterial strain *Bacillus* sp. 1A1 produced metabolites that exhibited antibacterial activity, mainly against Gram positive test strains. Based in 16S rRNA sequence it was found to be related to *B. amyloliquefaciens*, a species name given and revised by Priest *et al.* (1987) with high affinity to *B. subtilis*. Although percentage of similarity between both strains reach to 98% but the isolated soil strain was able to utilize D-xylose and hydrolyze DNA. *Bacillus amyloliquefaciens* was found to be neither able to utilize this type of sugar nor degrade DNA (O'Donnell *et al.*, 1980; Priest *et al.*, 1987). Therefore, strain 1A1 is a distinct species in the genus *Bacillus*.

Bacillus sp. 1A1 produced bioactive crude extract that through bioactivity-guided fractionation resulted in having three bioactive sub-fractions. S3 fraction was the only promising fraction that may harbor metabolites causing pronounced inhibition for growth of tested Gram-positive bacteria. On the other hand, Gram-negative test strains showed resistance to applied metabolites which may be attributed to presence of the low permeable outer membrane and the lipopolysaccharide barrier for the hydrophobic compounds.

In accordance with the present results, it was documented that *Bacillus* species are considered a source of interesting bioactive secondary metabolites and the most promising candidates for microbial biocontrol agents (Arguelles-Arias *et al.*, 2009; Benitez *et al.*, 2012). Several antibacterial agents were isolated and identified from several *Bacillus* species among which were the *B. subtilis* group of bacteria including *B. amyloliquefaciens* (Cao *et al.*, 2011; Wulff *et al.*, 2002). Moreover, they are known as a source of antifungal compounds (Athukorala *et al.*, 2009).

Bacillus strains may produce a large number of antimicrobial peptides with different chemical structures, such as bacteriocins, bacteriocin-like substances and lipopeptides (Balhara *et al.*, 2011). Lipopeptide antibiotics like iturin A and surfactin were reported to have antagonistic and repressive activity over plant pathogens; Bacteriocins like subtilin from *B. subtilis* and subtilosin A from some strains of both *B. subtilis* and *B. amyloliquefaciens* are ribosomally synthesized antimicrobial peptides that inhibit or kill microorganisms. The production of such agents was reported to be growth phase dependents. For instance surfactin and iturins are typical secondary metabolites produced during the stationary growth phase. During this work, the production of bioactive crude extract started early in exponential phase which coincided with production kinetics observed for subtilin, subtilosin and bacteriocin-like substances that were synthesized by different species of *Bacillus* during the exponential phase (Barboza-Corona *et al.*, 2007; Stein, 2005). Nevertheless, spraying of TLC plate with chromogenic reagents indicated none of partially purified fraction was amino acid, organic acid or phospholipids in nature. Consequently, this work confirmed that *Bacillus* species are still considered as a proliferative agent for antimicrobial compounds.

CONCLUSION

This study confirmed that *Bacillus* species are still considered as a proliferative agent for antimicrobial compounds. They could be used as a producer of antimicrobial and biocontrol agents in pharmaceutical and agricultural fields.

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