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Plasmid Mediated Antibiotic and Heavy Metal Resistance in Bacillus Strains Isolated from Soils in Rize, Turkey

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Keywords
Bacillus
Plasmid
Antibiotic resistance
Metal resistance
Transformation

Abstract: Fifteen *Bacillus* strains which were isolated from soil samples were examined for resistance to 17 different antibiotics (ampicillin, methicillin, erythromycin, norfloxacin, cephalotine, gentamycin, ciprofloxacin, streptomycin, tobramycin, chloramphenicol, trimethoprim-sulfamethoxazole, tetracycline, vancomycin, oxacilin, neomycin, kanamycin and, novabiocin) and to 10 different heavy metals (copper, lead, cobalt, chrome, iron, mercury, zinc, nickel, manganese and, cadmium) and for the presence of plasmid DNA. A total of eleven strains (67%) were resistant to at least one antibiotic. The most common resistance was observed against methicillin and oxacillin. The most resistance strains were found as *Bacillus* sp. B3 and *Bacillus* sp. B11. High heavy metal resistance against copper, chromium, zinc, iron and nickel was detected, but mercury and cobalt resistance was not detected, except for 3 strains (B3, B11, and B12) which showed mercury resistance. It has been determined that seven *Bacillus* strains have plasmids. The isolated plasmids were transformed into the *Bacillus subtilis* W168 and it was shown that heavy metal and antibiotic resistance determinants were carried on these plasmids. These results showed that there was a correlation between plasmid content and resistance for both antibiotic and heavy metal resistance.

Rize'deki Topraklardan İzole Edilen Bacillus Suşlarında Plazmit Kaynaklı Antibiyotik ve Ağır Metal Direnci

Anahtar kelimeler
Bacillus
Plazmit
Antibiyotik direnci
Metal direnci
Transformasyon

Özet: Toprak örneklerinden izole edilen 15 adet *Bacillus* suşu 17 farklı antibiyotiğe (ampisilin, metisilin, eritromisin, norfloksasin, sefalotin, gentamisin, siprofloksasin, streptomisin, tobramisin, kloramfenikol, trimetoprim-sulfametaksozol, tetrasiklin, vankomisin, oksasilin, neomisin, kanamisin ve novabiosin) ve 10 farklı ağır metale (bakır, kurşun, kobalt, krom, demir, cıva, çinko, nikel, manganez ve kadmiyum) karşı dirençliliği ve plazmit DNA içeriği bakımından incelenmiştir. Toplam olarak 11 suş (%67) en azından bir antibiyotiğe dirençli bulunmuştur. En yaygın direnç metisilin ve oksasilin'e karşı gözlemlenmiştir. En yaygın dirençli suşlar ise *Bacillus* sp. B3 ve *Bacillus* sp. B11 olarak tespit edilmiştir. Bakır, krom, çinko, demir ve nikel'e karşı direnç belirlenmesine rağmen, cıva direnci gösteren 3 suş hariç (B3, B11 ve B12) cıva ve kobalta karşı direnç belirlenmemiştir. Yedi adet *Bacillus* suşunun plazmit içerdiği tespit edilmiştir. İzole edilen plazmitler *Bacillus subtilis* W168 suşuna transforme edilmiş, ağır metal ve antibiyotik direnç belirleyicilerinin plazmit üzerinde taşındığı bulunmuştur. Bu sonuçlar hem antibiyotik hem de ağır metal direnci ile plazmit içeriği arasında bir ilişki olduğunu göstermektedir.

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1. Introduction

"Heavy metal" is a general collective term, which applies to the group of metals and metalloids with atomic density greater than 4000 kg m^{-3}, or 5 times more than water and, most heavy metals are transition elements with incompletely filled d orbitals (Nies, 1999; Hookoom and Puchooa, 2013). Low concentrations of certain transition metals such ascobalt, copper, nickel, iron, manganese and zinc are essential for many cellular processes of bacteria. Essential metals function as catalysts for biochemical reactions, are stabilizers of protein structures and bacterial cell walls, and serve in maintaining osmotic balance. Essential transition metals like iron, copper, and nickel are involved in redox processes. Still other essential metals like magnesium and zinc stabilize various enzymes and DNA through electrostatic forces. However, higher concentrations of these metals are often cytotoxic for bacterial cells (Bruins et al., 2000; Abou-Shanabet al., 2007).

