# Tolerance of Heavy Metals by Gram Positive Soil Bacteria

I. V. N. Rathnayake, Mallavarapu Megharaj, Nanthi Bolan, and Ravi Naidu

*Abstract*—With the intention of screening for heavy metal tolerance, a number of bacteria were isolated and characterized from a pristine soil. Two Gram positive isolates were identified as *Paenibacillus sp*. and *Bacillus thuringeinsis*. Tolerance of $Cd^{2+}$, $Cu^{2+}$ and $Zn^{2+}$ by these bacteria was studied and found that both bacteria were highly sensitive to $Cu^{2+}$ compared to other two metals. Both bacteria showed the same pattern of metal tolerance in the order $Zn^+ > Cd^{2+} > Cu^{2+}$. When the metal tolerance in both bacteria was compared, *Paenibacillus sp*. showed the highest sensitivity to $Cu^{2+}$ where as *B. thuringiensis* showed highest sensitivity to $Cd^{2+}$ and $Zn^{2+}$. These findings revealed the potential of *Paenibacillus sp*. in developing a biosensor to detect $Cu^{2+}$ in environmental samples.

*Keywords*—Heavy metals, bacteria, soil, tolerance.

## I. INTRODUCTION

HEAVY metals are often defined as a group of metals whose atomic density is greater than 5 g/cm³ [1],[2]. Metals play a vital role in the metabolic processes of the biota. Some of the heavy metals are essential and are required by the organisms as micro nutrients (cobalt, chromium, nickel, iron manganese and zinc etc.) and are known as 'trace elements' [3]. They are involved in redox processes, in order to stabilize molecules through electrostatic interactions, as catalysts in enzymatic reactions, and regulating the osmotic balance [2],[4]. On the other hand some other heavy metals have no biological role and are detrimental to the organisms even at very low concentration (cadmium, mercury, lead etc.). However, at high levels both of the essential and non essential metals become toxic to the organisms.

These heavy metals influence the microbial population by affecting their growth, morphology, biochemical activities and ultimately resulting in decreased biomass and diversity [5]. Heavy metals can damage the cell membranes, alter enzymes specificity, disrupt cellular functions and damage the structure of the DNA. Toxicity of these heavy metals occurs through the displacement of essential metals from their native binding sites or through ligand interactions [3]. Also, toxicity can occur as a result of alterations in the conformational structure of the nucleic acids and proteins and interference with oxidative phosphorylation and osmotic balance [6],[3].

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Due to the selective pressure from the metal in the growth environment, microorganisms have evolved various mechanisms to resist the heavy metal stress. Several metal resistance mechanisms have been identified: exclusion by permeability barrier, intra and extra cellular sequestration, active transport, efflux pumps, enzymatic detoxification, and reduction in the sensitivity of the cellular targets to metal ions [6],[3].

Heavy metal contamination in the environment has become a serious problem due to the increase in the addition of these metals to the environment. Natural sources as well as the anthropogenic sources account for this contamination, which has become a threat to public health. Cadmium, copper and zinc are among those heavy metals that are being released to the environment [7].

In this perspective many approaches have been used to assess the risk posed by the contaminating metals in soil, water bodies etc. At present the tolerance of soil bacteria to heavy metals has been proposed as an indicator of the potential toxicity of heavy metals to other forms of biota [9],[10]. Therefore, there is a dramatic increase in the interest on studying the interactions of heavy metals with microorganisms. The favoured approach now is selecting the organisms that can be used to develop tools to assess the metal levels in the environment. The objective of this study was to isolate and identify the bacteria from uncontaminated soil to determine their tolerance to cadmium, copper, and zinc.

## II. PROCEDURE

### A. Test Chemicals and Media

Stock solutions of cadmium, copper and zinc were prepared by dissolving the respective nitrate salt (Sigma) in MilliQ water. Working test metal solutions were prepared by diluting the concentrated stock solutions as required, and were sterilized by filtration. All glassware was acid washed before use to avoid binding of metal. All the media used in the experiments were dissolved in MilliQ water and sterilized by autoclaving.

### B. Isolation of the Bacteria

Bacterial cultures were isolated from a pristine soil sampled from Mt. Lofty, South Australia using the standard dilution plate technique. The soil was characterised in our previous study which was found to contain the metals levels below the National Environment Protection (Assessment of site contamination) Measure (NEPM) ecological investigation levels (EILs) for the metals in soil [11]. A 10-fold dilutions of fresh soil (1 g) were made in phosphate buffered saline and

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0.1 ml from each of these dilutions were spread on triplicate trypticase soy agar plates. Plates were incubated at $25^{\circ}$ C for 2-3 days. Colonies with different morphological appearance were selected from these culture plates and purified by further subculturing in the same media. All the cultures were stored at -80ºC in the trypticase soy broth with 20% glycerol.

