Arab Gulf Journal of Scientific Research, 21 (3), pp 134- 139 (2003)

ORIGINAL PAPER

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Antimicrobial Substances Produced by Air Flora

Abstract. Bacterial strains identified as *Bacillus megaterium* NB-3, *Bacillus cereus* NB-4, *Bacillus cereus* NB-5, *Bacillus subtilis* NB-6 and *Bacillus circulans* NB-7, were isolated from the air of Jerash Private University, Jerash, Jordan. They were found to produce several antimicrobial substances that exhibited potent antifungal activity against filamentous fungi and yeasts. *Bacillus subtilis* NB-6 showed activity against filamentous fungi, yeasts and gram-positive bacteria, while *Bacillus megaterium* NB-3 exhibited a broad spectrum of activity against filamentous fungi, yeasts, gram-positive bacteria and gram-negative bacteria. In batch culture, the bacterial isolates produced the antibiotics late in the growth phase and accumulated a main portion in the culture fluids. The data presented in this study exhibit a novel source for the isolation of antimicrobial substances producing microorganisms in Jordan, which were not previously described. The isolation of these strains suggests their potential as a source of novel antibiotics.

Keywords: Air flora, antibiotics, *bacillus*, cereus, circulans, megaterium.

الأحياء الدقيقة المنتجة للمضادات الحيوية الموجودة في الهواء

ناصر البنا

المستخلص: تم عزل عدة سلالات بكتيرية من الهواء في مختبر جامعة جرش الأهلية في الأردن. وعرفت على أنها *Bacillus circulans* NB-7 . NB-4, *Bacillus cereus* NB-5, *Bacillus subilis* NB-6, *Bacillus megaterium* NB-3, *Bacillus cereus* تفرز هذه السلالات البكتيرية عدة مضادات حيوية، ذات نشاط فعال ضد الفطريات والخمائر. *Bacillus subilis* NB-6 تظهر نشاط ضد الفطريات، الخمائر والبكتيريا موجبة صبغة غرام، بينما *Bacillus megaterium* NB-3 تظهر نشاط واسع المدى وفعال ضد الفطريات، الخمائر، البكتيريا موجبة صبغة غرام والبكتيريا سالبة صبغة غرام. وجد في المزرعة المغلقة في داخل المختبر، بأن هذه السلالات تنح المضادات الحيوية في وقت متأخر من النمو خارج الخلايا في السائل المحيط.

تبين معلومات هذه الدراسة مصدراً جديداً لعزل سلالات بكتيرية منتجة لمضادات حيوية في الأردن، وهذا لم يتم وصفه من قبل. إذ يركز هذا المصدر الجديد لعزل مثل هذه السلالات، على قدرة هذه السلالات على إنتاج مضادات حيوية جديدة.

كلمات مدخلية: سلالات بكتيرية، مضاد حيوي، فطريات، خمائر.

Introduction

Microorganisms live in a wide variety of natural environments. They are found in water, air and soil (Saadoun and Al-Momany, 2000; Khyami-Horani *et al.* 1999). The microbes most frequently encountered in the air are aerobic and anaerobic spore-forming bacilli, saprophytic cocci, yeasts, molds and actinomycetes (Obeidat *et al.* 2000).

After the discovery of penicillin in 1929 by Alexander Fleming, a systematic screening for antibiotics was initiated. Thousands of different antibiotics were discovered and isolated from different microorganisms (Moyne *et al.* 2001; Tsuge *et al.* 1999), but only a few of them were eventually used as therapeutic substances because of their serious side effects (Masafumi *et al.* 2001; Pinchuk *et al.* 2001; Toshikazu *et al.* 1989). Thus, screening programmes continued to look for new antimicrobial substances from natural sources. due to the development of bacterial resistance as well as the emergence of new diseases that could not be cured with known antibiotics.

