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Bioremediation of Industrial Wastewater. II- Isolation and Characterization of Heavy Metal Ions Tolerant Genes in Gram-positive Bacteria

Z.A. Abo El-Kheir*, O.A. Khafagi*, K.S. Abdallah, H.I. El-Kassas*** and A.I. Alawady****

**Botany Department, Faculty of Science (Girls), Al-Azhar University, **Agricultural Genetic Engineering Research Institute (AGERI), Agricultural Research Centre and ***Institute of Environmental Studies and Research, Ain-Shams University, Cairo, Egypt.*

THE THREAT of heavy metals pollution to public health and wild life led to an increased interest in developing systems that can remove or neutralize its toxic effects in industrial effluents and municipal wastewater. The present study was conducted to identify the bacterial isolates with superior ability to accumulate heavy metal ions, and to analyze some genes encoding metal resistance in such isolates using PCR and DNA sequencing. Bacteria were isolated from the industrial ponds of Sadat City, Egypt, and their heavy metal resistance was assessed. Identification indicated that some isolates were belonging to genus *Bacillus* and showed remarkable resistance to cadmium, zinc and cobalt. Specific DNA sequences could be amplified from the genomic DNA of the tested isolates using polymerase chain reaction (PCR). Amplification of ~ 400bp fragment was performed using the primer pair CzcD1 and CzcD2, and it was identical to the expected amplified fragment encasing the *Czc* gene. Alignment indicated that there is 99% similarity ratio between pAczc1 clone of the isolated *Bacillus cereus* and *Czc* gene of *Alkaligenes* sp., *Ralstonia* sp. and *Ralstonia metallidurans*.

Keywords: Heavy metal, Wastewater, *Czc*D resistant gene, Gram-positive bacteria, PCR, DNA sequencing.

Pollution is a change in the physiological, chemical, radiological or biological quality of the resource (air, land or water) caused by man or due to man's activities that is injurious to existing, intended, or potential uses of the resource (Salomons *et al.*, 1995). The main cause of water pollution is the discharge of solid or liquid waste products containing pollutants on to the land surface or into surface or coastal water. The wastes that contribute to water pollution may be broadly grouped into sewage, industrial, and agricultural types (Dix, 1981). A number of sites contaminated by heavy metals around the world are associated with human activities such as discharge of wastes into natural waterways,various metallurigical industries, accidental spills or mining (Trajanovska *et al.*, 1997).

As environmental pollutants pose a great risk to natural bacterial populations, the adaptive responses of bacteria in contaminated environments have been studied extensively. Such investigation have indicated that bacteria which survive and, indeed, flourish in such environments have developed resistance mechanisms that lead to the selection of resistant variants that can tolerate metal toxicity (Yilmaz, 2003). Bacteria resistant to cadmium, zinc, cobalt, chromium, copper, arsenic and nickel have been isolated from several contaminated sites and natural deposits.

Microorganisms in soils, sediments and natural waters influences the transport and fate of trace and contaminant metals through :(1) incorporation into biomass during growth, (2) metal complexation by cell/spore surfaces, extracellular polymers and diffusible exudates, (3) alteration of chemical microenvironment, (4) redox reactions with metal electron donors and acceptors, and (5) mineral formation and dissolution (Bargar *et al.*, 2000; Kraemer *et al.*, 2002; Nelson and Lion, 2003; Kemner *et al.*, 2004 and Toner *et al.*, 2006).

Investigators have studied the mechanism of heavy metal biosorption using pure microbial species. One microbial group, which possesses high metal sorption capacity consists of the Gram-positive bacteria (Yilmaz, 2003). A considerable amount of work has been done to study the ability of *Bacillus* species to remove metals (El-Helow *et al,* 2000). The members of this genus are easy to culture and have shown high tolerance to heavy metal toxicity. Thus, the present study was conducted to identify the bacterial isolates with superior ability to accumulate heavy metal ions and analyze some genes encoding metal resistance in such isolates using PCR and DNA sequencing.

Material and Methods

1- Isolation and characterization of bacteria

Samples of wastewater from ponds of industrial Sadat City, Egypt, were collected, and were transported to lab in an icebox for bacteriological investigation. The bacteria were isolated using plate dilution method and Luria-Bertaini agar plates (LB plates) supplemented with 0.5 mM of CdC12, $Zn(NO_3)_2$, $Co(NO_3)_2$, then incubated at 37°C for 3 days. The bacterial isolates which showed resistance to these heavy metal concentrations were selected and identified based on morphological feature by Gram-stain, and biochemical properties including potassium hydroxide test according to Halebian *et al.* (1981); catalase production (Fortin *et al.*, 2003); glucose fermentation, urease test & IMViC test (James and Natalie, 1989). Growth at different levels of temperature and at NaOH (7%) was also identified.