### B. Identification of Bacteria

Two isolates were selected based on the Gram's reaction and further characterized using morphological (shape, motility, presence of endospores), biochemical (catalase activity, oxidase activity, acid production from glucose, oxidation fermentation reaction (OF) characteristics) properties [12].

Based on preliminary screening 2 isolates were selected for further taxonomic identification using molecular techniques. The crude DNA extracts were prepared from bacterial cultures grown in Luria Bertani medium. The cultures were harvested by centrifugation at 2000 x g for 10 minutes and washed twice with 1 ml of sterile TE buffer (10 mM Tris, 1m M EDTA). After the final wash the pellet was re-suspended in 50 uL of sterile TE buffer containing 1% Triton X-100. The tubes were then incubated at $70^{\circ}$C for 30 mins, vortexed and placed on ice. To collect cellular debris the tube was centrifuged at 10 000 x g for 5 min., and the supernatant was collected [13]. The crude DNA extract was diluted in sterile water just prior to carrying out PCR under the following conditions. A 25 uL PCR mixture contained 1 x concentration of Taq DNA polymerase buffer, 2.5 mM $MgCl_2$, 200 uM betaine, 0.2 mM of each deocynucleoside trisphosphate, 25 pmol of each forward and reverse primers, 1 U of DNA polymerase (Promega), and 1 uL of the diluted DNA extract as template. Almost complete 16S rRNA genes were amplified with the forward primer E8f (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse primer 1541r (5'-AAGGAGGTGATCCANCCRCA-3') [14]. The DNA was amplified with a iCycler thermocycler (BioRad, Sydney) with the following program: 5 min of preheating at 95°C, 30 cycles of 30 s of denaturation at 95°C, 30 s of primer annealing at 55°C, and 2 min of elongation at 72°C. A final extension step of 10 min at 72°C was included. Successful amplification of a ~1525 bp DNA fragment was confirmed by running 5uL of the PCR reaction on a 1% agarose gel. PCR reactions were purified using MoBio UltraClean PCR Purification Kit (Geneworks, Adelaide), before being submitted for sequencing at the Flinders DNA sequencing facility (Adelaide).

All 16S rRNA gene sequences from the clone libraries were aligned with the "align" tool as available on greengenes website [15]. Most similar 16S rRNA genes sequences from the greengenes database [16] were also included in the alignment, and a phylogenetic tree constructed with MEGA, version 4.0 [17].

### C. Determination of the Effect of Metals on Bacterial Growth

Toxicity of the selected metals to the bacterial isolates was determined using seven concentrations of each metal. These concentrations ranged from 0 to 8.0 mg/L medium. Several 48 well sterile polystyrene microplates (Iwaki polystyrene, sterile, non treated, flat bottom with lid) were used in this study as growth vessels. Sterile MES buffered minimal medium (developed in our previous study) was amended with each heavy metal and inoculated with exponentially growing cultures (24 h old, optical density of 0.090 at 600 nm) of bacterial isolates prepared in the same medium. Medium without metal but the bacterial inoculum (bacterial growth control) and medium with metal but without bacteria (abiotic control) served as controls. All the experiments were conducted in triplicate. Microplates were then closed with their lids with condensation rings and sealed using additional laboratory film (Parafilm® M). The test microplates were incubated at 25ºC on an orbital shaker at 100 rpm. Bacterial growth was measured in terms of optical density at 600 nm for 4 days at 24 hour intervals using the Bio-Tek® Synergy™ HT Multi-Detection Microplate Reader with equipped with KC4 software.

### D. Statistical Analysis

Non linear regression analysis was performed with the statistical program Grapher 7, to fit the data obtained for the heavy metal toxicity experiments to the logistic model [18]. $EC_{50}$ values (statistically derived estimate of a concentration of a substance resulting in 50% reduction of the growth within a specified time) were estimated and presented as mean $EC_{50}\pm$ standard deviation based on the dose-response data obtained from 3 replicate samples.

