
ISOLATION AND CHARACTERIZATION OF NEW METALLOTOLERANT BACILLI STRAINS

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ABSTRACT

A total of ten mesophilic and thermotolerant metallotolerant bacilli were isolated from water and soil samples and identified up to species. The morphological, physiological and biochemical properties of metallotolerant strains were described, as well as 16S rDNA sequence analyses were carried out. The stability of isolates was demonstrated in the presence of different concentrations of Cd²⁺, Cu²⁺, Zn²⁺ and Ni²⁺ in growth media. The ability of isolates to accumulate mentioned metals was studied.

Keywords: accumulation of metals, metal tolerance, metallotolerant bacilli

Introduction

Metallotolerant microbes capable of tolerating and detoxifying high levels of dissolved heavy metals are a group of extremophiles of interest for both fundamental and applied researches (7, 12, 22). Polluted soils and waters with untreated industrial and urban wastes and samples of natural environment with high concentration of metals are the best source for isolation of metallotolerant microbes. Metallotolerant microbes have been found in all bacterial groups studied, mostly among aerobic and facultative aerobic chemo-heterotrophic microorganisms of *Staphylococcus*, *Escherichia*, *Pseudomonas* and *Bacillus* genera (3, 4, 6, 12, 15, 19).

Developed heavy metal resistance systems are mostly plasmid-mediated (18, 20). Among the various adaptation mechanisms, metal sorption, mineralization, uptake and accumulation, extracellular precipitation, enzymatic oxidation or reduction to a less toxic form, and efflux of heavy metals from the cell has been reported (2, 4, 7, 12, 15, 17, 19, 20). Cell wall of some representatives studied of the genus *Bacillus* contains the carboxyl, phosphate, and hydroxyl functional groups, which bind various metals and provide metal absorption to bacterial surface (5).

Many metallotolerant microbes may play an important role in the biogeochemical cycling of toxic heavy metals (21, 22). They could be new resources for active biosorbents of heavy metals and could have several applications in bioremediation of metal-polluted sites. Frequency of appearance of bacteria resistant to specific heavy metal can be used as biological monitors of environmental contamination. It was reported that thermophilic microorganisms in particular, due to their unique metabolic properties and cell wall structure, could be prospective in biotechnological application (9, 10, 21).

The aim of this study was isolation and characterization of heavy metal resistant mesophilic and thermotolerant bacilli of hot mineral spring Hankavan, heavy metal-polluted silt and water samples of Lake Yerevan, and soil samples collected at area of Zangezur Copper and Molybdenum Combine, Kajaran (Armenia).

Materials and Methods

Sampling and study sites

Samples from 3 above mentioned locations were placed into sterile screw cap bottles. Geographical coordinate of studied sites was determined using JPS. Temperature, pH and mineralization were determined *in situ* using a combined pH/EC/TDS/Temperature electrode (HANNA HI98129/HI98130). The water, silt and soil samples from

different sites were transported immediately after collection to the laboratory for analysis.

Enrichment and isolation

To isolate aerobic mesophilic and thermotolerant bacilli from samples, the latters (1 ml of water or 1 g of sediment) were inoculated in enrichment medium and incubated overnight at 37°C and 55°C with shaking at 240 rpm. Before inoculation all samples were subjected to 80°C for 10 min to isolate only spore-forming microorganisms. Basal medium had following composition: yeast extract 5 g/l, peptone 5 g/l, NaCl 5 g/l, glucose 1 g/l. The mixture of heavy metals (CuSO₄·5H₂O 10 mg/l, NiCl₂·6H₂O 7 mg/l, ZnSO₄·7H₂O 6 mg/l, CdCl₂·xH₂O 3 mg/l) were supplied into the basal medium after sterilization (autoclaving at 121°C for 15 min) and cooling. Cultures were then purified by streaking samples on the same medium supplemented with agar (2%, w/v). All colonies obtained on plates were picked and purified by streaking onto same medium at least three times. The subcultures were considered pure after microscopic observation - one type of bacterium per culture.

Phenotyping analysis

Morphological properties and staining

Cellular morphology and motility were determined by light microscope (Olympus CH2). Gram stain was made by the common method (14). For revealing of endospores the cells were stained according to the Peshkov's method (14).