Other heavy metals such as lead, cadmium, mercury, silver and chromiumare toxic even at low concentrations, have no known beneficial effects to bacterial cells and, they are nonessential. Nonessential metals bind with greater affinity to thiol-containing groups and oxygen sites than do essential metals. Toxicity results from alterations in the conformational structure of nucleic acids and proteins and interference with oxidative phosphorylation and osmotic balance (Bruins et al., 2000; Nies, 2004).

Contamination of the environment by heavy metal ions is a serious pollution problem for all living organism. Heavy metals are stable and persistent environmental contaminants since they cannot be degraded or destroyed. Therefore, they tend to accumulate in soils and sediments (Sevgi et al., 2010). Microbial survival in polluted soils depends on intrinsic biochemical and structural properties, physiological, and/ or genetic adaptation including morphological, changes of cells, as well as environmental modifications of metal speciation. Microbes apply various types of resistance mechanisms in response to heavy metals. These mechanisms include the efflux of metal ions outside the cell, accumulation and complexation of the metal ions inside the cell, and the reduction of the heavy metal ions to a less toxic state. These mechanisms may be encoded by chromosomal genes, but more usually loci conferring resistance are located on plasmids (Montuelle et al., 1994; Abou-Shanabet al., 2007; Sevgiet al., 2010). Plasmids are known to carry resistance genes for antibiotics and heavy metals (Matyar et al., 2008).

Bacillus genus is gram-positive and spore forming bacteria that are ubiquitous in the environment (Sevim et al., 2013). The aim of this study was to determine the heavy metal and antibiotic resistance of *Bacillus* strains isolated from soil samples in Rize, Turkey. The plasmid contents of *Bacillus* strains and transferable heavy metal and antibiotic resistance were also investigated.

2. Material and Methods

2.1. *Bacillus* strains

Bacillus strains used in this study were previously isolated from different soils in Rize, Turkey and they were identified by different techniques by Şensoy Karaoğlu et al. (2014). Some soil samples were taken from industrial areas which have heavy traffic, while some samples were taken away from industrial areas. The detailed information about *Bacillus* strains is given in Table 1.

Table 1. *Bacillus* species and their isolated soil location.

Strain	Species	Location of soil samples
B1	*Bacillus pumilis*	A*
B2	*B. subtilis*	A
B3	*Bacillus* sp.	B**
B4	*B. subtilis*	A
B5	*B. subtilis*	A
B6	*Bacillus* sp.	B
B7	*B. subtilis*	B
B8	*B. mojavensis*	B
B9	*Bacillus* sp.	A
B10	*B. amyloliqufaciens*	A
B11	*Bacillus* sp.	B
B12	*B. thuringiensis*	B
B13	*B. cereus*	B
B14	*B. thuringiensis*	B
B15	*Bacillus* sp.	A

*A, away from heavy traffic and industrial area
**B, heavy traffic and industrial area

2.2. Antimicrobial susceptibility test

The susceptibilities of the *Bacillus* strains and their transformants against different antibiotics were determined by standard disk diffusion method (Bauer et al., 1966). Each *Bacillus* strain was grown on Muller Hinton Agar (MHA) at 35°C, and then the turbidity was adjusted to the density of 0.5 McFarland by suspending in sterilized distilled water. The suspended strains were spread on MHA plates and the antibiotic disks were placed, then the plates were incubated at 35°C for 24 h. At the end of the incubation period, the diameter of the inhibition zones was measured and the strains were classified as resistant (R), and susceptible (S) (CLSI, 2006; formely NCCLS). The following antibiotic disks (Oxoid, UK) were used: ampicillin (10 µg), methicillin (5 µg), oxacillin (1 µg), cephalothin (30 µg), vancomycin (30 µg), erythromycin (15 µg), streptomycin (10 µg), tobramycin (10 µg), neomycin (30 µg), gentamicin (10 µg), kanamycin (30 µg), tetracycline (30 µg), chloramphenicol (30 µg), norfloxacin (10 µg), ciprofloxacin (5 µg), novobiocin

(30 µg) and trimethoprim/sulfamethoxazole (1,25 µg/23,75 µg).