In Jordan, several attempts were made to screen for antimicrobial substances from different sources. Khyami-Horani *et al.* (1999) isolated antibiotics producing bacilli from soil and water, Mahasneh and Wahbeh (1985) from a 45°C thermal spring and Saadoun and Al-Momani (2000) and Saadoun *et al.* (1999) from the soil of nothern Jordan.

The majority of antibiotic producing microorganisms were isolated from the soil and water (Abussaud, 2000; Khyami-Horani *et al.* 1999), and there is no study about the isolation of antibiotic producing microorganisms from air-borne microbial flora. Thus, the goal of this investigation was to screen the air microbial flora for the production of antimicrobial substances.

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Materials and Methods

Isolation of antibiotic producing bacteria

Both antagonistic and non-antagonistic strains were isolated from the air from Jerash Private University, Jerash, Jordan. Several petri dishes of nutrient agar medium contained (per liter) 5g peptone, 5g sodium chloride, 1.5g yeast extract, 1.5g beef extract and 15g agar (HiMedia Laboratories Pvt. Limited, Bombay-400 086, India) were placed next to each other on a horizontal surface at different places at Jerash Private University, and were exposed to the air for 30 min. The plates were incubated at 27°C for 72h. Single colonies were isolated and screened for antimicrobial activity using the petri plate assay.

Petri plate assay

All bacterial isolates were initially screened for the ability to inhibit fungal growth on nutrient agar plates using *Fusarium oxysporium* SQ 11 as a preliminary test microorganism. Single colonies were selected and patched along the perimeters of plates on which 30µl of suspension of *Fusarium oxysporium* SQ 11 was placed at the center and spread over the entire surface of the plate. The plates were incubated at 27°C for 48h, and the antifungal activity was determined by measuring zones of fungal growth inhibition (Jayaswal *et al.* 1990).

Identification of the producing strains

The isolates were identified by the use of dichotomous keys and Bergey's Manual of Systemic Bacteriology (Cappuccino and Sherman, 1992). A variety of biochemical tests were carried out.

Shake flasks culture

All experiments dealing with the growth and antibiotic production were carried out in 500ml Erlenmeyer flasks containing 100ml nutrient broth , this consisted of (per liter) 5g peptic digest of animal, 5g sodium chloride, 5g yeast extract and 1.5g beef extract (HiMedia Laboratories Pvt. Limited, Bombay-400 086, India) inoculated with 1ml of 24h preculture. Inoculated flasks were incubated at 27°C on a rotary shaker (Sanyo Gallenhamp PLC, Leicester, LE 3 2uz, UK) at 180rpm for 42-48h.

Growth measurement

The growth of bacteria was measured spectrophotometrically as an increase of optical density at 600nm (Roitman *et al.* 1990).

Agar diffusion test

Extraction of the active substance from the supernatants and cells grown in liquid cultures (100ml) was performed. Cells were pelleted by centrifugation and extracted with acetone, and the supernatant was extracted with ethylacetate. Both extracts were evaporated by a rotary evaporator (Heidolph Instruments, GmbH and Co KG Vertrieb, Kelheim, Germany) at <50°C, and the dry substances were dissolved in 0.5ml methanol. The antimicrobial activity of these extracts was measured against *Fusarium oxysporium* SQ 11 and *Eschericshia coli* SQ 22 by agar diffusion test (El-Banna and Winkelman, 1998).

Biotest plates preparation

Using bacteria and yeasts as test microorganisms, cell suspensions of 24h precultures were prepared ($O.D_{578}$=1), and 0.5ml of this suspension was used to inoculate 250ml soft agar medium (20ml per plate, Arab food and Media Appliances Co ltd. Zarka Industrial Area, Jordan). In the case of spore forming fungi (as test microorganisms), flasks with potato dextrose agar containing (per liter) 200g potato infusion, 20g dextrose and 15g agar (HiMedia Laboratories Pvt. Limited, Bombay-400 086, India) were inoculated with fungi and incubated at 27°C for 10 days. After sporulation, the spores were harvested using tween 80-saline (0.1% tween, 0.9% NaCl). The spores were then washed and resuspended in normal saline. 250ml of test media (soft agar) was inoculated with 1ml of spore suspension (10^7spore/ml).