Physiology and biochemical properties

The temperature range for growth was determined after incubation of isolates at 5 to 80°C with 5°C intervals. The pH dependence of growth was tested at pH range from 5 to 12. The range of NaCl concentrations for growth was determined by adding from 0 to 10% NaCl to the incubation medium. Catalase activity was determined by bubble formation in a 3% hydrogen peroxide solution. Anaerobic growth of the isolates was tested by growth in solid anaerobic stab cultures covered by paraffin layer. Reduction of nitrate to nitrite, Voges-Proskauer reaction, formation of dihydroxyacetone was determined according to Gordon et al. (8). The utilization of citrate and propionate was determined using the Simmon's medium (14). The casein and starch hydrolyses were tested by streak plate technique (1, 14). The gelatin liquefaction was determined by inoculating the bacilli into gelatin nutrient medium (gelatin 10 g; distilled water 100 ml) and incubation at 37°C for 24 hours (1, 14). The lipolytic activity was determined by the hydrolysis of Tweens 20 (polyoxyethylene sorbitanmonolaurate), 40

(polyoxyethylene sorbitanmonopalmitate), 60 (polyoxyethylene sorbitanmonostearate), 80 (polyoxyethylene sorbitanmonooleate) (1, 14). The basal medium with inverted Durham tubes was used to test acid and gas production from D-(+)-glucose, L-(+)-arabinose, D-(+)-xylose and D-(-)-mannitol.

Antibiotic sensitivity

Sensitivity of the strains to antibiotics was tested using the basal solid medium and sensitive discs. The following antibiotics were used: erythromycin (15 µg), tetracycline (30 µg), gentamicin (120 µg), rifampicin (5 µg), streptomycin (30 µg), ampicillin (10 µg), cefalotin (30 µg), kanamycin (30 µg), zinacef (30 µg), augmentin (30 µg) and ceftriaxone (30 µg).

Genotypic analysis

Nucleic acid extraction and polymerase chain reaction (PCR)

For the extraction of Genomic DNA from bacilli isolates a GenElute™ Bacterial Genomic DNA Kit (Sigma) was used according to the manufacturer's recommendations. Extracted DNA was used as templates for amplification 16S rRNA gene sequences by PCR. The "universal" oligonucleotide primers 16SF (5'-GAGTTTGATCCTGGCTCAG-3') and 16SR (5'-GAAAGGAGGTGATCCAGCC-3') were used for amplification of bacterial 16S rRNA genes.

PCR mixtures used for amplification of sequences contained 1 µl DNA, 5 µl 10×PCR buffer, 5 µl 10 mM dNTP, 1 µl each primer (25 pmol/µl), 2 µl MgCl₂, 0.2 µl *Taq* DNA polymerase, 0.1% bovine serum albumin, and sterile water to a final volume of 50 µl. PCR amplification was done with the following program: 96°C for 3 min; 30 cycles of denaturation at 96°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2.30 min; and a single final extension at 72°C for 10 min.

Sequencing and phylogenetic analysis

PCR products were purified with GenElute™ PCR Cleanup Kit. Sequencing was performed on ABI PRISM capillary sequencer according to the protocol of the ABI Prism BigDye Terminator kit (Perkin Elmer). Homology searches from the nucleic acid sequences were performed using the Blast algorithm (Altschul et al. 1990) at the NCBI (National Center for Biotechnology Information: <http://www.ncbi.nlm.nih.gov/Blast>). All sequences were compared with other sequences in the GenBank database to identify closest relatives.

Phylogenetic tree was made by DNASTAR and

Metal tolerance

Determination of minimal inhibition concentration (MIC)

MIC of each metal was determined by plating cultures on solid basal medium with different concentrations of separate metals Cu²⁺, Cd²⁺, Zn²⁺ and Ni²⁺ (from 10 to 300 mg/l). Plates were incubated at 37°C for mesophiles and 55°C for thermotolerant strains during 24-72 hours (16).

Influence of complex of heavy metals on bacteria growth

The influence of mixture heavy metals was determined by cultivation in basal medium containing from 1 to 10 mg/l for each metal at 37°C, with shaking. The extent of growth was estimated by determination of the optical density (Spectrophotometer-26, λ=595 nm) of the culture liquid after each 4 hours of incubation (6, 17).

Heavy metal accumulation by isolates

Bacterial cultures were grown in the 250ml flasks (final concentration of culture liquid was 1 liter) containing basal medium with mix heavy metals salts in concentration of 1mg/l for each metal. Flasks were incubated at 37°C for 24 hours, with shaking (200 rpm). Bacterial cells were harvested by centrifugation at 6000 g for 20 min at 4°C. Pellet was washed twice with distilled water, and then centrifuged at 6000 g for 20 minutes. Pellet was dried at 80°C overnight (13). Concentration of heavy metals in the dried cells was measured by means of spectral analysis (XRF spectrum analyzer NITON 700 sense).

Results and Discussion

A total of ten metalotolerant aerobic chemoorganotrophic bacilli strains were isolated from water and soil samples (Table 1).