2.3. Determination of heavy metal resistance

All *Bacillus* strains and their transformants were investigated in terms of heavy metal resistance profiles. Minimal Inhibition Concentrations (MIC) of heavy metals was determined by Microtitre Broth Dilition Method (Amsterdam, 1996). Following heavy metal salts were used in 0, 12, 25, 50, 100, 250, 500, 750, 1.000, 1.500, 2.000, 2.500 and, 3.000 µg/ml concentrations: $CuSO_4$, $Pb(NO_3)_2$, $CoCl_2$, $CrCl_2$, $FeSO_4$, $HgCl_2$, $ZnCl_2$, $NiCl_2$, $MnCl_2$ and, $Cd(NO_3)_2$. 96 well sterile microplates were used for growth dish. All strains were grown on MHA agar at 35°C, and then the turbidity was adjusted to the density of 0.5 McFarland by suspending in sterilized distilled water. 50 µl of the suspended cultures was added microplate wells including different concentration of heavy metals. The microplates were closed with lids to prevent evoparation. The microplates were incubated at 35°C on an orbital shaker at 180 rpm overnight. The bacterial growth was spectrophotometrically measured at 600 nm wavelength. Two different control groups were used. The medium without metal but bacteria was used as the bacterial growth control and, the medium without bacteria but metal was used as metal control. The lowest concentration of metal that completely prevented bacterial growth was termed the MIC. Since there are no standard acceptable heavy metal concentrations to mention metal resistance in bacteria, the bacterial strains which were not inhibited by 12 µg/ml were considered tolerant (Nieto et al., 1989).

2.4. Plasmid isolation and their transformation into *Bacillus subtilis* W168

Plasmid profile of *Bacillus* strains were detected on PAL (protoplast alkaline lysis) miniprep procedure according to the Voskuil and Chambliss (1993).

The obtained plasmid DNA of *Bacillus* strains were used for transformation into the *B. subtilis* W168 which is known to sensitive antibiotics and heavy metals used in this study. The transformation procedure was performed according to the one-step method modified by Kunstet al., (1994). Overnight culture of W168 in LB medium was inoculated (%10 v/v) in freshly prepared modified competence medium containing 100 Mm phosphate buffer (pH 7.0), 3 mM trisodium citrate, 3 mM magnesium sulfate, 2% glucose, 22 µg/mL ferric ammonium citrate, 0.1% casein hydrolysate and 0.2% potassium glutamate and then incubated at 37 °C for 60 min with shaking. When the OD_{600} nm reached around 0.6, 1 mL of the culture was removed to a test tube and plasmid DNA was added. This culture was incubated at 37 °C for 1 h. After incubation, cells were plated on LB-agar with appropriate antibiotics. Plates were incubated at 37 °C over night and the transformants were selected (Boylanet al., 1972). To confirm the transformation, the susceptibility tests of transformants against all used antibiotics and heavy metals were determined as described before. Moreover, plasmid-DNA isolation was performed for all strains including wild type and transformants and then, they were loaded on agarose gel (0.8%) including ethidium bromide and run at 100 V for 30 min. After running, they were visualized under UV light.

3. Results

3.1. Antibiotic resistance

To determine antibiotic resistance, all *Bacillus* strains were tested against different antibiotics. The most common resistance (8 of 15 strains (53%)) was observed against methicilin and oxacilin (Figure 1). Resistance to ampicillin and cephalotine was observed in 6 (40%) of 15 strains. Also, Resistance against tobramicin and trimethoprim/sulfamethoxazole was observed in 4 (27%) of 15 strains. The lowest resistance was obtained against three antibiotics (tetracycline, vancomycine, kanamycine) in 2 (13%) of 15 strains. Any resistance was not observed against other antibiotics (erythromycin, norfloxacin, gentamicin, ciprofloxacin, streptomycin, chloramphenicol, neomycin, novabiocin).