### III. RESULTS AND DISCUSSION

#### A. Identification of the Isolated Bacteria

The results of the morphological and biochemical identification experiments are shown in the Table I. Both of these bacterial isolates are rod shaped, spore formers. They showed almost same response to the biochemical reactions tested during the study. Based on the molecular analysis data, a phylogenetic tree was constructed by comparing nucleotide sequences with available 16S rRNA sequences. The two bacterial isolates were identified as *Paenobacillus sp.* and *Bacillus thuringiensis*. Constructed phylogenic tree was presented in the Fig. 1. *Paenobacillus sp.* was originally grouped within the genus *Bacillus*. Both of these bacterial genera represent the common soil bacteria and have been reported as soil inhabitants [5],[19],[20].

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Vol:3, No:5, 2009

TABLE I
MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF BACTERIAL ISOLATES

| Code | Shape | Motility | Gram's reaction | Endospores | Oxidase | Catalase | Acid from glucose | OF test |
|------|-------|----------|-----------------|------------|---------|----------|-------------------|---------|
| $B_3$ | short rod | + | + | + | - | + | + | O |
| $B_7$ | rod | + | + | + | - | + | + | O |

+ - positive; ─ – negative; O – Oxidative

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        68 ┌ Paenibacillus sp. str. DSM 1352 (AJ345017)
   100 ┌──┤ B3
        └ Paenibacillus sp. H08-06 (AM162336)
   ┌ Bacillus sp. str. cp-h12 (EU584533)
   ┤ Bacillus thuringiensis str. TCCC11336 (FJ393313)
   83 └ B7
   0.01
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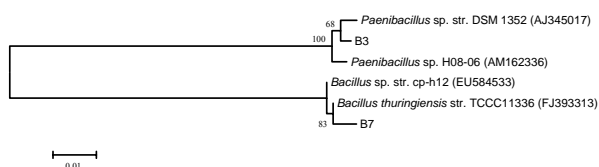
Fig. 1 Phylogenetic tree constructed from the 16S rRNA gene sequences determined from genomic DNA extracted from bacteria isolates and similar sequences as available on Greengenes. Original tree was constructed with a neighbour joining algorithm from an alignment of 1271 nucleotides. Accession numbers of corresponding sequences are given in parenthesis and the scale bar represents 1 base substitution per 10 nucleotide positions. The bootstrap probabilities calculated using neighbour joining, maximum likelihood and parsimony algorithms from 1000 replications with values greater than 60 are indicated at nodes
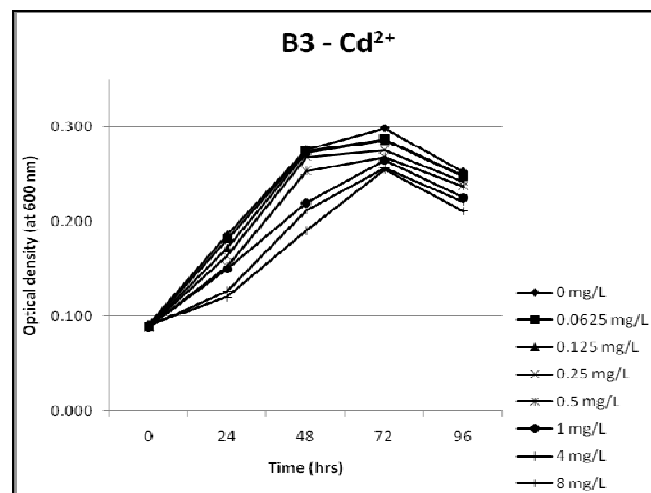
*B. Determination of the Effect of Metals on Bacterial Growth*

The metal response experiments were carried out in a minimal medium (formulated in our previous study) which maintains a high free metal concentration in solution. Two Gram positive isolates exhibited different growth patterns in the presence of different heavy metals. The growth curves for *Paenobacillus sp.* and *B. thuringiensis* in the presence of different metal concentrations are shown in the Figure 1 & 2. *Paenobacillus sp.* exhibited a growth curve similar to the typical bacterial growth curve over the experiment time period (96 hrs). *Paenibacillus sp.* increased its growth and reached its maximum growth at 72 hrs. A decrease in growth (measured in terms of optical density) was observed upon increasing metal concentration at any given time interval compared to the control without metal amendment. The lower optical density values revealed that the bacterial growth was affected due to the presence of metal in the growth medium. On the other hand in the case of *B. thuringiensis* the growth increased steadily over the entire 96 h experimental period. However, the reduction of the growth in the presence of increased concentration of the metals used in the study was evident throughout the experiment compared to the control without

metal. But its growth was not affected much in the presence of $Zn^{2+}$. A similar finding was reported elsewhere [21].