Results

In the present study, emphasis was laid on the isolation of antimicrobial substance producing bacteria from Jerash Private University. The screening of antibiotics from bacteria isolated from the air of Jerash Private University yielded a number of bacterial isolates. The antimicrobial activity of these isolates was tested by petri plate assay against *Fusarium oxysporium* SQ 11.

Strains that inhibited the growth of the fungal strain were then tested against other known test microorganisms. A zone of inhibition (mm) indicated antimicrobial activity, and the strains were selected and used for further study.

All the isolated strains were identified by the use of dichotomous keys and Bergey's Manual of Systemic Bacteriology as shown in (Table 1.) They are gram-positive, aerobic spore-forming rod shaped bacteria. On the basis of these, the isolated strains were identified as *Bacillus megaterium* NB-3, *Bacillus cereus* NB-4, *Bacillus cereus* NB-5, *Bacillus subtilis* NB-6 and *Bacillus circulans* NB-7.

Table 1. Taxonomic identification of the isolated strains*

Tests	Strain NB-3	Strain NB-4	Strain NB-5	Strain NB-6	Strain NB-7
Gram-stain	+	+	+	+	+
Spore formation	+	+	+	+	+
Catalase	+	+	+	+	+
Urease	+/-	+/-	+/-	-	-
Amylase	+	+	+	+	+
Gelatinase	+	+	+	+	+/-
Lipase	--	+	+	-	-
Caseinase	+	+	+	+	+/-
Indole	--	--	--	--	-
Methyl red	+	+	+	+	+
NaCl (7%)	+	+	+	+	-
NaCl (10%)	+	-	-	+/-	-
Nitrate reduction	+	+	+	+	+
Voges-Proskauer	-	-	-	+	-
Manitol	+	-	-	+	+
Acid from glucose	+	+	-	+	+
Gas from glucose	-	-	-	-	-

* Identified by the use of dichotomous keys and Bergey's Manual of Systemic Bacteriology.

At the end of fermentaion, the cells and the supernatants were extracted with acetone and ethylacetate, respectively. Agar diffusion test showed that the isolates (NB-3, NB-4, NB-5, NB-6 and NB-7) produced the antimicrobial active substances in the culture supernatants. To determine and monitor the time course for the production of the antimicrobial substances in batch culture, agar diffusion tests were employed. The antimicrobial activity of the strains was first detected after 15-18h of incubation, corresponding to the late exponential phase, and continued to increase during stationary phase reaching maximal activity at 42-48h as shown in (Figures 1, 2, 3, 4 and 5.)

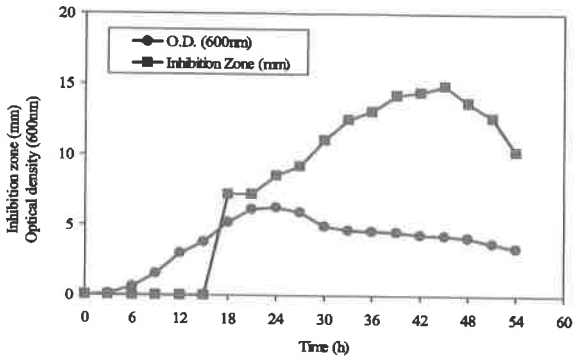

Fig. 2. Time course of antimicrobial substance produced by *Bacillus cereus* NB-4.

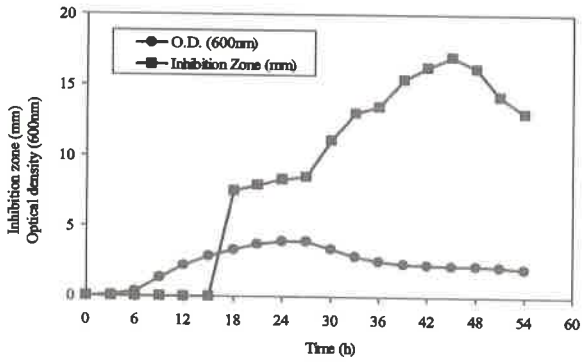

Fig. 1. Time course of antimicrobial substance produced by *Bacillus megaterium* NB-3.

