

Heavy Metal Resistant CZC Genes Identification in *Bacillus cereus*, *Enterobacter asburiae* and *Pseudomonas aeruginosa* Isolated from BHEL Industry, Tamilnadu

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ABSTRACT

Microbial interactions with metals may have several implications for the environment. The main resistance mechanisms for divalent heavy metals, such as Zn²⁺, Cu²⁺ and Ni²⁺ are active efflux transporters. This article explores the molecular basis of zinc resistance in the bacteria by determining the presence of znt-related genes and comparing the sequences obtained from these isolates with those published in the literature. It also investigates the adaptation of *Bacillus cereus* and expression of these znt genes. Heavy metal resistant genes were identified in *Bacillus cereus*, *Pseudomonas aeruginosa* and *Enterobacter asburiae*. CZC genes were observed in *Pseudomonas aeruginosa* and *Enterobacter asburiae*. Zinc transporting ATPase genes were observed in *Bacillus cereus*.

INTRODUCTION

Microbes may play a large role in the biogeochemical cycling of toxic heavy metals also in cleaning up or remediating metal-contaminated environments [1]. The term 'heavy metals' refers to metallic elements that have high atomic weights (>100) and a relative density >5. Some heavy metals, such as cobalt, copper and zinc, are essential micronutrients for biological systems, although they may be toxic in larger amounts. Other metals, such as mercury, cadmium and lead, are biologically non-essential and toxic in any quantity [2]. According to the Agency for Toxic Substances and Disease Registry (ATSDR, 2013), arsenic, lead and mercury comprise the top 3 in the list of top 20 hazardous substances, while cadmium is ranked 7th. Major sources of heavy metal contamination in the environment are the combustion of fossil fuels, the operation of smelters and other industrial activities such as mineral mining and processing, brewery and distillery wastes and the generation and use of agricultural chemicals [2]. Heavy metals do not degrade in soil and many are considered persistent bioaccumulative toxins (PBTs).

The risk to human and ecosystem health from land-application of PBTs in residuals depends on solubility and bioavailability of these contaminants in the residual-treated soil. Uncertainties exist in the effect of decomposition of soil organic matter complexes that bind metal and uncertainties of the effect of slower long-term reactions between metals adsorbed to inorganic oxide surfaces in soil on metal solubility and bioavailability. Recent research findings show heavy metal is sorbed to oxide phases of biosolids [3]. Heavy metals sequestered to oxide surfaces will likely remain sequestered longer than metal complexed by biosolids natural organic matter. However, the stability of metal sequestered by oxide depends on the metal and the mineral oxide surface. Long-term mineral crystallization reactions may "eject" metals from the solid phase into solution.

MATERIALS AND METHODS

PCR Analysis of the Presence of Zinc Resistance Genes based on the zntA Gene of *Escherichia coli*

To test for the presence of a typical Zinc-transporting ATPase/ cation-transporting ATPase, P-type, primers were designed. The zntA gene of *Escherichia coli* encodes a Zn(II)-translocating P-type ATPase were also used in the design of some primers [4]. Primer pairs were specifically designed to amplify overlapping fragments based on these zinc resistant genes for full sequence coverage of any amplified sections. The size and numbers of PCR fragments produced using the various primer pairs provided a preliminary estimation of which genes may be present or absent **Table 1**.

Table 1. PCR cycle conditions.

94 °C	94 °C	40-50 °C	72 °C	72 °C	4 °C
5 min	30 sec	30 sec	1.30 min	5 min	∞
10+35 Cycles					

PCR mix components: Sample gDNA: ~50 ng; Forward Primer: 400 ng; Reverse Primer: 400 ng; Reverse Primer: 400 ng; 10 mM dNTPs mix: 4 µl; 10 X AMTaq Pol. Buffer: 10 µl; AMTaq Polymerase enzyme: 3 U; PCR grade water: to make the volume up to 100 µl.