Two bacterial isolates showed different levels of tolerance to the metals under investigation and the dose-response data for these bacteria are shown in Table II.

*Paenibacillus sp.*, showed a higher sensitivity to $Cu^{2+}$ with 50 % growth inhibition observed at 0.011 mg/L of $Cu^{2+}$. *B.thuringiensis* was not as sensitive as *Paenibacillus* to $Cu^{2+}$ because the derived $EC_{50}$ value was 0.82 mg/L. A study carried out elsewhere reported a minimum inhibitory concentration of 3.5 mM of $Cu^{2+}$ on *Paenibacillus polymixa* [22]. The tolerance of $Cd^{2+}$ by the two bacterial isolates was different from each other, and the bacterium that was most sensitive to $Cd^{2+}$ was *B.thuringiensis.* However, there were reports on $Cd^{2+}$ resistant *B.thuringiensis* strains which were able to absorb cadmium [23]. They also reported that a strain of this species has relatively a long lag phase in the presence of $Cd^{2+}$. The levels of $Zn^{2+}$ tolerance among the bacterial isolates were different from one to the other showing the reduction of their growth to 50 % in the presence of 2.98 mg/L of $Zn^{2+}$ by *B.thureingiensis* and 7.02 mg/L of $Zn^{2+}$ by *Paenibacillus sp.* This finding revealed that *Paenibacillus sp.* can tolerate more than twice the level of $Zn^{2+}$ than *B.thuringiensis.* When the tolerance of three metals was compared with respect to each bacterium, it was evident that both of the bacteria were more sensitive to $Cu^{2+}$ than the other two metals. Both of the bacterial isolates were more tolerant to $Zn^{2+}$ than $Cd^{2+}$. Although there are several reports on the metal binding and biosorption capacity of these two bacterial genera [23],[22],[24], studies on the metal tolerance are scarce. However it is not easy to make a meaningful comparison with the findings reported in the literature due to the range of protocols and media used.

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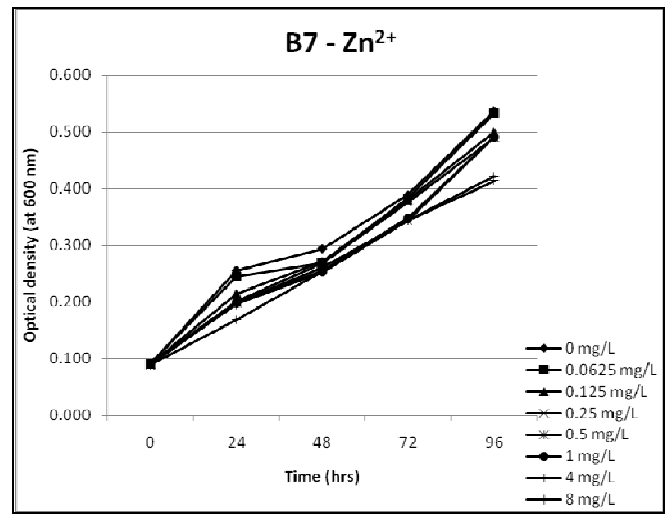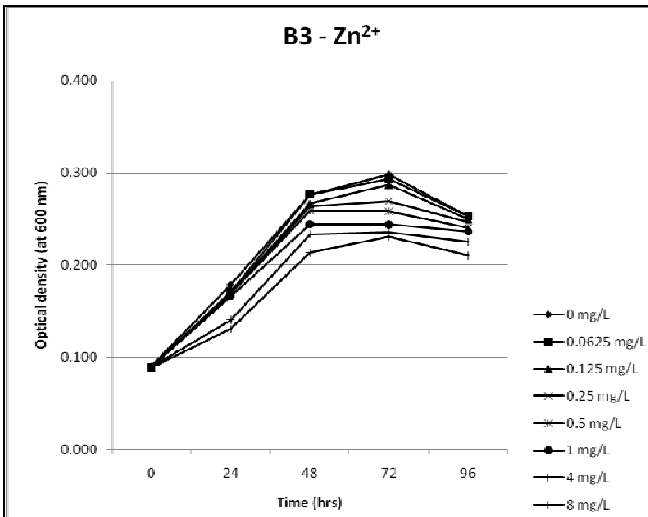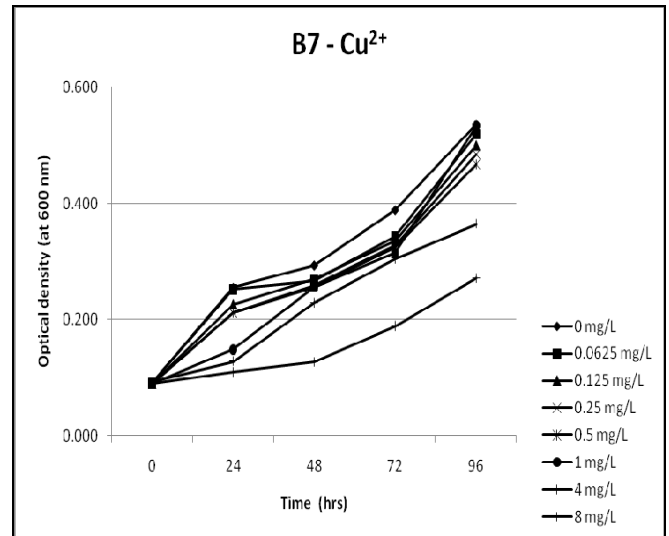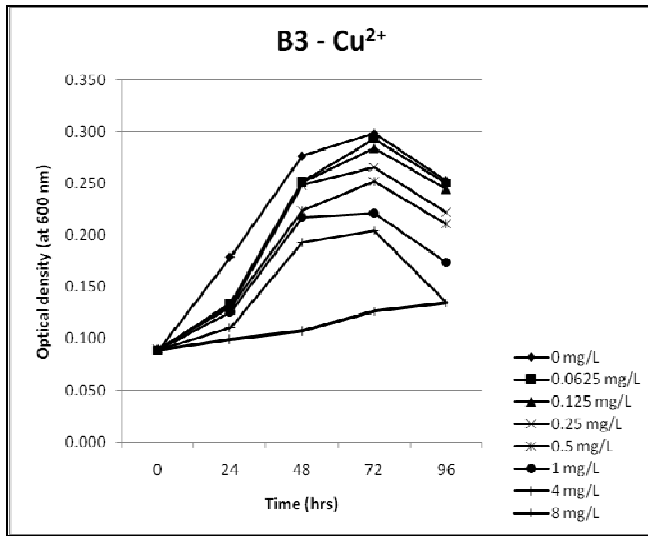Vol:3, No:5, 2009

