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Antimicrobial activities of some *Bacillus* spp. strains isolated from the soil

Mirac Yilmaz[a,*], Haluk Soran[a], Yavuz Beyatli[b]

[a]*Faculty of Education, Department of Science and Mathematics Fields in Secondary Education, Hacettepe University, 06532 Ankara, Turkey*
[b]*Faculty of Science and Arts, Department of Biology, Gazi University, Teknik Okullar, Ankara, Turkey*

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Summary

In this study a total of 29 *Bacillus* species isolated from the soil was analyzed using the agar diffusion method in terms of their general inhibition effects to some test bacteria. It has been found that isolates are effective against Gram-positive and Gram-negative bacteria whereas their extensive inhibition effect is particularly against Gram-positive bacteria. On the other hand, *B. cereus* M15 strain has an inhibitory effect against both Gram-positive and Gram-negative bacteria. Furthermore some isolates are more effective against test bacteria when compared to some antibiotics.
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Introduction

Polypeptide antibiotics which constitute the *Bacillus* bacteria have been gaining importance as a result of studies. The *Bacillus* species that produce antibiotics are *B. subtilis, B. polymyxa, B. brevis, B. licheniformis, B. circulans, B. cereus*. Polypeptide antibiotics produced by *Bacillus* that are used in medical treatments are bacitracin, gramycidin S, polymyxin, tyrotricidin (Morikawa et al., 1992; Perez et al., 1992, 1993; Drablos et al., 1999). In these studies it is stated that antibiotics produced by the *Bacillus* species are more effective for Gram-positive bacteria; however, the production of large spectrum and anti-fungal antibiotics that are effective for Gram-negative bacteria is relatively less (Morikawa et al., 1992; Perez et al., 1993; Eltem and Ucar, 1998). The *Bacillus* species have a wide range of antimicrobial activities since they are used as anti-fungal agents (Milner et al., 1995), anti-viral agents (Steller et al., 1999) anti-ameobocytic agents (Galvez et al., 1994) and anti-mycoplasma agents (Peypoux et al., 1999).

This study attempts at investigating the antimicrobial activity of 29 *Bacillus* species isolated

*Corresponding author. Fax: +90 312 299 20 83
E-mail addresses: miraycan3@hotmail.com, mirac@hacettepe.edu.tr (M. Yilmaz).

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from the soil against some pathogens and other test bacteria.

Materials and methods

Isolation, identification and growth conditions

Different grassland soil samples were taken from different eight regions of Ankara in Turkey. Each 1 g of the sample was suspended in 9 ml sterile distilled water and shaken vigorously for 2 min. The samples were heated at 60 °C for 60 min in a water bath. Then the liquid was serially diluted in sterile distilled water, and the dilution from 10^{-1} to 10^{-7} was plated on nutrient agar medium. Plates were incubated at 30 °C for 24–48 h (Chilcott and Wigley, 1993).

In the identification of isolated bacteria species, standard taxonomic descriptions from Sneath (1986) were used and *Bacillus* species were identified (Table 1).

The bacterial strains cultivated in nutrient broth (NB) which contained (per l) 1 g lab-lemco powder, 2 g yeast extract, 5 g peptone and 5 g NaCl. The pH was adjusted to 6.8 with 0.01 M HCl and 0.01 M NaOH. The temperature was maintained at 30 °C, and the agitation was maintained at 100 rpm. The cultures were inoculated into broth medium with 2% (v/v) inocula.