All studied strains were Gram-positive, motile rods with oval endospores. Optimal temperature of growth for strains 1A, 1B, 1C, 2A, 2B, 3A, Met1, Met5 was 50-55°C, for strains Cu6 and Cu7 – 35-40°C. The pH range for growth was 5.5-9 with the optimum at 7. The optimal NaCl concentration for 1A strain was 2.5-3.5 %, for strains 1C and Met1 was 5-6%. Growth was aerobic, although the 1B, 2A, 2B, 3, Cu6 and Cu7 strains were able to grow under anaerobic condition too. The catalase test was positive for all strains. They used citrate, assimilated D-glucose, L-arabinose and D-mannitol by forming acids, but did not produced gas from glucose. The strains 1A and 1C were positive according to Voges-Proskauer test. The isolates hydrolyzed gelatin, casein, starch, Tween 20 and Tween 40. Different phenotypic and biochemical characters of the strains are shown in Table 2.

TABLE 1

Isolated strains and physicochemical characteristics of sampling sites

Location	Geographic coordinates	Temperature (°C)	pH	Mineralization (mg/l)	Isolated strains
Hot mineral spring Hankavan	N40°37'57.55" E 44°29'04,65"	>44	7.0-7.2	6722.9	3A
Lake Yerevan	40°9'35.04"N 44°28'36.54"E / 40.15973333°N 44.4768167°E	>25	8.5-8.8	-	1A, 1C, 1B, 2A, 2B, Met1, Met5
Zangezur Copper and Molybdenum Combine (Kajaran)*	39°09'04"N 46°09'36"E / 39.15111°N 46.16°E	-	-	-	Cu6, Cu7

(-) - Not determined.

(*) - The mine of Kajaran contains 87% of copper and 8% of molybdenum.

TABLE 2

Phenotypic characters of isolates

Characteristics	1A	1B	1C	2A	2B	3A	Met1	Met5	Cu6	Cu7
Cell size > 10µm	-	-	-	-	-	-	+	-	+	+
Anaerobic growth	-	+	-	+	+	+	-	-	-	-
Voges-Proskauer test	+	-	+	-	-	-	-	-	-	-
pH in V-P broth	<6	>7	<6	>7	>7	>7	>7	>7	>7	>7
Acid from D-Glucose	+	+	+	+	+	+	+	+	+	+
L-Arabinose	+	+	+	+	+	-	+	+	-	-
D-Manitol	+	+	+	+	+	-	+	+	+	+
Hydrolysis of Casein	+	+	-	-	+	+	+	+	+	+
Gelatin	+	+	+	+	+	+	+	+	+	+
Starch	+	+	+	-	+	+	+	+	+	+
Tween 20; 40; 60; 80	+	+	+	+	+	+	+	+	-	-
Nitrate reduction to nitrate	-	-	-	-	-	-	-	-	-	-
Formation of Dihydroxyacetone	-	-	-	-	-	-	-	-	+	+

Following to the criteria of *Bergey's Manual of Determinative Bacteriology* (1, 11) the isolates were identified as *Bacillus subtilis* (1A, 1C, Met5), *B. megaterium* (1B, Met1, Cu6, Cu7), *B. brevis* (2B), *B. circulans* (3A) and *B. sp.* (2A).

The identification of strains 1A, Met1 and Cu6 was confirmed by 16S rDNA sequence analysis. The 16S rDNA sequence of strain 1A was almost identical to the 16S rDNA sequence of *Bacillus subtilis* (PCL1608) – 99%. For Cu6 16S rDNA sequence was nearly identical to the 16S rDNA sequence of *B. megaterium* (LCR43) – 99%, what conformed identification by phenotype characters. From 16S rDNA analysis results, strain Met1 was most related (89%) to *B. pumilus* (3L-10F), what differed from identification by phenotype characters and may be considered as a new bacillus strain. A phylogenetic tree including isolates and related species is given in **Fig. 1**.

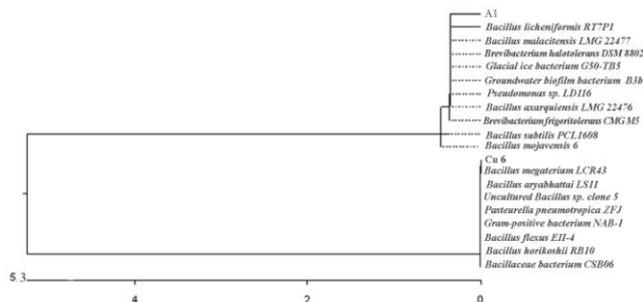


Fig. 1. Phylogenetic tree of isolates based on 16S rDNA comparison

The isolated metalotolerant bacilli showed high resistance to metals Cu^{2+} and Ni^{2+} . The MIC of Cu^{2+} for 1C, Met5, Cu6 and Cu7 strains was 200 mg/l, for strains 1A, 1B, 2A, 2B, 3A and Met1 it was 150 mg/l. MIC of Ni^{2+} for strain 1C was 250 mg/l, for strains 1A, Cu6 and Cu7 it was 200 mg/l, for strains 1B, 2A, 2B, 3A, Met1 and Met5 it was 150mg/l. MIC of Zn^{2+} for strains 1A, 1B, 1C, 2A, 2B, Met1 was 70 mg/l, for strains 3A, Met5, Cu6 and Cu7 it was 50 mg/l. MIC of Cd^{2+} – 10 mg/l.