2- Determination of the minimum inhibitory concentrations (MICs)

The minimum inhibitory concentrations (MICs) of each isolate were determined according to Lambert and Pearson (2000). The MIC is defined as the lowest concentration of metal ion of which no growth could be observed following overnight incubation at 37°C.

Analytical grades of metal salts were used to prepare 0.5-1M stock solutions, which was filter-sterilized. Each isolated species were grown overnight LB flask supplemented with serial concentrations of heavy metals, with shaking at 37°C, 2000 rpm. The range of the used concentrations was 18.3 to 293.3 ppm for cadmium chloride, 32.3 to 226.1 ppm for zinc nitrate and 11.89 to 475.8 ppm for cobalt nitrate. The least concentration of metal that completely prevented growth was the minimal inhibitory concentration (MIC). In general, the greater the turbidity, the larger the population size; and this measured by spectrophotometer using turbidity measurements as indicators of growth. Experiments were conducted in triplicate and results represent average values.

3-Analysis of metal accumulation and evaluation of biosorption performance

Analysis of metal accumulation was performed as described by Steven *et al.* (1997) and Yilmaz (2003). Biosorption metal values (%) were calculated by taking differences between the metal concentration of cultures at time zero and the time of sampling.

4- Preparation of the total genomic DNA and plasmid DNA

Genomic DNA preparation was done according to manufacturer specifications (Promega, USA, 1991). A single bacterial colony was transferred into 5 ml of LB-plate in a loosely capped 10 ml tube and incubated overnight at 37°C with vigorous shaking. One ml of the culture was centrifuged at 13.000-16.000 xg for 2 min, 240µl of 10 mg/ml lysozyme was added and gently pipetted to mix then incubated at 37°C for 30-60 min and centrifuged. Six hundred µl of nuclei lysis solution were added, incubated at 80°C for 5 min, after cooling 3 µl of RNase solution 10 mg/ml were added and incubated at 37°C for 30 min. Two hundred µl of protein precipitation solution were added to the RNase-treated cell lysate and vortexed vigorously at high speed for 20 seconds then incubated on ice for 5 min and centrifuged at 13.000 xg for 3 min. Six hundred of isopropanol eas added to the supernatant, gently mixed until the thread-like strands of DNA formed a visible mass, then centrifuged at 13.000-16.000 xg for 2 min and the ethanol carefully aspirated. The pellet allowed to air-dry for 10-15 min and the genomic DNA was dissolved in 100 µl of DNA rehydration solution and stored at -20°C.

Plasmid DNA preparation was done as described by Sambrook *et al.* (1989). The cell pellets were suspended in 100 µl ice-cold Miniprep lysis buffer (50 mM glucose, 25 mM Tris-HCl, pH 8.0 and 10 mM EDTA, pH 8.0) by vigorous vortex. Two hundred µl of freshly prepared solution containing (0.2 N NaOH, 1% SDS) were added, after inversion 3 times, 150 µl of ice-cold potassium acetate were added, pH 4.8. Aqueous phase was obtained by centrifugation and equal volume of TE-saturated phenol/ chloroform was mixed and recentrifuged. The DNA was precipitated from solution by sodium acetate and ethanol. Following 30 minutes incubation at -20°C, the pellet was washed with 1 ml of prechild 70 % ethanol and vacuum dried. The dried pellet was dissolved in 50 µl of TE at pH 8.0 and stored at -20°C.

5- Amplification of cadmium, zinc, & cobalt (Czc) resistance gene

The oligonucleotide primers targeting the resistance determinants of Czc were synthesized at the Agricultural Genetic Engineering Research Institute (AGERI), using ABI 392 DNA/RNA synthesizer (Applied Biosystems, USA). The primer sequence used was designed to amplify region containing *pczcD* operon of *pczc* gene and the base composition was chosen in such that they had similar annealing temperature as published. PCR amplification was generated using forward primer (5' CAGGTCACTGACACGACCAT 3') and reverse primer (5' CATGCTGATGAGATTGATGATC 3'). Templates for PCR amplification included total genomic DNA from bacterial isolates & plasmid blue script SK (+). The used primers were predicated to yield a ~ 400bp product. The gene amplification PCR reagent system, from Perkin Elemer, was source of fragment with Hot start *Taq* DNA polymerase stored in a buffer. Amplifications were performed in 100 µl reaction volumes for 30 cycle consisted of denaturation at 95°C for 30 sec , annealing at 50°C for 30 sec and extension at 72°C for 2 minutes. The procedure was performed according to the manufacturer specifications (Promega, USA, 1991).

6- Cloning of PCR products obtained from the investigated isolates

Cloning was carried out with PCR-Script SK(+) cloning Kit (statgene, USA) and the experimental conditions were those recommended by the manufacturers.

7- Transformation protocols

The ligated reaction were transformed into *E.coli* as the domestic host for that purpose. Competent cells of XL1-blue *E.coli* were prepared for transformation of recombinant plasmid by trituration procedure protocols and application guide (Promega, 1991), and the detection of recombinants was done by blue/white colony screening.