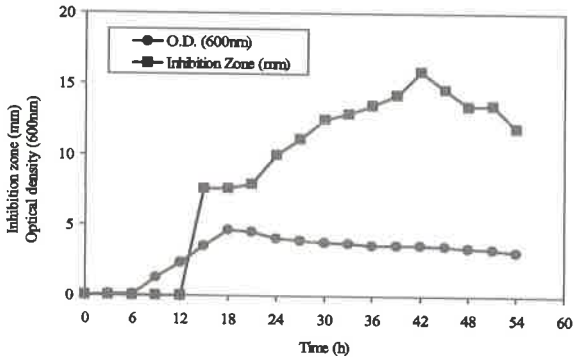

Fig. 3. Time course of antimicrobial substance produced by *Bacillus cereus* NB-5.

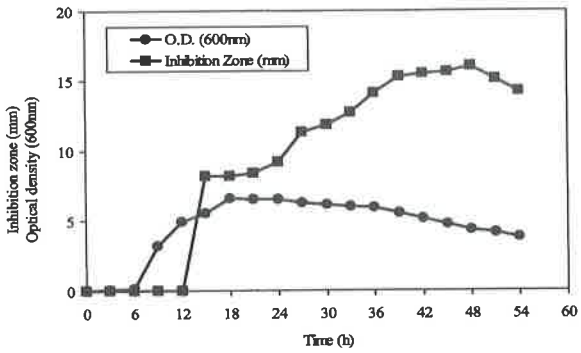

Fig. 4. Time course of antimicrobial substance produced by *Bacillus subtilis* NB-6.

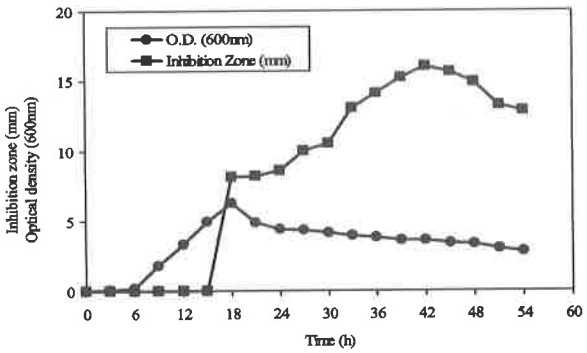

Fig. 5. Time course of antimicrobial substance produced by.*Bacillus circulans* NB-7.

The antimicrobial activity of the active substance produced by the isolates was determined by agar diffusion test as shown in (Table 2.) The strains (NB-4, NB-5 and NB-7) exhibited an antifungal activity, while the strain NB-6 showed activity against filamentous fungi, yeasts and gram-positive bacteria. In addition to the antifungal activity, *Bacillus megaterium* NB-3 was the only isolate that inhibited the growth of gram-negative bacteria. It had a weak activity against gram-positive bacteria.

Table 2. Antimicrobial spectrum of the active substance produced by the isolated strains.

Test microorganisms****	Inhibition zone (mm) *				
	Strain NB-3	Strain NB-4	Strain NB-5	Strain NB-6	Strain NB-7
Bacillus megaterium SQ 5	w.a.***	n.a.**	n.a.	12.6	n.a.
Bacillus cereus SQ 6	w.a.	n.a.	n.a.	13.6	n.a.
Staphyllococcus aureus SQ 9	w.a.	n.a.	n.a.	10.5	n.a.
Streptococcus pyogens SQ 10	w.a.	n.a.	n.a.	11.2	n.a.
Eschericshia coli SQ 22	17.0	n.a.	n.a.	n.a.	n.a.
Klepsiella spp SQ 33	16.0	n.a.	n.a.	n.a.	n.a.
Pseudomanas mallei SQ 34	w.a.	n.a.	n.a.	n.a.	n.a.
Aspergillus niger SQ 40	w.a.	14.7	15.7	16.0	16.0
Fusarium oxysporium SQ 11	16.6	15.0	15.8	16.0	16.0
Verticillium dehliae SQ 42	17.1	14.9	w.a.	15.8	15.5
Saccharomyces cerevisiae SQ 46	13.6	11.0	w.a.	12.4	10.6
Candida albicans SQ 47	12.8	11.2	w.a.	12.4	11.0