11 strains (67%) of 15 *Bacillus* strains were resistant to at least one antibiotic. While *Bacillus* sp. B3 and *Bacillus* sp. B11 strains was the most resistance against 9 (53%) of 17 antibiotics, any resistance pattern was not observed in *B. subtilis* B2, *Bacillus* sp. B6, *B. subtilis* B7, *Bacillus* sp. B9 and *B. amyloliquefaciens* B10 strains (Table 2).

3.2. Minimal inhibitory concentration (MIC) to heavy metals

Minimal inhibition concentrations (MIC) of various heavy metals were determined for *Bacillus* strains which were found resistance against metals. All *Bacillus* strains exhibited high heavy metal resistance to $CuSO_4$, $CrCl_2$, $ZnCl_2$, $FeSO_4$, $NiCl_2$ and, less resistance to $CoCl_2$ and $HgCl_2$. When compared with *B. subtilis* W168 (wild type), *Bacillus* sp. B3, *Bacillus* sp. B11 and *B. thrungiensis* B12 were the most resistance strains against most of heavy metals ($CuSO_4$, $Pb(NO_3)_2$, $CrCl_2$, $FeSO_4$, $ZnCl_2$, $NiCl_2$, $Cd(NO_3)_2$) used in this study (Table 3).

3.3. Plasmid profile and transferable plasmids of *Bacillus* strains

The PAL (protoplast alkaline lysis) method was used for plasmid isolation for *Bacillus* strains. According to electrophoresis results, it has been determined that eight strains had plasmids to one or more copies

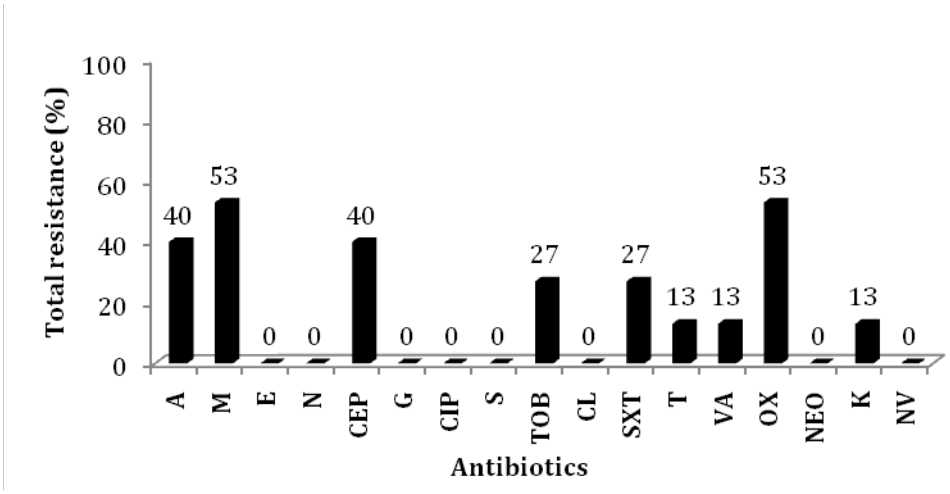

Figure1. Total resistance against antibiotics. A, Ampicillin; M, Methicillin; E, Erythromycin; N, Norfloxacin; CEP, Cephalotine; G, Gentamicin; CIP, Ciproflaxacin; S, Streptomycin; TOB, Tobramicin; CL, Chloramphenicol; SXT, Trimethoprim/Sulfamethoxazole; T, Tetracycline; VA, Vancomycin; OX, Oxacilin; NEO, Neomycin; K, Kanamycin; NV, Novabiocin.

Table 2. Antibiotic susceptibility profiles of *Bacillus* strains.