8-Isolation and purification of recombinant plasmid DNA from transformed cells

To confirm the cloning steps and to identify each clone, purified plasmid DNA was used as template for PCR using the same primer pairs used before, and the resulting product was analyzed along side the original total-community PCR products.

Preparation of recombinant plasmid DNA was done according to Sambrook *et al.* (1989), and as described before using a single bacterial colony of *E.coli* from each bacterial isolate.

Before restriction samples of white colonies were chosen for examination and screening using PCR technique, bacterial colony was touched with toothpick and resuspended in 50 µl sterile water then boiled for 5 minutes. To a PCR tube, 4 µl of cell extract was added to 25 µl PCR reaction containing the 2.5 µl 10X buffer, 10 pmol primer forward, 200 µM dNTPs, 10 pmol primer reverse, 1 unit *Taq* polymerase and water to 25 µl volume. PCR cycle was carried out at 95°C for 30 sec., 50 °C for 30 sec. and 72 °C for 2 minutes, for 30 cycles.

Recombinant plasmids were digested with the required restriction enzymes, which were purchased from New England Biolabs (Beverly, MA, USA), as described by the manufacturer. A total volume of 20 µl reaction mix containing 1 µg plasmid DNA, 2 µl of the 10X buffer and 10 units of both *EcoRI* and *HindIII* restriction endonuclease. The reaction mix was incubated at 37°C for 2 h, followed by heat inactivation at 65°C for 10 minutes. Agarose gel (1.2 %) was prepared whereas the restriction products were electrophoresed and run in 1X TAE buffer containing 0.1 µl /ml Ethidium bromide to stain the DNA for visualization with the UV transilluminator (λ= 375). Phages XI74 DNA digested with *Hae* III was used as size marker.

9- Sequencing of the cloned genes

The dideoxyribonucleoside chain termination procedure originally developed by Sanger *et al.* (1977), was employed for sequencing the double-stranded recombinant DNA plasmids obtained during the cloning procedure. The DNA sequence was determined by automated DNA sequencing method using ABI PRISM Big Dye terminator cycle sequencing ready reaction kit (PE applied Biosystems, USA), in conjunction with ABI PRISM (310 Genetic Analyzer). Cycle sequencing was performed using the Gene Amp 2400 Thermal Cycler, and the reaction was conducted in a total volume of 20 µl, containing 8 µl of terminator ready reaction mix, 1 µg of plasmid DNA, and 3.2 pmol of Ml3 universal forward primer (provided with the kit). The cycle sequencing program was 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes, repeated for 25 cycles with rapid thermal ramping. The nucleotide sequence was determined automatically by the electrophoresis of the cycle sequencing reaction product on 310 Genetic Analyzer. The data were provided as fluorimetric scans from which the sequence was assembled using the sequence analysis software. Alignment of DNA sequences was performed using the BLAST comparison with GenBank (Altschul *et al.* 1997). All chemicals and reagents mentioned under materials and methods were of the highest purity available and obtained from Sigma (Saint Louis, USA), FisherBiotech (FairLawn, USA), Acros (New jersey, USA), and Difco (Sparks, USA).

Results and Observations

1-Isolation and identification of bacterial isolates for heavy metals resistance

Two bacterial isolates designated A1 and A2 with remarkable resistance to Cd, Zn and Co were selected to the classical identification.

Preliminary identifications indicated that, the three isolates were Gram positive bacilli and spore forming. The results of biochemical analysis were compared according to Bergey's Manual of Systematic Bacteriology Sneath *et al.* (1986). The obtained data indicated that A1 and A2 are most probably species of *Bacillus cereus* sub group and *Bacillus megaterium* sub group respectively.

2- Determination of the minimum inhibitory concentrations (MICs)

The minimum inhibitory concentrations (MICs) of each isolate was determined by using spectrophotometer for estimating the size of populations, to determine the effect of metal salts on growth of cells, and consequently to evaluate the levels of resistance. The growth was monitored spectrophotometrically at 600 nm. MIC of cadmium, zinc, and cobalt for *Bacillus cereus* and *Bacillus megaterium* are presented in Table 1.

As shown in Table 1, the MICs of *B. cereus* for cadmium, zinc, and cobalt were approximately 293.3, 129.2 and 333.1 ppm, respectively, while those of *B. megaterium* were 146.4, 193.8, and 333.1 ppm for cadmium, zinc, and cobalt, respectively. Comparison of the obtained data indicated that each isolate exhibited a characteristic level of resistance to cadmium and zinc and they showed similar MICs to cobalt. *Bacillus cereus* was found to be more resistant to cadmium than the other isolate (Table 1).

TABLE 1. MICs of *Bacillus cereus* and *Bacillus megaterium* for $CdCl_2$, $Zn(NO_3)_2$ and $Co(NO_3)_2$.

