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# Identification and Characterization of Aluminium Tolerant Bacteria Isolated from Soil Contaminated by Electroplating and Automobile Waste

**H. Bisht* and N. Kumar*†**

*Department of Biotechnology and Bioinformatics, NIIT University, Neemrana-301705, Rajasthan, India

†Corresponding author: N. Kumar; narayan.kumar@niituniversity.in

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## ABSTRACT

Due to anthropogenic activities and the advancement of industries, metal contamination is growing globally. Aluminum toxicity is seriously endangering plants, animals, and humans by rapidly rising in soil and water. Even though some fungi can tolerate aluminum, researchers are interested in finding bacteria that are resistant to aluminum. The current state of knowledge on bacteria resistant to aluminum is extremely limited. In the present study, bacterial isolates from soil near a metal electroplating and automobile industry in Punjab, India, were isolated and then screened for high aluminum metal tolerance. The aluminum tolerant bacterial isolate was identified as *Cedecea davisae* M1, a member of the *Enterobacteriaceae* family, using morphological, biochemical, and 16srRNA gene sequence analyses. The spectroscopic results indicate that the strain may tolerate up to 150 ppm of aluminum. Antibiotic resistance of *Cedecea davisae* M1 was determined using disks on Luria agar plates, and the bacteria were found to be resistant to vancomycin, ampicillin, carbenicillin, and rifampicin. The findings of the study indicated that the strain might be able to remove aluminum toxicity from the environment, which needs to be further explored.

## INTRODUCTION

Over the last few decades, the population, industrialization, urbanization, and agricultural practices have all increased considerably. These industries' waste products and toxic effluents are dumped into the environment without being treated (Ashraf et al. 2019). As a result, contamination of the air, land, and water is increasing day by day around the planet. It is a real global matter of concern, as it is hazardous to plants, animals, and humans. Because inorganic pollutants such as metals, salts, and minerals cannot be destroyed like other pollutants, they are considered the most hazardous. They tend to accumulate along the food chain and try to ruin our ecosystem (Kobya et al. 2005). Aluminium metal (Al) is one of the hazardous inorganic contaminants and is identified as the third most abundant metal in the earth's crust. Due to the disposal of Al trash by construction activities, the aerospace and automobile sector, electroplating, solid rocket fuels, pharmaceuticals and cosmetics, and other packaging industries, Al toxicity in soil and water is increasing rapidly (Igbokwe et al. 2019). Excessive Al is particularly neurotoxic in animals and has been linked to a variety of bone deformities and neurodegenerative diseases in humans (Bondy 2014). Acidification of soil due to Al affects plants by limiting root growth and cell division, thus reducing cation uptake, such as $Ca^{2+}$, and decreasing stomatal opening and photosynthetic efficiency, lowering plant growth and yield (Panda et al. 2009).

To detoxify or recycle Al waste, many physiochemical procedures, such as adsorption, absorption, and chemical precipitation are used, but these methods are quite expensive and can produce even more harmful contaminants (Dada et al. 2015). As compared to traditional approaches, bioremediation is the most promising strategy for detoxifying or recycling the Al waste present in soil and water these days since it is environmentally safe, cheaper, lower maintenance, faster, and produces fewer toxic by-products (Jan et al. 2014, Purwanti et al. 2017). Microorganisms are considered the most suited and effective bioremediation agents for reducing Al toxicity and studying Al tolerance mechanisms (Haytham 2016). *Pseudomonas aeruginosa*, *Brochothrix thermosphacta*, and *Vibrio alginolyticus* have been reported to be resistant to Al exposure in acidic conditions (Kurniawan et al. 2018). Ji et al. (2016) isolated four strains of bacteria: *Chryseobacterium* sp. B1, *Brevundimonas diminuta* B3, *Hydrogenophaga* sp. B4, and *Bacillus cereus* B5 from activated sludge, which were capable of tolerating up to 20 mM concentration of Al. In another study, dead biomass of *Aspergillus oryzae* was isolated from waste sites of Al mills and biosorption of Al ions at low concentrations of 10-50 $mg.L^{-1}$ was observed (Omeike et al. 2013). Several

ectomycorrhizal fungi, including *Pisolithus tinctorius* and *Lactarius deliciosus*, emerged as promising candidate species for high Al tolerance and for understanding the role of Al immobilization and accumulation (Gu et al. 2021).

Most studies focus on the isolation and identification of Al-tolerant fungi. To our knowledge, just a few studies on Al-tolerant bacteria have been conducted. The purpose of this research is to identify Al metal-tolerant bacteria from the metal-contaminated sites to reduce Al toxicity in the environment. Our in-vitro research found a bacterial strain from soil samples that showed high metal tolerance when exposed to Al and exhibited multiple antibiotic resistance.

## MATERIALS AND METHODS

### Sample Collection

The soil sample was collected from a metal-contaminated waste disposal location near an electroplating and automobile workshop in Patiala, Punjab. Sampling was done and transferred to sterilized polyethylene bags, which were then stored at 4°C before analysis and evaluation.