Identification of Heavy Metal Resistant Genes in *Pseudomonas aeruginosa* and *Enterobacter asburiae*

The gene expression of heavy metal resistant genes in *Pseudomonas* and *Enterobacter* species was studied. RNA Isolation by TRIZOL Method [5]. 1 ml of TRI Reagent was added per 10 cm² of glass culture plate to lyse the cells directly. The lysate was gently mixed by pipetting several times until a homogenous suspension was formed. The samples were transferred to a new 2 ml eppendorf tube and allowed to stand at room temperature for 5 minutes. 200 µl of chloroform was then added for each ml of TRI reagent used for lysis. The microfuge tube was vortexed vigorously for few seconds and allowed to stand for 12-15 min at room temperature for phase separation. The samples were centrifuged at 12000 xg for 15 minutes at 2-80 °C. Centrifugation separated the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colorless upper aqueous phase (containing RNA). The upper aqueous phase was transferred into a fresh 1.5 ml eppendorf tube and 0.5 ml of isopropanol per ml of TRI reagent was added to it, followed by gentle pipetting. The samples were allowed to stand for 5-10 minutes at room temperature and centrifuged at 12,000 xg for 10 minutes at 40 °C. The supernatant was carefully aspirated off the tube, leaving behind the precipitated RNA pellets on the sides of the tube. The RNA pellet was washed by adding a minimum of 1 ml of 75% ethanol per 1 ml of TRA reagent. The sample was then vortexed and then centrifuged at 7500 xg for 5 minutes at 2-80 °C. Note: Samples can be stored in ethanol at 2-80 °C at upto 1 year at -200 °C. The RNA pellet was air-dried and re-suspended in 20-30 µl of RNase free water. Preparation of RNA - (No DNA Contamination)

Added to an RNase-free tube

RNA: 1 µg; 10 X reaction buffer with MgCl₂: 1 µl; DNase 1, RNase-free: 1 µl; DEPC-treated water (#R0601): 10 µl

The tubes were incubated at 370 °C for 30 minutes. 1 µl of 50 mmol EDTA was added and incubated at 650 for 10 minutes. It was then preceded to cDNA conversion **Tables 2 and 3 and Figure 1.**

Table 2. *Bacillus cereus*- Genes for Metal Resistance.

10X RT Buffer, 1 ml	1 tube
10X RT Random Primers	1 tube
25X dNTP mix(100 mM)	1 tube, 0.2 ml
Multiscribe TM Reverse Transcriptase, 50 U/µl	2 tubes, 0.1 ml
RNase inhibitor, 100 µl	2 tubes

Table 3. Details of Primers used in *Pseudomonas aeruginosa* and *Enterobacter asburiae*.

#	ID	Sequence, 5' to 3'	Oligo Type	Scale	Purification	# bases
1	CzcR - FP	gtcatcaccggagcgcagatcat	DNA	25 nmole	Desalt	23
2	CzcR - RP	gtagccgacgccggaatggtat	DNA	25 nmole	Desalt	23
3	pcoA gene FP	ACYTACTGGTAYCACAGCCATTC	DNA	25 nmole	Desalt	23
4	pcoA gene RP	CCACATVCCRTGCAGGTG	DNA	25 nmole	Desalt	18
5	nccA gene FP	ACGCCGACATCACGAACAAG	DNA	25 nmole	Desalt	21
6	nccA gene RP	CCAGCGCACCGAGACTCATCA	DNA	25 nmole	Desalt	21
7	β-Actin gene FP	GTGGGCCGCTCTAGGCACCAA	DNA	25 nmole	Desalt	21
8	β-Actin gene RP	CTCTTTGATGTCACGCACGATTTTC	DNA	25 nmole	Desalt	24
9	CopA1	TCCATACACTGGCACGGCAT	DNA	25 nmole	Desalt	20
10	CopA2	TGGATCGGGTGAGTCATCAT	DNA	25 nmole	Desalt	20
Total						214