	MIC of the tested metals					
Isolates	**$CdCl_2$**		**$Zn(NO_3)_2$**		**$Co(NO_3)_2$**	
	(ppm)	**OD**	**(ppm)**	**OD**	**(ppm)**	**OD**
B. cereus	18.3	1.9	32.3	2.11	47.6	2.6
	54.9	1.6	48.5	1.9	95.2	2.1
	109.8	1.5	64.6	1.7	142.7	2.1
	164.9	1.4	80.8	1.43	190.3	1.9
	219.9	1.35	96.9	1.3	237.9	1.4
	274.9	0.9	113.1	0.4	285.5	0.9
	293.3	0.0	129.2	0.0	333.1	0.0
B.megaterium	18.3	2.2	8	1.9	11.9	2.8
	36.6	2.1	32.3	1.7	47.6	2.5
	54.9	1.7	46.5	1.5	95.2	2.1
	73.2	1.5	96.9	1.3	142.7	1.9
	91.5	1.3	129.2	0.9	190.3	1.5
	109.8	0.8	161.5	0.5	237.9	0.4
	128.1	0.3	193.8	0.0	333.1	0.1
	146.4	0.1				

3- Evaluation of biosorption performance

The amount of metal biosorption of the investigated isolates is shown in Table 2. It is clear that *B. cereus* showed the highest level of resistance to Cd, Zn, and Co, the biosorption reaching approximately 82.6, 97.9 and 99 % respectively. The highest amount of biosorption *of B. cereus* for the tested heavy metals was observed with Co, while the lowest one was recorded with Cd (Table 2).

TABLE 2. Metal biosorption by the investigated isolates treated with tested heavy metals for four hours.

Isolates	Initial Cd 56.2 ppm*		Initial Zn 26.2 ppm*		Initial Co 5.9 ppm*	
	ppm**	%***	ppm**	%***	ppm**	%***
B. cereus	46.4	82.6	25.6	97.8	5.9	99
B.megaterium	14.6	31.3	17.4	66.4	5.8	97.6

*Initial concentration at zero time, **Residual metal concentration. ***Percentage of heavy metals removal.
Results represented average values

4-Isolation of genomic and plasmid DNA from the selected isolates

Genomic DNA was prepared by using Wizard® Genomic DNA Purification Kit. While Plasmid DNA was prepared by using Wizard® plus Minipreps DNA Purification Systems. Figure 1a shows intact DNA for each isolate, Fig. 1b shows the isolated plasmids for each isolate. The size of genomic DNA of each investigated isolate was ~ 13 Kb, while those of the plasmids were ~ 8.0 Kb. It is clear from the previous figure that each investigated isolate harboured only one plasmid.

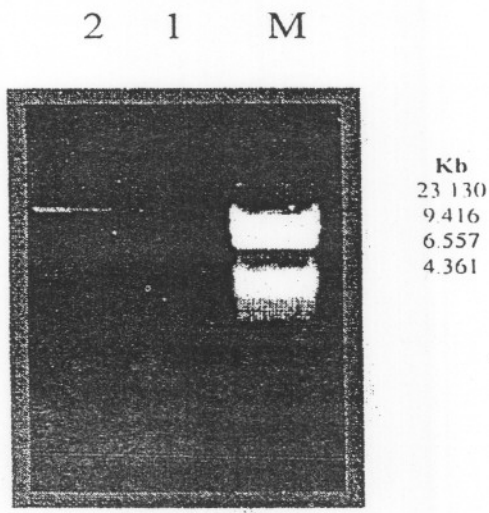

Fig. 1a. Agarose gel electrophoresis of genomic profile. M: DNA marker lambda DNA digested with HindIII marker, Lanes 1, 2 genomic profile of *Bacillus cereus* & *Bacillus megaterium*, respectively.

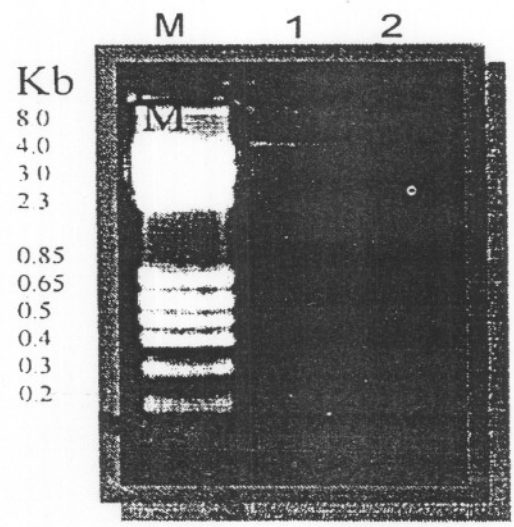

Fig. 1b. Agarose gel electrophoresis of plasmid profile. M: AGE1 markers, Lanes 1, 2 plasmid profile of *Bacillus cereus* & *Bacillus megaterium*, respectively.