Test microorganisms

The test bacteria (*Micrococcus luteus* NRLL-B 4375, *Micrococcus flavus*, *Escherichia coli* NRRL B-704, *Yersinia enterocolitica* ATCC 1501, *Staphylococcus aureus* ATCC 25923, *Pseudomonas fluorescens* RSKK 240, *Pseudomonas aeruginosa* ATCC 27853 *Bacillus megaterium* RSKK 578, *Bacillus thuringiensis* RSKK 380, *Bacillus subtilis* F1, *Bacillus cereus* F2) used in this study were obtained from Culture Collections of the Biotechnology Laboratory at the Department of Biology in Gazi University. While *M. luteus* NRLL-B 4375 and *M. flavus* were incubated at 30 °C, other bacteria were

Table 1. Isolated species and their geographical origin

Number	Strain	Species	Origin
1	M2	*Bacillus brevis*	Beytepe Grassland, Ankara
2	M3	*Bacillus sphaericus*	Beytepe Grassland, Ankara
3	M4	*Bacillus brevis*	Beytepe Grassland, Ankara
4	M5	*Bacillus cereus*	Beytepe Grassland, Ankara
5	M6	*Bacillus brevis*	Keçiören Grassland, Ankara
6	M8	*Bacillus coagulans*	Keçiören Grassland, Ankara
7	M10	*Bacillus cereus*	Sincan Grassland, Ankara
8	M14	*Bacillus megaterium*	Eryaman Grassland, Ankara
9	M15	*Bacillus cereus*	Eryaman Grassland, Ankara
10	M16	*Bacillus circulans*	ODTU Grassland, Ankara
11	M17	*Bacillus subtilis*	ODTU Grassland, Ankara
12	M18	*Bacillus circulans*	ODTU Grassland, Ankara
13	M19	*Bacillus licheniformis*	Ostim Grassland, Ankara
14	M20	*Bacillus licheniformis*	Ostim Grassland, Ankara
15	M21	*Bacillus megaterium*	Ostim Grassland, Ankara
16	M22	*Bacillus megaterium*	Ostim Grassland, Ankara
17	M23	*Bacillus circulans*	Ostim Grassland, Ankara
18	M24	*Bacillus subtilis*	Ostim Grassland, Ankara
19	M25	*Bacillus coagulans*	Yıldız Grassland, Ankara
20	M26	*Bacillus megaterium*	Yıldız Grassland, Ankara
21	M27	*Bacillus licheniformis*	Yıldız Grassland, Ankara
22	M28	*Bacillus megaterium*	Yıldız Grassland, Ankara
23	M29	*Bacillus subtilis*	Yıldız Grassland, Ankara
24	M30	*Bacillus licheniformis*	Yıldız Grassland, Ankara
25	M31	*Bacillus circulans*	Ahlatlıbel Grassland, Ankara
26	M32	*Bacillus circulans*	Ahlatlıbel Grassland, Ankara
27	M33	*Bacillus subtilis*	Ahlatlıbel Grassland, Ankara
28	M34	*Bacillus circulans*	Ahlatlıbel Grassland, Ankara
29	M35	*Bacillus coagulans*	Ahlatlıbel Grassland, Ankara

Table 2. Antimicrobial activity of *Bacillus* strains on test bacteria

Inhibition zone (diameter, mm) against tested bacteria						
	S. aureus ATCC 25923*	*P. fluorescens* RSKK 240*	*M. flavus**	*B. megaterium* RSKK 578*	*B. thuringiensis* RSKK 380*	*B. cereus* F2*
Bacillus Strains						
B. brevis M4	NI	NI	NI	NI	NI	3.1±0.3
B. brevis M6	16±1.2	NI	NI	NI	10.8±1.0	NI
B. cereus M5	NI	NI	NI	NI	NI	2.4±0.4
B. cereus M10	NI	NI	NI	5.6±0.0	8.8±1.6	3.1±0.7
B. cereus M15	NI	10.5±1.3	6.4±2.4	NI	6.7±1.5	NI

NI: no inhibition.
*Values are the means± standard deviations of triplicate measurements.

incubated at 37 °C and all were activated by incubating for a period of 24 h in a NB (Oxoid).