### Isolation and Screening of Al-Resistant Bacteria

The soil sample was serially diluted and plated on Luria-Agar (LA) medium supplemented with a particular concentration of Al. Incubation was done at 37°C for 24-48 hrs. Aluminium chloride ($AlCl_3$) was used as a metal stock solution. Colonies were picked and streaked on another LA plate enriched with a predetermined dose of Al. To allow for comparison, control plates were made using LA medium without Al metal. Plate streaking was repeated until morphologically separate colonies were seen, resulting in the isolation of pure cultures. A bacterial isolate was chosen and stored at -80°C for future analysis.

### Identification of Bacterial Isolate: Morphological and Biochemical Tests

Various morphological and biochemical tests, such as oxidase, catalase, urease, indole, starch hydrolysis, gelatin hydrolysis, citrate utilization, and Gram staining were carried out to identify the bacterial isolate, according to Bergey's Manual of Determinative Bacteriology (Krieg & Holt 1984).

### Identification of Bacterial Isolate: 16srRNA Gene Amplification and Sequencing

The modified Rapid One-Step Extraction method (ROSE) was used to isolate genomic DNA (Steiner et al. 1995). The extracted DNA was PCR amplified using universal primers (8F: 5' AGA GTT TGA TCC TGG CTC AG 3' and 1492R: 5' CGG TTA CCT TGT TAC GAC TT 3') in a gene AMP PCR system (Applied Biosystems, California, USA) (Xiang et al. 2005). For 16srRNA gene amplification, the PCR conditions were as follows: 5 minutes at 95°C for pre-denaturation, 1 minute at 95°C for denaturation, 1 minute at 56°C for annealing, 1 minute at 72°C for extension, and 5 minutes at 72°C for the final extension. The cycle was repeated 30 times from denaturation to extension. The purified PCR product was sequenced by Amnion Biosciences, Bangalore, India. The resultant sequence was corrected with Seq-Man V 4.1 software (Swindell & Plasterer 1997) before being compared to existing sequences in the GenBank database. The 16srRNA gene sequence was compared using NCBI-BLASTN (Altschul et al. 1990). We retrieved highly comparable sequences from the NCBI GenBank database (www.ncbi.nlm.nih.gov.) and compared them using multiple sequence alignment. The sequencing result of the 16s rRNA gene of a bacterial isolate was submitted to the GenBank database (KF146959).

### Maximum Tolerance Concentration of Al-Resistant Bacteria

The maximum tolerance concentration (MTC) of the Al-resistant bacterial isolate was determined as follows: The bacteria were inoculated in LB medium with varying concentrations of filter-sterilized Al (50 ppm, 75 ppm, 100 ppm, 150 ppm, and 200 ppm), and incubated at 37°C with agitation (150 rpm). As a control, the same bacterial isolate was cultured in the LB medium without Al. The OD600 of bacterial culture was determined using a UV VIS Spectrophotometer (HITACHI U-2900) at every 12 hr interval (0 hr, 12 hrs, 24 hrs, 48 hrs, and 72 hrs).

### Antibiotic Resistance

The bacterial isolate was tested for antibiotic susceptibility against eight different antibiotics using the disk diffusion method (Bauer et al. 1966). Antibiotic disks used in this study were Streptomycin (10 mcg), Vancomycin (30 mcg), Tetracycline (30 mcg), Kanamycin (30 mcg), Ampicillin (10 mcg), Carbenicillin (100 mcg), Rifampicin (5 mcg), and Chloramphenicol (30 mcg). With the help of a sterile swab, 100 µL of overnight-grown culture was taken and spread evenly on LA plates. Disks of different antibiotics were placed on the plate and incubated at 37$^{o}$C for 24 hrs. The results were depicted according to the guidelines of the Clinical Laboratory Standard Institute (CLSI 2018). Based on the zone of inhibition, the isolated strain was classified as resistant, intermediate, or sensitive with respect to the specific antibiotic. The diameter of the zone of inhibition was measured with the help of a ruler in millimeters.

### Statistical Analysis

All the experiments were done in triplicates with the selected isolate against different concentrations of Al. The mean and standard deviation were calculated in Microsoft Excel software, version 2010.