	***Bacillus* strains**															
Antibiotics	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**	**B9**	**B10**	**B11**	**B12**	**B13**	**B14**	**B15**	**WT***
A	S**	S	R	S	S	S	S	S	S	S	R	R	R	R	R	S
M	S	S	R	R	S	S	S	R	S	S	R	R	R	R	R	S
E	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
N	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
CEP	S	S	R	S	S	S	S	S	S	S	R	R	R	R	R	S
G	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
CIP	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
TOB	R	S	R	S	R	S	S	S	S	S	R	S	S	S	S	S
CL	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
SXT	S	S	R	S	S	S	S	S	S	S	R	R	R	S	S	S
T	S	S	R	S	S	S	S	S	S	S	R	S	S	S	S	S
VA	S	S	R	S	S	S	S	S	S	S	R	S	S	S	S	S
OX	S	S	R	S	R	S	S	R	S	S	R	R	R	R	R	S
NEO	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
K	S	S	R	S	S	S	S	S	S	S	R	S	S	S	S	S
NV	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

* WT, *Bacillus subtilis* W168; ** S, Sensitive, R, Resistance.

A, Ampicillin; M, Methicillin; E, Erythromycin, N, Norfloxacin; CEP, Cephalotine; G, Gentamicin; CIP, Ciproflaxacin; S, Streptomycin; TOB, Tobramicin; CL, Chloramphenicol; SXT, Trimethoprim/sulfamethoxazole; T, Tetracycline; VA, Vancomycin; OX, Oxacilin; NEO, Neomycin; K, Kanamycin; NV, Novabiocin.

Table 3. Minimal Inhibitory Concentration (MIC) of heavy metals for *Bacillus* strains.

Strain	**CuSO4**	**Pb(NO2)2**	**CoCl2**	**CrCl2**	**ZnSO4**	**FeSO4**	**HgCl2**	**Cd(NO2)2**	**MnCl2**	**NiCl2**
					µg/ml					
B1	1000	1000	100	1000	2000	1500	-	250	1000	250
B2	1000	1000	50	1000	250	1500	-	25	750	250
B3	1500	3000	100	2000	2000	2000	25	500	1000	1000

B4	1500	1000	100	1000	250	1500	-	12	750	500
B5	1500	1000	100	1000	250	1500	-	12	750	500
B6	1000	2500	100	1000	250	1000	-	25	1000	500
B7	1500	2500	50	1000	250	1500	-	25	500	500
B8	1500	2500	50	1000	250	1500	-	25	1000	500
B9	1000	1000	100	1000	250	1500	-	25	750	500
B10	1000	3000	100	1000	250	1500	-	25	1000	250
B11	1500	3000	100	1000	500	1000	25	500	1000	1000
B12	1500	2500	100	1000	500	1000	25	500	1000	2000
B13	1000	2500	100	1000	500	1000	-	250	1000	1000
B14	1000	1000	100	1000	750	1000	-	500	1000	1000
B15	1000	1000	50	1000	250	750	-	12	750	250
WT*	100	1000	100	100	100	100	-	25	1000	100

* WT, *Bacillus subtilis* W168.

(Figure 2). Seven *Bacillus* strains (*Bacillus* sp. B3, *B. subtilis* B5, *B. mojavensis* B8, *B. thuringiensis* B11, *B. thuringiensis* B12, *B. cereus* B13 and *Bacillus* sp. B15) have only one copy plasmid, but *B. thuringiensis* B14 have two copies plasmid (Figure 2).

