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Research article

# Tolerance and growth kinetics of bacteria isolated from gold and gemstone mining sites in response to heavy metal concentrations

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ABSTRACT

Response and growth kinetics of microbes in contaminated medium are useful indices for the screening and selection of tolerant species for eco-friendly bio-augmentative remediation of polluted environments. In this study, the heavy metal (HM) tolerance, bioaccumulation and growth kinetics of seven bacterial strains isolated from mining sites to 10 HMs (Cd, Hg, Ni, Al, Cr, Pb, Cu, Fe, Mn and Zn) at varied concentrations (25–600 $mgL^{-1}$) were investigated. The isolates were phylogenetically (16S rRNA gene) related to *Lysinibacillus macroides*, *Achromobacter spanius*, *Bacillus kochii*, *B. cereus*, *Klebsiella pneumoniae*, *Pseudomonas mosselii* and *P. nitroreducens*. Metal tolerance, effects on lag phase duration and growth rates were assessed using the 96-well micro-titre method. Furthermore, metal bioaccumulation and quantities within cells were determined by transmission electron microscopy and electron dispersive x-ray analyses. Tolerance to Ni, Pb, Fe and Mn occurred at highest concentrations tested. Growth rates increased with increasing Fe concentrations, but reduced significantly ($p < .05$) with increasing Zn, Cu, Hg, Cd and Al. Significantly higher ($p < .05$) growth rates (compared to controls) was found with some isolates in Hg (25 $mgL^{-1}$), Ni (100 $mgL^{-1}$), Cr (150 $mgL^{-1}$), Mn (600 $mgL^{-1}$), Pb (100 $mgL^{-1}$), Fe (600 $mgL^{-1}$) and Al (50 $mgL^{-1}$). Lag phase urations were isolate- and heavy metal-specific, in direct proportion to concentrations. *A. spanius* accumulated the most Mn and Zn, while *B. cereus* accumulated the most Cu. Metals accumulated intra-cellularly without cell morphology distortions. The isolates' multi-metal tolerance, intra-cellular metal bioaccumulation and growth kinetics suggest potentials for application in the synergetic biodegradation and bioremediation of polluted environments, especially HM-rich sites.



## 1. Introduction

Heavy metal (HM) pollution originating from anthropogenic sources is fast becoming a global burden. Heavy metal pollution results in soil degradation, nutrient depletion, impairment of water and air quality, and specifically, poses risk to human health and safety (Kaplan et al., 2011; Oladipo et al., 2016a, 2016b). Conventional methods, including physical and chemical approaches, have been employed as HM clean-up strategies. However, these technologies present technical and economic constraints, such as high operational costs, increased energy consumption and generation of huge toxic sludge (Azubuike et al., 2016). While the reduction of environmental HM releases is advocated, a cost effective, eco-friendly and efficient strategy is required for the remediation of heavy metal polluted environments.

In recent times, there has been a surge in the exploration of microbial treatment (bioremediation) options for the clean-up of HM-contaminated environments. Microbial treatment is considered a relatively cost-effective, environmentally friendly and sustainable strategy compared to physical and chemical remediation technologies (Azubuike et al., 2016; Gadd, 2010; Valls and Lorenzo,

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2002). Furthermore, the higher metal uptake capacity, ease of application and maintenance (requires less technical support) and minimal sludge production are additional advantages over other remediation strategies (Valls and Lorenzo, 2002). Previous studies indicate that bacteria and fungi possess inherent capacities, which enable them to scavenge HM from their environment (Giudice et al., 2013; Oladipo et al., 2016a, 2017).

In multimetal-stressed environments (especially when heavy metal threshold limits are exceeded), bacterial cells develop, and/or trigger diverse detoxifying physiological, morphological and adaptive survival mechanisms. These include bioaccumulation (Jayanthi et al., 2017), biosorption (Fashola et al., 2016; Fomina and Gadd, 2014) and biotransformation (Gadd, 2010; Uroz et al., 2009). Other heavy metal tolerance mechanisms include intracellular and extracellular precipitation (Mohamed and Farag, 2015), enzymatic oxidation or reduction, active efflux pumps and secretion of siderophores (Gaonkar and Borkar, 2017; Ianeva, 2009). One or more of these mechanisms are responsible for bacterial tolerance to numerous heavy metals such as iron, nickel, manganese, aluminium, copper, zinc, mercury, selenium, cadmium, lead, chromium, and arsenic, among others (Burkhardt et al., 2011; Jackson et al., 2012; Jayanthi et al., 2017; Manegabe et al., 2017; Neethu et al., 2015).

Of more importance are bacterial strains isolated from HM-contaminated sites which are known to exhibit exceptional metal-resistance traits when compared to same species from uncontaminated sites (Muñoz et al., 2012; Pereira, 2017; Rauwane et al., 2017). For this reason, contaminated sites such as mineral ore mining environments are thus regarded as principal sources of multi-metal tolerant species (Egamberdieva, 2017; Muñoz et al., 2012; Pereira, 2017). The response and growth kinetics of microbes isolated from such contaminated sites to HM concentrations may serve as useful bio-indices for the screening and selection of HM tolerant species.