Seq Name	Sequence (5'->3')	Length	Yield [OD]	Yield [µg]	Yield [nmol/ml]	Volume 100 pmol/ul	Tm [°C]	MW [g/mol]	GC content	Purification
1202948-Fwd	ATGGCTGAAGCGTTAGTAAAAAGAAACT	29	10.5	346.5	38.4	384	57.3	9023	38%	Desalted
1202948-Rev	CCTATAAATCTTCACTCGTAATACACGCAT	31	11.2	369.6	39.4	394	56.4	9388.2	32%	Desalted
1202806-Fwd	ATGAACTCAGAAGTAAAAACGGTTACACGCA	30	9.6	316.8	34.3	343	57.5	9225.1	37%	Desalted
1202806-Rev	CGTTATTTGCTCTTTTAGTAATCGTAAGC	31	12.5	412.5	43.6	436	57.8	9457.2	35%	Desalted
11111914-Fwd	ATGACCACTCAACGCCCTTAGCTACGA	26	10.5	346.5	43.9	439	59.5	7884.2	50%	Desalted
11111914-Rev	TTAGCATCCCGCTGCGATGTAATCGC	26	12.4	409.2	51.7	517	61.1	7922.2	54%	Desalted

Figure 1. Primer details of genes present in *Enterobacter asburiae*

Gene-1: (Zinc-transporting ATPase/cation-transporting ATPase, P-type) Primers Used

Forward Primer: ATGGCTGAAGCGTTAGTAAAAAGAACT

Internal Primer: GTCGGTGGAGATATCGTTTGGAGA

Reverse Primer: CTTATAAATCTTTCCTACTCGTAATACACGCAT

Gene-2: (Zinc uptake P-type ATPase) Primers Used:

Forward Primer: ATGAACTCAGAAGTAAAAACGTTACACGCA

Reverse Primer: CGTTATTTGCTTCCTTTTAGTAATCGTAAGC

RESULTS AND DISCUSSIONS

Identification of (Zinc-transporting ATPase/cation-transporting ATPase, P-type and Gene-2: (Zinc uptake P-type ATPase) in *Bacillus cereus*

Chromatogram results of genes identified in supplementary 1. Real-time PCR was performed on an ABI 7900 Fast HT Sequence Detector (Applied Bio systems, Foster City, Calif.) (Figures 2a and 2b). Q-PCR mixtures contained SYBR green master mix. Annealing temperatures, primer concentrations, and MgCl₂ concentrations were optimized. Fluorescence measurements were taken during the final temperature ramp. RT PCR amplification is done using these specific primers to study the gene expression as Delta CT Values. The genes CZC, PCOA, COP & NCC with endogenous control B actin. An endogenous control gene is a gene whose expression level should not differ between the samples, such as a housekeeping or maintenance gene. Comparison of the CT value of a target gene with that of the endogenous control gene allows the gene expression level of the target gene (CZC, PCOA, COP & NCC) to be normalized to the amount of input RNA or cDNA. The CZC gene expression is up regulated (Figures 3 and 4) in both these bacteria, which are indicated by the CT values, while in case of other genes it is not expressed (down regulated).