Inhibitory effect by the agar-well diffusion method

The determination of the inhibitory effect of isolates on test bacteria was carried out according to the agar-well diffusion method. All bacteria were cultured on NB medium and incubated at the appropriate temperature for 24 h. Nutrient agar medium (20 ml) was poured into each sterile Petri dish (100 mm diameter). Suspensions (100 μl) of target strain cultured for 24 h were spread on the plates, and wells of 6 mm diameter were punched in the agar with a sterile steel borer. The *Bacillus* cultures were centrifuged at 6000*g* for 15 min to remove cell debris. After centrifugation, supernatant samples (100 μl) were filled into the wells of agar plates directly. Each sample (100 μl) was then filled into the wells of agar plates inoculated with target strains. The inoculated plates were incubated for 24 h at their optimum growth temperatures, and the diameter of the inhibition zone was measured with calipers as mm. The measurements were done basically from the edge at the zone to the edge of the wall (Reinheimer et al., 1990). Besides, the antimicrobial activity of bacteria was compared with antibiotics. Standard antibiotic discs used for control are as follows: Erythromycin (E; 15 mg, Oxoid), Vancomycin (VA; 30 mcg, Difco), Cephazolin (CZ; 30 mg, Oxoid), Azithromycin (AZM; 15 mcg, Difco), Penicillin-G (P; 10U, Oxoid), Chloramphenicol (C; 30 mcg, Difco), Sulbactam+Ampicillin (SAM; 10 mcg/10 mcg, Difco), Cephoxitin (FOX; 30 mg Oxoid), Cephadroxil (CFR; 30 mg, Oxoid), Trimethoprim-Sulfamethoxazole (SXT; 1.25 mcg/23.75 mcg, Difco), Oxacillin (OX; 5 mcg, Difco), Cephamandole (MA; 30 mcg, Difco).

Results

As a result of the identification tests, 29 *Bacillus* spp. strains were identified as 3 *B. brevis*, 1 *B. sphaericus*, 3 *B. cereus*, 5 *B. megaterium*, 6 *B. circulans*, 4 *B. subtilis*, 4 *B. licheniformis* and 3 *B. coagulans*.

This study examined the antimicrobial activity of 29 *Bacillus* spp. strains against tested bacteria. Some isolates (5 *Bacillus* spp. isolates) show antimicrobial activity (Table 2), but other isolates did not show antimicrobial activity. Table 2 shows the inhibitory effect of strains against *S. aureus* ATCC 25923, *P. fluorescens* RSKK 240, *B. megaterium* RSKK 578, *B. thuringiensis* RSKK 380, *M. flavus* and *B. cereus* F2.

The sensitivity levels of some isolates are more in comparison to some antibiotics. Diameters of inhibition zone (mm) exhibited against test bacteria of standard antibiotics are shown in Table 3.

In our study, it is found that *S. aureus* ATCC 25923 is resistant to Vancomycin, Cephazolin, Azithromycin, Cefoxitin and Cefamandole antibiotics, whereas *B. brevis* M6 has better antimicrobial activity against *S. aureus* ATCC 25923 than these antibiotics. It was found that *B. brevis* M6 had a higher level of antimicrobial activity (16 mm) than the antibiotic Chloramphenicol (14 mm). It appears that *P. fluorescens* RSKK 240 is resistant towards Erithromycin, Vancomycin, Cephazolin, Penicillin G, Chloramphenicol, Sulbactam+Ampicillin, Cefoxitin, Oxacillin, Cefamandole and Trimethoprim+Sulphamethoxazole antibiotics and that *M. flavus* is resistant to Penicillin G. On the other hand, *B. cereus* M15 has better inhibitory effect than these antibiotics. *B. thuringiensis* RSKK 380 is resistant to

Table 3. Diameters of inhibition zone (mm) exhibited against test bacteria of standard antibiotics