5- Amplification of cadmium, zinc, & cobalt (Czc) resistance genes

A partial length *Czc* resistant gene have been detected and amplified from *Bacillus cereus* which showed high level of resistance to the tested heavy metals.

Amplification of ~ 400bp fragment was performed using the primer pair czcDl and czcD2 for genomic DNA of *Bacillus cereus.* Fig. 2 shows that the size of amplification product is identical to the expected amplified fragmented (~ 400 bp) encompassing the *Czc* gene lane 2.

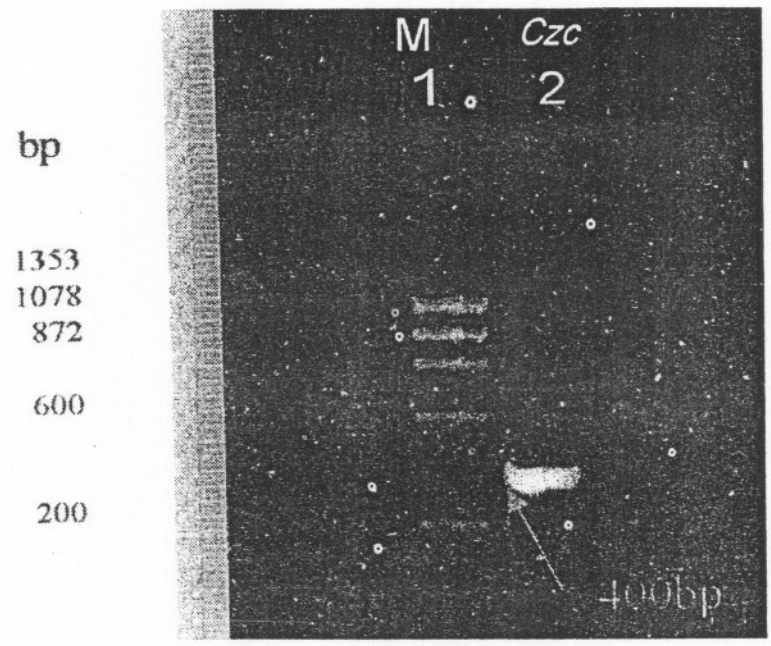

Fig. 2. Agarose gel electrophoresis of PCR amplification products of *Czc* gene, lane 1: M ΦXl74/HaeIII marker, lane2: (*Czc*) cadmium, zinc and cobalt resistance gene.

6- Cloning and transformation of PCR products

The previous PCR products of the putative *Czc* genes were purified and ligated into PCR-script SK (+) plasmid in the multiple cloning sites. Following ligation, plasmids were transformed into *E.coli* competent cells (XL-1 blue strain). The transformants were plated on LB-ampicillin plates containing X-gal & IPTG for identification of recombinants by the blue/ white colony screen. Some white colonies were picked and plated on a master plate, and others cultured in liquid media for DNA minipreps. To confirm the cloning step, white colonies were picked up and used as a template in the PCR reaction using *CzcD* primers. Figure 3 shows the amplified products, which have the same predicted size(~ 400 bp) for PCR product of *Czc* gene and was identical to the expected fragment in the positive control (lane 2). This fragment corresponds to the *CzcD* gene of the *Czc* operon. Blue colonies (negative control, lane 3, 4 and 5) showed no band, indicating no transformation and therefore no recombination (Fig.3).

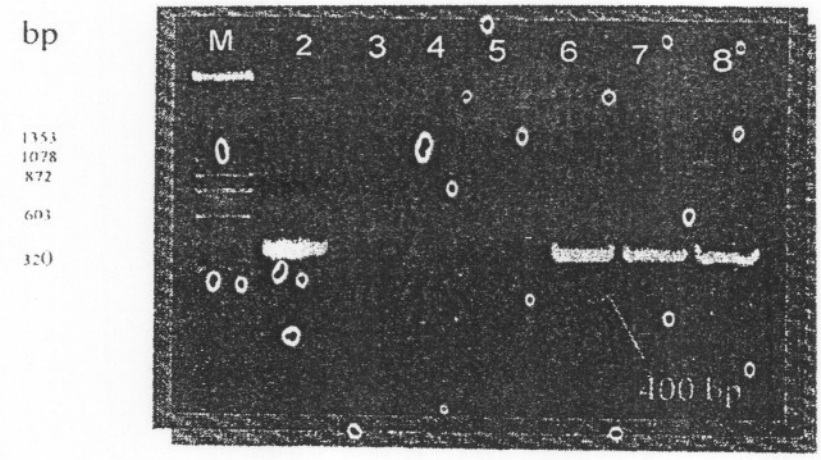

Fig. 3. Agarose gel electrophoresis of PCR screening of different white colonies of Czc resistant genes in *Bacillus cereus*. Lane 1: ΦX174 Lane 2: positive control, lanes 3, 4, 5: negative control blue colonies. Lanes 6,7, 8: amplified products from white colonies .