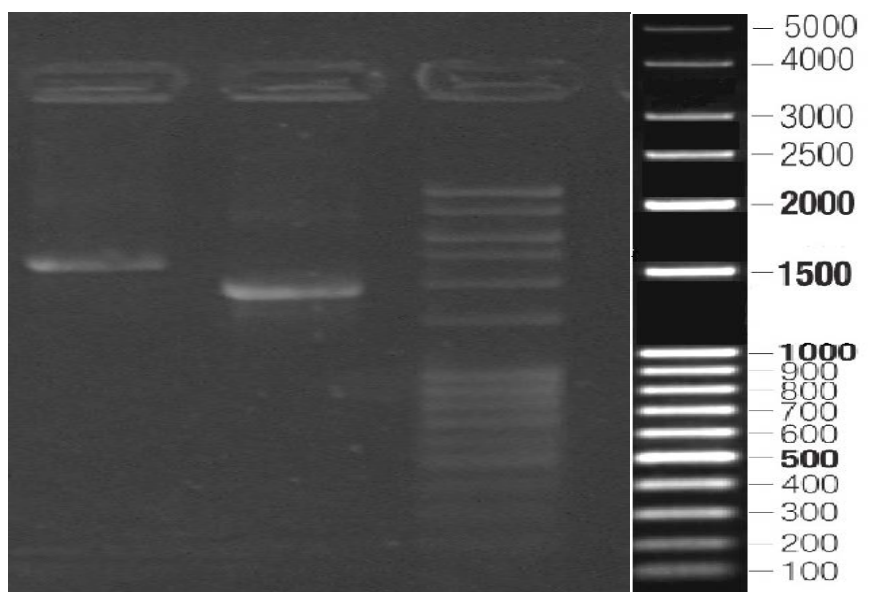


Figure 2. *Bacillus cereus*- Genes for metal resistance.

CT<29 are strong positive reactions indicative of abundant target nucleic acid in the sample

CT of 30-37 is positive reactions indicative of moderate amounts of target nucleic acid.

CT of 38-40 are weak reactions indicative of minimal amounts of target nucleic acid which could represent an infection state or environmental contamination genes related to heavy metal tolerance within the genome of isolated strain.

DISCUSSION

Zinc Resistant Genes in *Bacillus cereus*

The genes screened included zinc-transporting ATPase gene, zinc uptake P-type ATPase gene in *Bacillus cereus* and CZC genes in *Pseudomonas aeruginosa* and *Enterobacter asburiae*. The integral membrane P-type ATPases are an important class of ion transport proteins that serve to maintain suitable ionic conditions. The term “P-type” refers to the formation of a phosphoenzyme intermediate in the reaction cycle. The energy released by the removal of the gamma-phosphate from ATP is coupled to the translocation of an ion across biological membranes. Substrates are inorganic cations such as H⁺, Na⁺, K⁺, Mg²⁺, Ca²⁺, Cu⁺, Ag⁺, Zn²⁺, Cd²⁺, Co²⁺ and Pb²⁺. P-type ATPase-based resistance to zinc has been reported in *E. coli* and in *P. putida* strain

S4 [5]. The *ZntA* P-type ATPase of *E. coli* has been predicted to be the first example of a zinc-specific transporter, based upon the close sequence homology of this ATPase to the *CadA* ATPase of *Staphylococcus aureus* and *Helicobacter pylori* enzymes, which affect the cellular zinc content. P-type ATPases mediating cadmium resistance also bring about zinc efflux in most cases, while the P-type ATPases transport zinc only across the cytoplasmic membrane [6]. P-type ATPases constitute a superfamily of transport proteins that transport ions against the concentration gradient using energy provided by ATP hydrolysis.

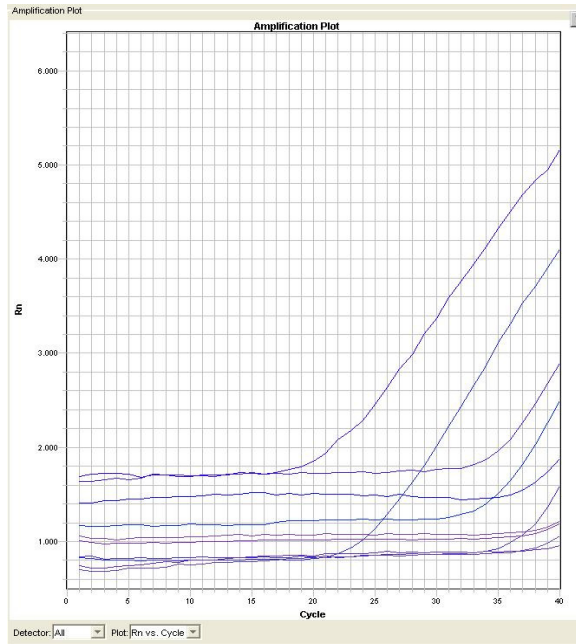


Figure 3. Represents the amplification plot for all the genes studied in *Pseudomonas aeruginosa* and *Enterobacter asburiae*