Antibiotics	E	VA	CZ	AZM	P	C	SAM	FOX	CFR	SXT	OX	MA
Test Bacteria												
S. aureus ATCC 25923	17^{I}	8^{R}	9^{R}	10^{R}	R	14^{I}	ND	12^{R}	9^{R}	16^{S}	17^{S}	30^{S}
P. fluorescens RSKK 240	R	R	R	12^{R}	R	6^{R}	R	R	R	2^{R}	R	R
M. flavus	31^{S}	20^{S}	18^{S}	11^{R}	R	35^{S}	22^{S}	29^{S}	30^{S}	22^{S}	7^{R}	44^{S}
B. megaterium RSKK 578	18^{I}	12^{S}	9^{R}	27^{S}	R	15^{I}	12^{I}	8^{R}	14^{R}	5^{R}	7^{R}	26^{S}
B. thuringiensis RSKK 380	R	R	2^{R}	9^{R}	R	20^{S}	R	ND	ND	ND	ND	ND
B. cereus F2	41^{S}	10^{I}	7^{R}	32^{S}	R	29^{S}	R	4^{R}	16^{I}	R	R	6^{R}

R: resistant, S: sensitive, I: semi-sensitive, ND: not detected.

Vancomycin, Cephazolin ve Azithromycin but *B. brevis* M6 has better activity than these antibiotics and *B. thuringiensis* RSKK 380 is resistant to Cephazolin, Cefoxitin and Cefamandole antibiotics but *B. cereus* M10 and *B. cereus* M15 has better inhibition effect than these antibiotics.

Discussion

In this study, was determined that *B. brevis* M6 showed an inhibition zone diameter of 16 mm against *S. aureus* ATCC 25923 and that *B. cereus* M15 showed an inhibition zone diameter of 6.4 mm against *M. flavus* (Table 2). Perez et al. (1992, 1993) reported that *B. subtilis* MIR 15 strain displayed antimicrobial activity against *P. aeruginosa, E. coli* and *M. luteus*. Aslim et al. (2002) found that four strains belonging *B. thuringiensis, B. subtilis* and *B. megaterium* were active against *E. coli* and *Y. enterocolitic*a. This study concluded that the isolates used have no inhibitory effects regarding *E. coli* NRRL B-704, *Y. enterocolitica* ATCC 1501, *P. aeruginosa* ATCC 27853, *M. luteus* NRRL-B 4375 and *B. subtilis* F1. Various strains are reported as effective against *S. aureus*: *B. subtilis* ATCC 6633 (Perez et al., 1993), *B. megaterium* Y6 (Aslim et al., 2002). *Bacillus* spp. TEM-FA-19 (Eltem and Ucar, 1998). Furthermore, Aslim et al. (2002) concluded that 14 *Bacillus* strains were influential against *M. flavus*.

The other significant findings of our study are as follows: *B. cereus* M15 has the inhibition zones of 6.4 and 6.7 mm diameters against *M. flavus* and *B. thuringiensis* RSKK 380, respectively, and it has 10.5 mm inhibition zone diameter against *P. fluorescens* RSKK 240. Oscariz et al. (1999) isolated and identified a bacteriocin-producing strain of *B. cereus* from a soil sample. The strain was active against most Gram-positive but not Gram-negative bacteria. The findings of the present study indicate that *Bacillus* isolates have antimicrobial effects particularly against the Gram-positive test bacteria. However, *B. cereus* M15 has inhibitory affect both against Gram-positive and Gram-negative bacteria.

In our study, it is found that *B. brevis* M6 has better antimicrobial activity against *S. aureus* ATCC 25923, *B. thuringiensis* RSKK 380; *B. cereus* M10 has better activity against *B. thuringiensis* RSKK 380 and *B. cereus* M15 has better inhibitory effect against *P. fluorescens* RSKK 240, *M. flavus* and *B. thuringiensis* RSKK 380 than some antibiotics. It is also found that our isolates have much better inhibitory effects against the test bacteria in contrast to some antibiotics. Ugur et al. (2001) found that *S. aureus* (MRSA), which was resistant to Methicillin, was also sensitive to Muğla royal jelly. Mercan et al. (2002) found that Karaman royal jelly has inhibitory effect on *M. flavus*, which is resistant to the antibiotic Azithromycin.

The benefits of our isolates and the chemical characterization of the antimicrobials determined are subject to further studies.

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