The effect of mixture of heavy metals on growth of 1A and 1C cultures showed the stimulation of bacterial growth in the presence of metals in concentration 1 mg/l and inhibition in the presence – 4 mg/l (**Fig. 2**).

For strain Cu6 was used basal medium with Cu^{2+} in concentrations from 10- to 50 mg/l. Cu^{2+} had stimulation effect on Cu6 strain growth in concentrations up to 50 mg/l (**Fig. 3**). The higher concentrations had inhibitory effect.

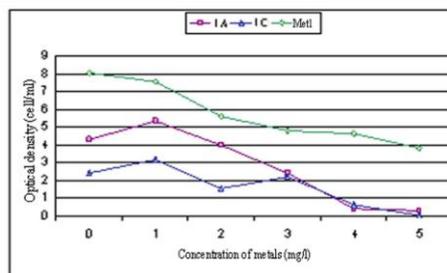


Fig. 2. Influence of mix metals on growth of 1A, 1C, Met1 strains

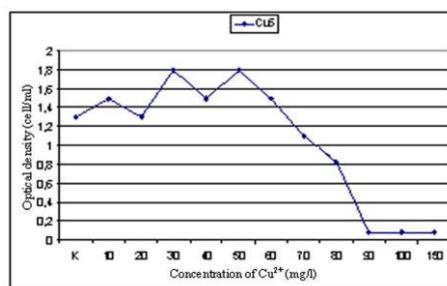


Fig. 3. Influence of Cu^{2+} on growth of Cu 6 strain

The stimulation of cultures' growth by traces of heavy metals could be explained by known Arndt-Shultz effect consisting in the intensification of bacterial metabolism. The accumulation of not lethal concentrations of heavy metals on the cell surfaces changed the membrane permeability and disrupted their barrier function, due to which the nutrient uptake as well as the cell metabolism were increased (7).

For strain Met1 stimulation effect was not detected, and besides the inhibition effect was not expressed so obvious.

The study of the ability of isolates to accumulate heavy metals showed the high accumulation of Cd^{2+} and Cu^{2+} (**Fig. 4**).

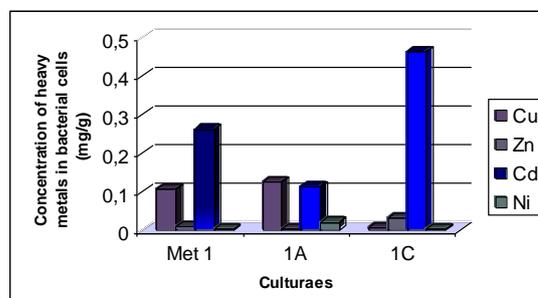


Fig. 4. Accumulation of heavy metals by 1A, 1C, Met1 strains

Ability of strains to accumulate the metals is characterized as follows: for 1C - $\text{Cd}^{2+} > \text{Zn}^{2+} > \text{Ni}^{2+}$, for 1A - $\text{Cu}^{2+} > \text{Cd}^{2+} > \text{Ni}^{2+}$, for Met1 - $\text{Cd}^{2+} > \text{Cu}^{2+} > \text{Zn}^{2+}$. Ni^{2+} and Zn^{2+} almost were not accumulated. According to primary results the strains 1C and Met1 can be considered as the

highest Cd²⁺ accumulators.

Metal resistance is often associated with antibiotic resistance (3, 19). Resistance to these both factors can be transferred between organisms via conjugation or transduction. In some cases resistance to certain antibiotics and metals is mediated by the same plasmids (7, 19, 20). However in our experiments we found the high sensitivity of isolates to the tested antibiotics, so the correlation between metal tolerance and antibiotic resistance in studied isolated strains was not revealed. Resistance to the ampicillin, zinacef, augmentin and ceftriaxone was detected only for strains 1B, 2B, 2A, 3A.

All isolated strains are maintained in the culture collection of the Department of Microbiology and Biotechnology of YSU and will be used in the further research to study their potential in bioremediation and biotechnology.

Conclusions

The main conclusions of our investigation are follows:

- a) Ten mesophilic and thermotolerant metallotolerant bacilli were isolated and identified up to species.
- b) All isolates were demonstrated high stability in the presence of different concentrations of Cu²⁺ and Ni²⁺ in growth media.
- c) Strains *Bacillus subtilis* 1C and *B. megaterium* Met1 can be considered as the highest Cd²⁺ accumulators.

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