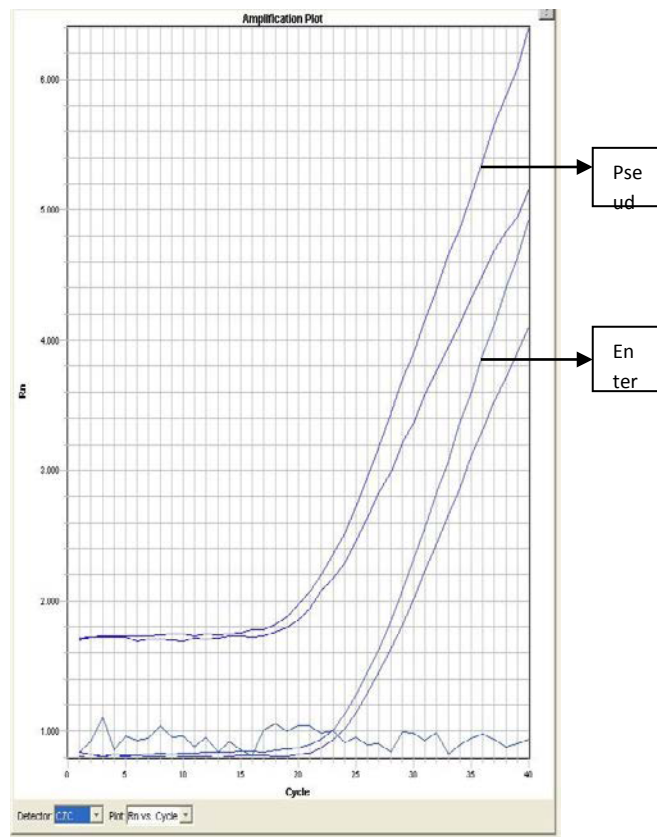


Figure 4. Represents the amplification of CZC gene in *Pseudomonas aeruginosa* and *Enterobacter asburiae*.

Gene Expression of Heavy Metal Resistant Genes in *Pseudomonas* and *Enterobacter* Species

Analysis of changes in gene expression is an important part of fundamental and applied research. Northern blot, slot/dot blot, DNA arrays, and reverse transcription (RT)- PCR analyses are established techniques to estimate gene-expression rates, e.g., under different growth conditions. The genes *CZC*, *PCOA*, *COP* & *NCC* with endogenous control B actin were studied in

Pseudomonas aeruginosa and *Enterobacter asburiae*. Presence of CZC genes were expressed in *Pseudomonas aeruginosa*. The three structural genes *czcCBA* are transcribed as a tricistronic mRNA of 6200 nucleotides [7]. These three genes are encoded in the structural gene region located in the center of the *czc* determinant. Two regulatory regions flank the structural genes, the upstream regulatory region, and the downstream regulatory region. Nine *czc* genes are transcribed in the same direction. This has also revealed that the CZC determinant was not species specific and is widely spread among bacteria isolated from entirely distinct geographical location. The best studied zinc resistance mechanism is the Czc system operating in *Ralstonia*, a Gram-negative soil bacterium. The Czc system conferring resistance to cadmium, zinc and cobalt in *R. eutrophus* strain CH34, functions as cation/proton antiporter effluxing cations from the cells [8-9]. The *czc* determinant encodes resistance to Cd²⁺, Zn²⁺ and Co²⁺ by metal-dependent efflux [10] driven by the proton motive force [11].

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