Clones containing *Czc* fragment *(pACzc)* were chosen for insert release. These samples were grown overnight in order to prepare plasmid minipreps for restriction digestion. Two restriction endonucleases, *EcoRl* and *Hind* III, were used for double digestion in the multiple cloning sites of the vector on either side of the cloning insert to allow releasing the inserted DNA fragment. Figure 4 shows the result obtained from the double digestion of *pACzc* clones. As indicated from this figure, the performed restriction digestion revealed that clones No. 4, 5 and 6 revealed insert release of the expected size for *Czc* gene (~ 400 bp). Bands of size 2900 bp (lanes 4, 5, 6) are the profile of vector without insert.

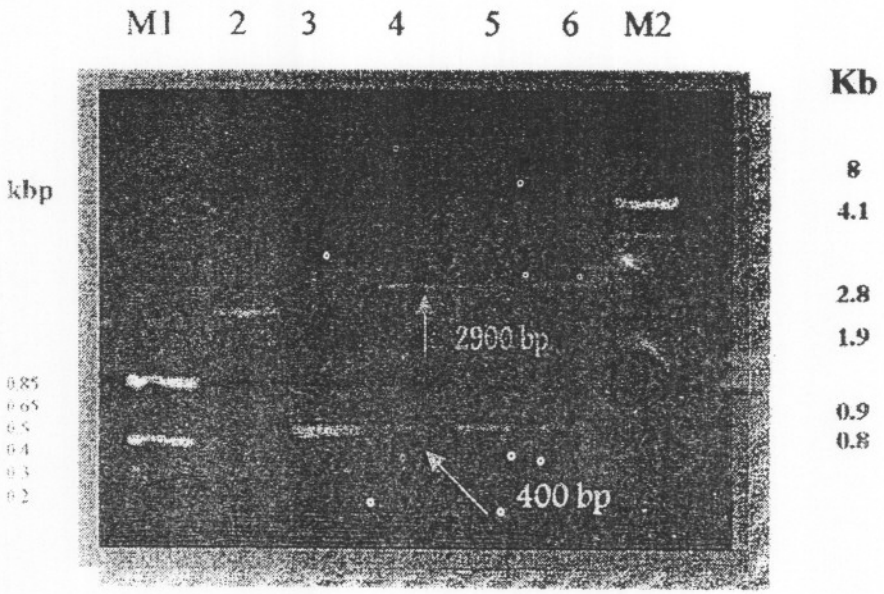

Fig. 4. Agarose gel electrophoresis of restriction digestion *of Czc* clones. M1: DNA marker (AGEI). Lane 2: undigested pA*Czc* clone, lane3: positive control of *Czc* PCR product, lanes 4, 5, 6: digested pA*Czc* clone with *EcoRl* and *HindIII* restriction endonuclease. M2: DNA marker (AGEII).

7- Sequencing of pACzc clones and computer analysis

The second step of confirmation was sequencing of pA*Czc*l clones to confirm the correlation between the isolated clones and published sequence in the-GenBank. Purified plasmid was used as a template in the sequencing reaction using Big Dye terminator ready sequencing Kit and M_{13} forward primers. The obtained sequence of pA*Czc*l clone was compared to those in public database. The result of this comparison is summarized in Table 3, and the graphical overview of the deduced nucleotide sequence is shown in Fig. 5. The alignment showed that there is 99 % similarity ratio between pA*Czc*l clone *of Bacillus cereus* and *Czc* gene of *Ralstonia* sp., *Ralstonia metallidurans* and *Alcaligenes sp.*

TABLE 3. Summary of sequence database search results for*Czc* genes.

Gene name	Source	DNA	
		Accession number	Identify %
Czc genes	*Ralstonia* sp.	X98451.1	99
Czc genes	*Ralstonia metallidurans*	AAAI01000324	99
Czc genes	*Alcaligenes sp.*	D67044.1	99