* Agar difusion test.
** No activity.
*** Weak activity without halo.
**** All microorganisms were obtained from Jerash Culture Collection of Microorganisms.

Discussion

During a screening program for antimicrobial substance producing microorganisms from a new source, several bacterial strains were isolated from the air of Jerash Private University. They were identified by the use of dichotomous keys and Bergey's Manual of Systemic Bacteriology as *Bacillus megaterium* NB-3, *Bacillus cereus* NB-4, *Bacillus cereus* NB-5, *Bacillus subtilis* NB-6 and *Bacillus circulans* NB-7.

Bacillus strains belonging to the family *Bacillaceae*, and are ubiquitous bacteria commonly recovered from water, soil, air and decomposing plant residues (Obeidat *et al.* 2000) The bacteria produce an endospore that allows them to endure extreme conditions of heat and desiccation in the environment. During the past decades of antibiotic screening, members of the genus *Bacillus* have proven to be the most fruitful of all in the order Eubacteriales in the search for new antibiotics. Strains of *Bacillus* produced many kinds of peptide

antibiotics. Among them, plipastatin (Tsuge *et al.* 1999), surfactin (Cosmin *et al.* 1993) and cerein (Naclerio *et al.* 1993).

In batch culture, some processes leading to the production of antibiotics are sequential, i.e, they exibit a distinct growth phase (trophophase) followed by a production phase (idiophase). In other processes, trophophase and idiophase overlap (Martin and Demain, 1980). The isolated strains (NB-3, NB-4, NB-5, NB-6 and NB-7) seem to produce the antimicrobial substances in a fair amount in the culture fluids. Under the conditions used, the active substance accumulated late in the growth cycle, (i.e. in the stationary phase) in the laboratory media reaching a maximum at 42-48h. Naclerio *et al.* (1993) reported that the antibiotics of *Bacillus* were isolated from culture filtrates.

The fermentation time needed for maximal yield of the antibiotic production seems to be different among bacterial strains, 36-40, 72, 120, 144 and 168h have been reported (Zheng and Slavik, 1999; Janisiwsz and Roitman, 1988; El-Banna and Winkelmann, 1998; Meyer *et al.* 1973; Moyne *et al.* 2001).

The antimicrobial spectra of the active substances isolated from culture filtrates of *Bacillus cereus* NB-4, *Bacillus cereus* NB-5 and *Bacillus circulans* NB-7, determined by agar diffusion method, exhibited potent antifungal activity against filamentous fungi and yeasts. *Bacillus subtilis* NB-6 showed activity against filamentous fungi, yeasts and gram-positive bacteria. *Bacillus megaterium* NB-3 exhibited a broad spectrum of activity against filamentous fungi, yeasts, gram-positive bacteria and gram-negative bacteria.

El-Banna (1989) reported that the strain isolated from Jordanian soil was active against filamentous fungi, yeasts and gram-positive bacteria and Mahasneh and Wahbeh (1985) showed that about half of the antibiotic producers were active against gram-negative bacteria and the other half against both gram-negative bacteria and gram-positive bacteria.

The data presented in this study show that gram-positive aerobic bacilli strains with different antimicrobial activities were isolated from air microbial flora. This is the first report that we are aware of which describes the antimicrobial activity of the air microbial flora in Jordan. The source of isolation of these strains suggests their potential as a source of novel antibiotics.

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Ref. 2218
Received 07/09/2002.
In revised form 06/06/2003