Fig. 2 Growth curves of *Paenibacillus sp.* in the presence of different heavy metals

Fig. 3 Growth curves of *B. thuringiensis* in the presence of different heavy metals
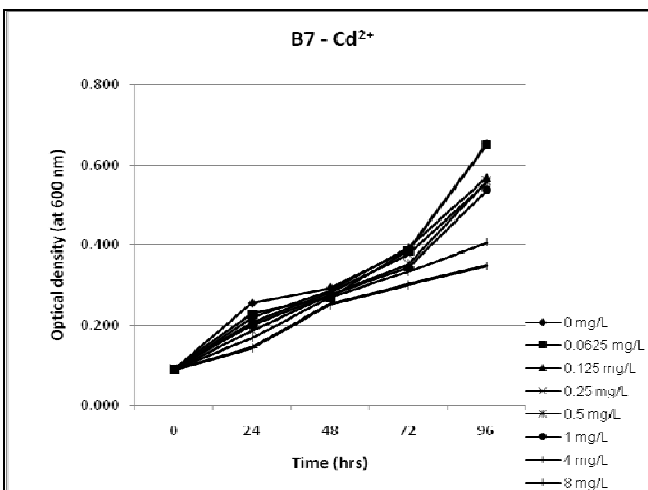


TABLE II
ESTIMATED EC$_{50}$ VALUES FOR *PAENIBACILLUS SP.*, AND *B.THURINGIENSIS*

| Bacterium | Cd$^{2+}$ (mg/L) | Cu$^{2+}$ (mg/L) | Zn$^{2+}$ (mg/L) |
|---|---|---|---|
| *Paenibacillus sp.* | 1.77± 0.16[*] | 0.011±0.003 | 7.02±0.44 |
| *B.thuringiensis* | 1.53±0.01 | 0.82±0.04 | 2.98±0.2 |

[*]Mean ±standard deviation

## IV. CONCLUSION

The current work demonstrated that the tolerance of heavy metals varied between bacteria even though they were isolated from the same soil. Both the Gram positive bacteria were highly sensitive to Cu$^{2+}$ than the other two metals. *Paenibacillus sp.* has great potential to be used as a biosensor to assess the Cu$^{2+}$ toxicity in the environment due to its high sensitivity to Cu.

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