In this study, we assessed the tolerance competence of seven bacterial species isolated from gold and gemstone mining sites to 10 heavy metals at varied concentrations. We further investigated the quantities and location of bioaccumulated heavy metals within bacterial cells of selected isolates. This study is important for bioprospecting and identification of heavy metal tolerant bacterial strains as bioremediation agents for heavy metal contaminated environments.

## 2. Materials and methods

### 2.1. Sources of isolates

Seven bacterial isolates were used in this study. They were isolated from soils of a gold mine located in Itagunmodi (7°30′N, 4°49′E) and gemstone mining site located in Awo (7°46′N, 4°24′E), South-West Nigeria. Details of the bacterial isolation methods and elevated heavy metal levels of the sites are presented in Oladipo et al. (2014).

### 2.2. Partial 16S rRNA gene-based identification of isolates

For this study, isolates were identified by sequencing the partial 16S rRNA gene. To achieve this, genomic DNA was extracted from isolates using the Nucleospin tissue kit (Macherey-Nagel, Bethlehem, PA, USA) and following the manufacturer's instruction. For the amplification of the 16S rRNA gene, universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) were used. PCR was performed in a C1000TM thermal cycler (Bio-Rad, Hercules, CA, USA). Each PCR reaction contained 12.5 μL of 2x Dream Taq PCR master mix (Thermo Scientific Technologies, Waltham, MA, USA), 50 ng DNA template, 0.25 μM of each forward (27F) and reverse (1492R) primers and nuclease-free water to a final volume of 25 μL. PCR conditions were exactly as described by Ezeokoli et al. (2016). PCR amplicons were purified prior to sequencing of the partial 16S rRNA gene (hypervariable region V1-V3) using primer 27F. For the taxonomic assignment, sequences were aligned against sequences in the GenBank using the EZBioCloud database (http://www.ezbiocloud.net). Phylogenetic association of sequences with closely related sequences in the GenBank were depicted by constructing a neighbour-joining tree as previously described (Oladipo et al., 2016a).

The partial 16S rRNA sequences are available in the GenBank under the accession numbers KT819126-KT819133.

### 2.3. Maximum metal tolerance assessment and effect on bacterial growth kinetics

Ten heavy metals were used in this study, including cadmium, mercury, nickel, aluminium, lead, chromium, copper, iron, manganese and zinc (Table 1). The heavy metals were divided into three groups (I, II and III) based on toxicity and/or concentrations commonly reported in previous tolerance studies. The concentration range for each group was selected (Table 1) so that it ranged from minimal concentrations to concentrations exceeding the world soil permissible limits (Kabata-Pendias, 2010). Each heavy metal salt was dissolved in appropriate quantities of sterile distilled water and filter-sterilized (0.2 μm pore filters).

For the heavy metal tolerance and growth kinetics assessment, a 24-h old suspension culture (approximately $10^6$ CFU/mL) of isolates in Mueller-Hinton broth (MHB) was used. The tolerance assessment was performed in a 96-well micro-titre plate. Briefly, each well contained a final volume of 220 μL, comprising 170 μL autoclaved sterile MHB, 30 μL filter sterilized metal salt solution (to give a final metal concentration indicated in Table 1), and 20 μL of the 24 h-old suspension culture of isolate. Experimental controls and blank wells were also setup; controls for each isolate comprised 200 μL MHB and 20 μL of the 24-h old isolate culture, while blanks contained only 220 μL MHB. All tests were performed in triplicates. The micro-titre plates were incubated at 30°C for 48 h (2880 min) in a Microplate HT reader (Powerwave Microplate spectrophotometer, BioTek Instruments Inc., Winooski, VT, USA). Automated measurement of optical density (OD) was taken at 520 nm every 30 min. Turbidimetric growth curves generated from OD measurements were then used for the determination of growth rate (μ) and duration of lag phase (λ) as described in the statistical analysis section below.

### 2.4. Quantification and localization of heavy metals within bacterial cells

Electron dispersive x-ray (EDX) and transmission electron microscopy (TEM) analyses were used for quantifying and detecting the location of selected heavy metals (Cu, Zn and Mn) within bacterial cells, respectively.

For EDX analysis, six bacterial isolates adjudged metal tolerant were selected for this test. The basis for this selection was founded in the outcome of the heavy metal tolerance assessment conducted in this study (findings are detailed in section 3.2 below). The isolates were inoculated onto nutrient agar (NA) supplemented with Cu, Zn and Mn at 200, 300 and 600 $mgkg^{-1}$, respectively, and incubated at 30°C overnight. After incubation, the bacterial cells were transferred into 1.5 mL micro-centrifuge tubes and washed with distilled water five times. Thereafter, the washed cells were transferred onto carbon coated aluminium stubs for viewing under