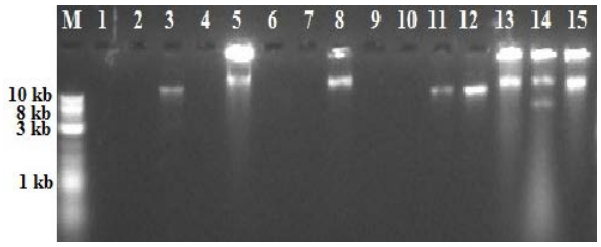

Figure 2. Plasmid profile of *Bacillus* strains. M, DNA Marker (Quick-Load®Purple 2-Log DNA Ladder, NEB); Lane 1, *B. pumilis* B1; Lane 2, *B. subtilis* B2; Lane 3, *Bacillus* sp. B3; Lane 4, *B. subtilis* B4; Lane 5, *B. subtilis* B5; Lane 6, *Bacillus* sp. B6; Lane 7, *B. subtilis* B7; Lane 8, *B. mojavensis* B8; Lane 9, *Bacillus* sp. B9; Lane 10, *B. amyloliqufaciens* B10; Lane 11, *Bacillus* sp. B11; Lane 12, *B. thuringiensis* B12; Lane 13, *B. cereus* B13; Lane 14, *B.thuringiensis* B14; Lane 15, *Bacillus* sp. B15.

For transformation experiment, the isolated plasmids were transformed into the *B. subtilis* W168 wild type using Kunst one-step modified medium (Kunst et al., 1994). At the end of the experiment, the plasmids of *B. thuringiensis* B11 and *B. thrungiensis* B12 strains was transformed into the wild type. As a result of the plasmid isolation from transformant colonies, it was detected that one copy plasmid of *Bacillus* sp. B11 and *B. thrungiensis* B12 were transferred into the wild type (Figure3). It was observed that transformant cells gained new heavy metal and antibiotic resistance properties which wild type strains carried (Table 4).

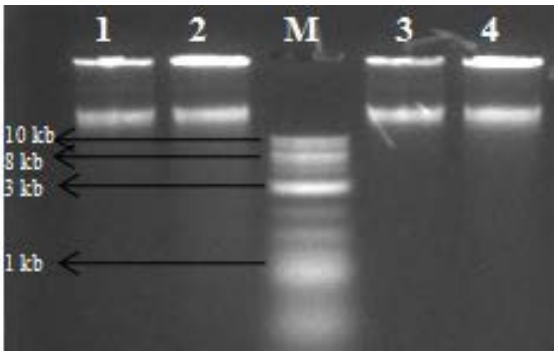

Figure 3. Transformed plasmid profile of *Bacillus* strains. Isolated plasmids of *Bacillus* strains were transformed into the *Bacillus subtilis* W168. Plasmid isolation was performed from transformant cell by using the PAL method. M, DNA Marker (Quick-Load®Purple 2-Log DNA Ladder, NEB); Lane 1, *B. thuringiensis* B11; Lane2, plasmid profile of transformant cells including plasmid of B11 wild type; Lane 3, *B. thuringiensis* B12; Lane 4, plasmid profile of transformant cells including plasmid of B12 wild type.

4. Discussion

In this study, *Bacillus* strains (*Bacillus* sp. B3, *Bacillus* sp. B11, *B. thrungiensis* B12 and *B. cereus* B13) were able to grow at high concentrations of

Table 4. Plasmids and plasmid mediated antibiotic and heavy metal resistance profiles of *Bacillus* strains.