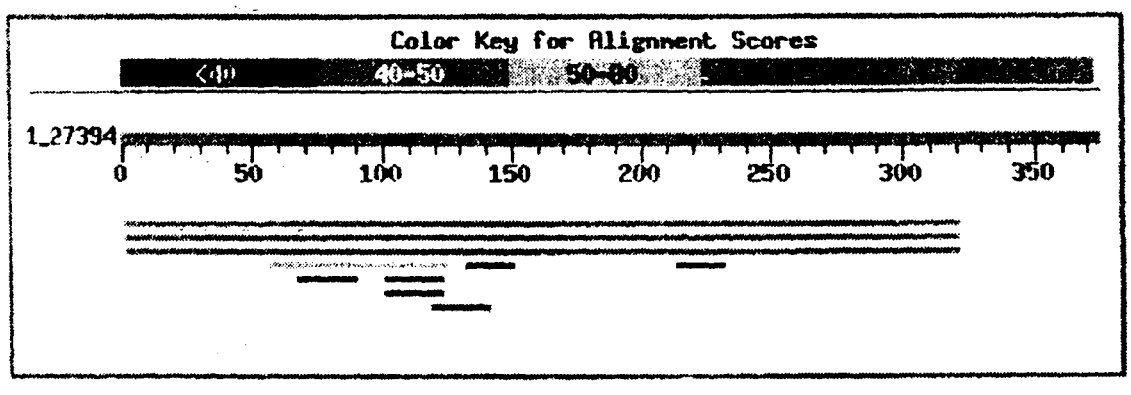

Fig. 5. Graphical overview of DNA alignment of pA*Czc*l clone scanned by BLASTn database.

Discussion

Wastewater from a chemical industry polluted by heavy metal ions represent a hazard for all living organisms. It can mean danger for ecosystem and human health. New methods are sought alternative to traditional chemical and physical processes. Active elimination process of heavy metals ions provided by living cells, their components and extracellular products represents a potential way of separating toxic heavy metals from industrial wastewater (Mikes *et al.*, 2005). The biological and chemical characteristics of these uptake processes are

important, not only as an aid in the understanding of the role of metallic ions in the basic cellular functions, but also as an aid to use for detoxification of industrial effluents because of possible use of biomass to protect the environment (Yilmaz, 2003).

The first identified member of the RND family was the *Czc*A protein from *Ralstonia metallidurans* (Nies, 2003). Resistance mediated by *Czc* is based on energy -dependent metal ion efflux (Nies and Silver, 1989). The *Czc* determinant contains three structural genes coding for subunits of the membrane- bound efflux complex *Czc*CB2A (Nies *et al.*, 1990; Rensing *et al.*, 1997b). The driving force for the export of heavy metal cations is not ATP, but the proton- motive force (Nies, 1995).

In Czc, as well as in other transenvelope transporters, one component transports the substrates across the cytoplasmic membrane ; this transporter may be a RND, an ABC or a MFS (major facilitator super-family) protein or protein complex. In the *Czc* system, this transporter is *Czc*A, *Czc*B and *Czc*C. Together, all three components could transport Co^{2+}, Zn^{2+} and Cd^{2+} across cytoplasmic membrane, periplasm and outer membrane (Rensing*et al.*, 1997b).

A considerable amount of work has been done to study the ability of *Bacillus* species to remove metals. The members of this genus are easy to culture and have shown high tolerance to heavy metal toxicity. *B. circulans* EB1 was able to grow at high concentrations of Cd, Co, Cu, Ni, Zn and Mn in liquid media, which might be important for the capacity of this bacterium to survive in different sources of pollution with elevated heavy metal levels (Yilmaz, 2003). Also the *mer* determinant was first described in *Bacillus cereus* RC607 from Boston Harbor, USA, and was then found in various *Bacillus* and related species in Japan, Russia and England (Bogdanova *et al.*, 2001).

Some *Bacillus* strains, from industrial wastewater oxidation ponds found in Sadat City, Egypt, were screened for their metal tolerance. The selection of the isolates was based on the ability to grow at high concentrations of Cd (274.9), Zn (193.8) and Co (452.1) ppm in liquid media.

The selected bacterial isolates were subjected to identification processes and biochemical investigations. The obtained data showed that the tested isolates are Gram-positive, bacilli and have most of the characteristics that resemble *Bacillus cereus* and *Bacilus megaterium* species, according to Bergy's Manual of Systematic Bacteriology (Sneath, 1986). MICs determination indicated that the isolated wastewater bacteria studied in the present investigation have developed resistance to the tested heavy metals (Cd, Zn and Co). MICs of *B. cereus* for Cd, Zn and Co were approximately 293.3, 129.2 and 333.1 ppm, respectively. The levels of tolerance shown by this bacterium were considerably greater than those reported by Hassen *et al.*, (1998) for *Bacillus thuringiensis* and *Pseudomonas aeroginosa.*

Mercury resistant marine bacterium, *Pseudomonas aeroginosa* CHO7 (NRRL B-30604), are found to be very promising in dealing with mercury (De *et al.*, 2006). The previous authors showed, in their study to investigate the effect of *P.aeroginosa* in removing Cd, that the culture was able to remove over 70% Cd from growth medium when supplemented with 100 ppm Cd.

Our results revealed that *Bacillus cereus* showed the highest level of biosorption of cadmium, zinc, and cobalt, reaching approximately 82.6, 97.9, and 99 %, respectively. Such values are confirmed by previous studies on the metal tolerance of *Bacillus* strains as reported by other investigators; among them El-Helow *et al.* (2000) and Yilmaz (2003).