Strain	Resistance properties of strains	Plasmids/ Copy number	Plasmids mediated properties
B1	TOB* $CuSO_4$, $CrCl_2$, $ZnCl_2$, $FeSO_4$, $Cd(NO_3)_2$, $NiCl_2$	-	
B2	- $CuSO_4$, $CrCl_2$, $FeSO_4$, $ZnCl_2$, $NiCl_2$	-	
B3	A, M, CEP, TOB, SXT, T, VA, OX, K $CuSO_4$, $Pb(NO_3)_2$, $CrCl_2$, $FeSO_4$, $HgCl_2$, $ZnCl_2$, $NiCl_2$, $Cd(NO_3)_2$	+/1	A*, M, CEP, TOB, SXT, T, VA, OX, K $CrCl_2$, $ZnCl_2$, $FeSO_4$, $Cd(NO_3)_2$, $NiCl_2$
B4	M, $CuSO_4$, $CrCl_2$, $FeSO_4$, $ZnCl_2$, $NiCl_2$	-	
B5	TOB, OX $CuSO_4$, $CrCl_2$, $FeSO_4$, $ZnCl_2$, $NiCl_2$	+/1	NT**
B6	- $CuSO_4$, $Pb(NO_3)_2$, $CrCl_2$, $FeSO_4$, $ZnCl_2$, $NiCl_2$	-	
B7	- $CuSO_4$, $Pb(NO_3)_2$, $CrCl_2$, $FeSO_4$, $ZnCl_2$, $NiCl_2$	-	
B8	M, OX $CuSO_4$, $Pb(NO_3)_2$, $CrCl_2$, $FeSO_4$, $ZnCl_2$, $NiCl_2$	+/1	NT
B9	- $CuSO_4$, $CrCl_2$, $FeSO_4$, $ZnCl_2$, $NiCl_2$	-	
B10	- $CuSO_4$, $Pb(NO_3)_2$, $CrCl_2$, $FeSO_4$, $ZnCl_2$, $NiCl_2$	-	
B11	A, M, CEP, TOB, SXT, T, VA, OX, K $CuSO_4$, $Pb(NO_3)_2$, $CrCl_2$, $FeSO_4$, $HgCl_2$, $ZnCl_2$, $NiCl_2$, $Cd(NO_3)_2$	+/1	A, M, CEP, TOB, SXT, T, VA, OX, K $CrCl_2$, $ZnCl_2$, $FeSO_4$, $Cd(NO_3)_2$, $NiCl_2$
B12	A, M, CEP, SXT, OX $CuSO_4$, $Pb(NO_3)_2$, $CrCl_2$, $FeSO_4$, $HgCl_2$, $ZnCl_2$, $NiCl_2$, $Cd(NO_3)_2$	+/1	A, M, CEP, SXT, OX $CrCl_2$, $ZnCl_2$, $FeSO_4$, $Cd(NO_3)_2$, $NiCl_2$
B13	A, M, CEP, SXT, OX $CuSO_4$, $Pb(NO_3)_2$, $CrCl_2$, $FeSO_4$, $ZnCl_2$, $NiCl_2$, $Cd(NO_3)_2$	+/1	NT
B14	A, M, CEP, OX $CuSO_4$, $CrCl_2$, $FeSO_4$, $ZnCl_2$, $NiCl_2$, $Cd(NO_3)_2$	+/2	NT
B15	A, M, CEP, OX $CuSO_4$, $CrCl_2$, $FeSO_4$, $ZnCl_2$, $NiCl_2$	+/1	NT

* A, ampicillin; M, methicillin; OX, oxacillin; CEP, cephalothin; VA, vancomycin; TOB, tobramycin; K, kanamycin; T, tetracycline; SXT, trimethoprim/sulfamethoxazole; ** NT, Nontransformant

the heavy metals such as Cu, Pb, Cr, Zn, Fe, Hg, Cd and Ni. At the same time, *Bacillus* sp. B3 and *Bacillus* sp. B11 strains were most antibiotic resistance strains. In many studies, antibiotic resistance and heavy metal tolerance have been observed together in *Bacillus* species and gram negative bacteria (Belliveau et al., 1991; Hassenet al., 1998; Ince Yılmaz, 2003; Kamala-Kannan and Lee, 2008; Christopheret al., 2014). In earlier studies, it has been shown that antibiotic and metal resistance determinants are commonly located on plasmids or transposons. As a results of these evidence, it has been suggested that these resistance genes were spread to divergent bacteria by horizontal gene transfer (Austinet al., 2006; Abou-Shanabet al., 2007). As a result of transformation experiment in our study, the plasmids isolated from *Bacillus* strains (*B. thuringiensis* B11 and *B. thrungiensis* B12) were transferred to the *B. subtilis* W168 wild type cell and, it have been found that the wild type cell which is sensitive to antibiotics and heavy metals used in this study become resistant against same antibiotic and heavy metals as in *B. thuringiensis* B11 and *B. thrungiensis* B12 cell (Table 4).

The electrophoresis results of plasmids from host cells and transformants showed that the transformed plasmids are approximately at the same size. Also, while heavy metals resistance determinants transferred are also the same for B11 and B12 strains, antibiotic resistance determinants aren't the same.

Up to now, resistance against heavy metals has been found in many gram positive and gram negative bacteria (Trajanovskaet al., 1997; Alamet al., 2011; Sinhaet al., 2013). In our study, *Bacillus* sp. B3, *Bacillus* sp. B11 and *B. thrungiensis* B12 are most resistance strains with respect to heavy metals and, it were found that these strains included various plasmids encoding heavy metal

determinants. It was also found that Cd, Ni, Cr, Zn and, Fe resistance were located on plasmids. In bacteria, resistance to cadmium is based on cadmium efflux system. Cadmium resistance can be mediated by chromosomes, plasmids, or transposons. The plasmid-encoded *cad* system is best characterized Cd(II) resistance efflux system in *S. aureus* (Smith and Novick, 1972; Bruins et al., 2000).

During this study, resistance to Cu, Zn, Fe and Cr was found in all *Bacillus* strains. All resistance determinants were transformed via plasmid, except for Cu. Transforment cells showed resistance against chromium, iron and zinc. Zinc and iron are essential metal ions at low concentration for bacterial cells and zinc plays important role in development, growth and differentiation for all living organisms (Nies, 1999; Choudhury and Srivastava, 2001). Chromium exists in different oxidation states, but Cr (VI) that is hexavalent form of chromium and Cr (III) that is trivalent form of chromium are the most common in the environment. Cr (VI) is more toxic and harmful than trivalent chromium (Chatuverdi, 2011; Das et al., 2014; He et al., 2014). Bacterial chromate resistance is commonly provided by plasmids (Campos et al., 1995).

In this study, the lowest heavy metal resistance in all *Bacillus* strains has been identified as mercury. Only three strains (*Bacillus* sp. B3, *Bacillus* sp. B11 and *B. thrungiensis* B12) are resistance to mercury. Detoxification of mercurial compound mediated by *mer* operon and mercury reductase is essentially enzyme for reduction in high toxic ionic Hg^{2+} into less toxic and, volatile Hg^{0}. The *mer* operon has been usually located on plasmid (Mahler et al., 1986; Mathema et al., 2011). Interestingly, we did not found resistance in transforment cells against mercury. Possibly, the mercury resistance determinates might be located on chromosome.

Methods for the removal of heavy metals from the environment can be divided into two groups: 1- biotic methods which are based on the accumulation of heavy metals by plants or microorganisms and, 2- abiotic methods which are based on the removal of heavy metals using physiochemical processes such as precipitation, coprecipitation, ion exchange and, adsorption of heavy metals by suitable adsorbent. However, physiochemical processes are very expensive and generate secondary products, thereby resulting in merely the transfer of the metal from one form into less mobile and available form, but not providing a definitive solution (Velusamy et al., 2011). Heavy metal resistant microorganisms play an important role in the bioremediation of heavy metal contaminated soils (Abou-Shanab et al., 2007). The *Bacillus* species was considered suitable agent to remove heavy metals since the members of this genus are easy to culture and have shown high tolerance to heavy metal toxicity (Ince Yılmaz, 2003). The result of our study, indicate that the isolated *Bacillus* strains (especially *Bacillus* sp. B3, *B. thuringiensis* B11 and *B. thuringiensis* B12) were found the most resistance against many heavy metals. These heavy metal resistant *Bacillus* strains could be a potential agent for bioremediation of heavy metals in many polluted environments.

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Abbreviations

DNA	Deoxyribonucleic acid
kg	Kilogram
m^{-3}	Meter cube
MHA	Muller Hinton Agar
CLSI	Clinical and laboratory Standards Institute
NCCLS	National Committee for Clinical Laboratory Standards
µg	Microgram
MIC	Minimal Inhibitory Concentration
µl	Microliter
rpm	Revolutions per Minute
nm	Nanometer
°C	Celsius degree
PAL	Protoplast Alkaline Lysis
LB	Laura Bertani
pH	Power of hydrogen
mM	Milimolar
mL	Mililiter
OD	Optical Density
UV	Ultra Violet
WT	Wild Type