The results of this study showed also the potential applicability of the recently isolated heavy metal-tolerant wastewater strains, especially *Bacillus megaterium* (A2) in the treatment of heavy metal containing solutions. Because efficient metal removal and growth over a wide range of heavy metal mixtures, under aerobic conditions are advantageous, this organism may be employed for metal remediation in simple reactors. Because of the only possible remediation methods of heavy metals are based on concentration and subsequent removal, this concentrated end product can afterwards be damped in a controlled way or recycled for metal recovery. Metals, which are readily available in wastewater, can be desolubilized via biologically- induced adsorption, precipitation, transformation or complexation processes (Diels *et al.*, 1999). The adsorptive treatment and bioprecipitation process are sensitive to ambient conditions while biosorption has been one of the most actively studied process. However, many aspects of metal-microbe interactions remain unexplored, further development and application are necessary.

Czc system of *Ralstonia* sp. was identified by van der Lelie *et al.* (1997). The CzcD protein is involved in the regulation of *Czc* system as reported by Anton *et al.* (1999). So, the amplification of *CzcD* from these isolates is a good indicator for detection of resistance to Cd, Zn and Co in the present study. In spite of the highly diverged *Czc* gene sequences among different bacteria (Trajanovska *et al.*, 1997), we had successfully amplified the *Czc* gene from *Bacillus cereus* using *CzcD* primer set. The lack of uniform amplification of various *Czc*-related sequences in other tested isolate, and/or the presence of fragments with sizes other than those obtained with positive control samples, suggest that either these sequences are present but highly diverged in such isolate, or they are absent in, at least, some of the tested strain. It is also possible that these bacteria have other genetic systems or unidentified genes that contribute resistance to the tested metals (Trajanovska *et al.*, 1997).

PA*Czc*I clones were confirmed by sequencing of these amplification products to confirm the correlation between the isolated clones and the published sequence in the GenBank. The major result of the present investigation is the demonstration of the regulatory genes responsible for Czc resistance, since the

alignment indicated that there is 99 % similarity ratio between *Bacillus cereus* of pACzcl clone and *Czc* gene of *Alcaligenes* sp., *Ralstonia* sp. & *Ralstonia metallidurans* (Kunito *et al.*, 1996 and van der Lelie *et al.*, 1997).

The possible avenue for future prospective can be summarized as follows: (I) Isolation of the full length gene of the isolated fragments of *Bacillus cereus* (Al). (II) Transformation of this gene into plant systems which can also be used for reduction of heavy metal contaminants in soils.(III) Investigations should continue to elucidate the genetic system of *Bacillus megaterium.*

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المعالجة الحيوية لمياه الصرف الصناعى .
٢- فصل و توصيف جين CzcD من بعض عزلات البكتيريا الموجبة الجرام

زكية أحمد أبو الخير*، أم محمد أحمد خفاجي* ، خالد صبري عبدالله، هشام ابراهيم القصاص*** و عبلة ابراهيم العوضي****
*قسم النبات- كلية العلوم (نبات) ، جامعة الأزهر، **معهد بحوث الهندسة الوراثية الزراعية - مركز البحوث الزراعية ***ومعهد الدراسات و البحوث البيئية – جامعة عين شمس – القاهرة - مصر .

في هذه الدراسة جمعت عينات مياه من ثلاث برك للصرف الصناعي من مدينة السادات بجمهورية مصر العربية. عزلت أنواع مختلفة من البكتيريا و تم اجراء التعريف لها بالطرق البكتيريولوجية و الكيمياء الحيوية كما قيمت قدرة العزلات على تراكم عناصر الكادميوم و الخارصين و الكوبالت.

أظهرت بعض هذه العزلات مثل *Bacillus cereus* (موجبة الجرام) مقاومة عالية للعناصر السابق ذكرها.

عين الجين المقاوم CzcD في هذا الكائن عن طريق استخدام تقنية تفاعل البلمرة PCR لتكبير جزء المادة الوراثية بوزن جزيئي حوالي ٤٠٠ زوج من القواعد و باستخدام $CzcD_1$، $CzcD_2$. كما تم إكثار و استنساخ الجزء المنتج من CzcD في خلايا الإيكولاي كمضيف و تم تعيين التتابع النيوكليوتيدي لـ DNA sequence.

قورن هذا التتابع بنظيره المنشور في بنك الجينات باستخدام برامج الحاسب الآلي. أظهرت المقارنة تطابق شديد لل CzcD تصل إلى ٩٩٪ بين *Bacillus cereus* (المعزول في هذه الدراسة) مع ذلك المعزول من أنواع *Ralstonia*.

تفتح نتائج هذا البحث المجال لمزيد من الدراسات على امكانية استخدام هذا الكائن في أنظمة المعالجة الحيوية لمياه الصرف أو نقل الجين لخلايا نباتية لتقليل الملوثات في التربة.