## RESULTS AND DISCUSSION

### Identification of an Al-Tolerant Bacterial Isolate Based on Morphological, Biochemical, and Molecular Analyses

Nowadays, Al toxicity in soil and water is a major concern. It is one of the hazardous metals that has been identified as having harmful effects on plants, animals, and human health (Kochian 1995). In this study, an Al-tolerant bacterial strain was isolated after screening from metal-contaminated soil and was named M1. Identification of isolate M1 was done at genus and species level by morphological, biochemical, and 16srRNA gene sequence analyses. It was milky white in appearance, rod-shaped, motile, and Gram-negative. It showed a positive reaction to catalase while a negative reaction to oxidase, indole, and urease. It was also found that the isolate was capable of fermenting citrate. Isolate M1 was checked for starch and gelatin hydrolysis tests and found a positive reaction for starch hydrolysis rather than gelatin. It produced convex colonies when grown on a medium at 37°C. Based on the morphological and biochemical analyses, it was determined that the isolate belongs to the *Enterobacteriaceae* family as given in Table 1. Similar results of morphological and biochemical tests, such as Gram negative, rod shaped, positive citrate utilization test, and negative oxidase, urease, and indole tests for the Enterobacteriaceae family, were also observed by some researchers (Bhagat et al. 2016, Singh et al. 2018). The negative oxidase test is a key test to differentiate families of Enterobacteriaceae from Pseudomonadaceae and Pasteurellaceae. In addition to the conventional phenotypic and biochemical methods, PCR amplification of the 16srRNA gene (Fig. 1) and its sequencing is an important method for the identification of bacteria at the species level. The unknown Al-tolerant bacterial isolate M1 was identified as *Cedecea davisae* (*C. davisae*) with the highest 99.46% homology based on 16srRNA gene sequencing and BLAST homology search. The identified isolate M1 was designated as *C. davisae* strain M1. The 16srRNA gene sequence of *C. davisae* strain M1 has been deposited in the GenBank with accession number KF146959.1 (Table 2).

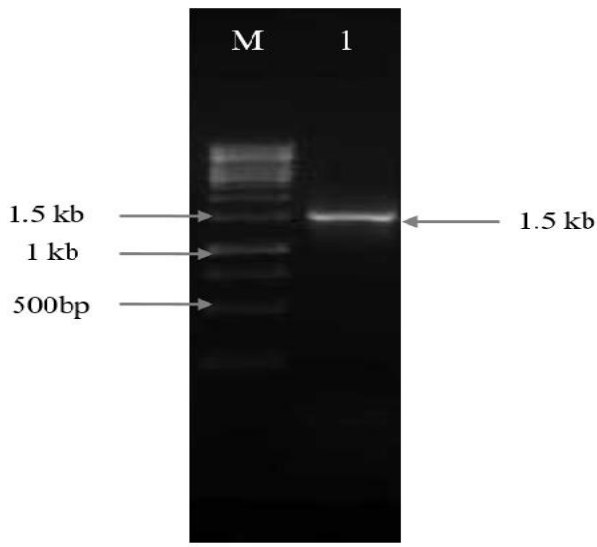
Table 1: Morphological and biochemical characterization of Al tolerant bacterial isolate M1.

| Characteristics | Bacterial Isolate M1 |
|---|---|
| Color | Milky white |
| Shape | Rods, Convex colonies |
| Motility | Motile |
| Gram Staining | - |
| Catalase test | + |
| Indole test | - |
| Citrate utilization test | + |
| Gelatin hydrolysis test | - |
| Oxidase test | - |
| Starch hydrolysis test | + |
| Urease test | - |

+: Positive; -: Negative

Table 2: Sequence similarity analysis of the isolate M1 based on BLASTn comparison to the GenBank database.

| Isolate | BLAST identification with accession no. | Similarity | Submitted in Genbank with accession no. |
|---|---|---|---|
| M1 | *Cedecea davisae* isolate ABRL062 (MZ597848.1) | 99.46% | *Cedecea davisae* M1 (KF146959.1) |




M: 1kb DNA ladder SM0312 (Thermo scientific); 1: Amplified product

Fig. 1: PCR amplification of 16srRNA gene of bacterial isolate M1.

### Maximum Tolerance Concentration Analysis

*C. davisae* M1 was examined for its maximum tolerance concentration for Al at various concentrations and time periods with the help of a UV-VIS spectrophotometer (Fig. 2). There was no research done on bacteria- *C. davisae* for Al tolerance. In our study, *C. davisae* M1 has grown in the culture media supplemented with different doses of Al (50 ppm, 75 ppm, 100 ppm, 150 ppm, and 200 ppm). We

Table 3: Antibiotic resistance profile of *C. davisae* M1.

| Antibiotics | Zone of Inhibition (mm) | Classified as | Interpretative criteria for *Enterobacteriaceae* according to CLSI Standard | | |
|---|---|---|---|---|---|
| | | | Sensitive (mm or more) | Intermediate (mm) | Resistance (mm or less) |
| Tetracycline (30 mcg) | 17 | S | 15 | 12-14 | 11 |
| Streptomycin (10 mcg) | 14 | I | 15 | 12-14 | 11 |
| Vancomycin (30 mcg) | NI | R | 17 | 15-14 | 13 |
| Kanamycin (30 mcg) | 14 | I | 18 | 14-17 | 13 |
| Ampicillin (10 mcg) | NI | R | 17 | 14-16 | 13 |
| Carbenicillin (100 mcg) | NI | R | | | |
| Rifampicin (5 mcg) | NI | R | | | |
| Chloramphenicol (30mcg) | 18 | S | 18 | 13-17 | 12 |

Diameter of disks: 6mm; NI: No inhibition; S: Sensitive; I: Intermediate; R: Resistant; mm: millimetres; mcg: microgram; CLSI: Clinical Laboratory Standard Institute; Zone of inhibition: diameter of the zone along with the disk.

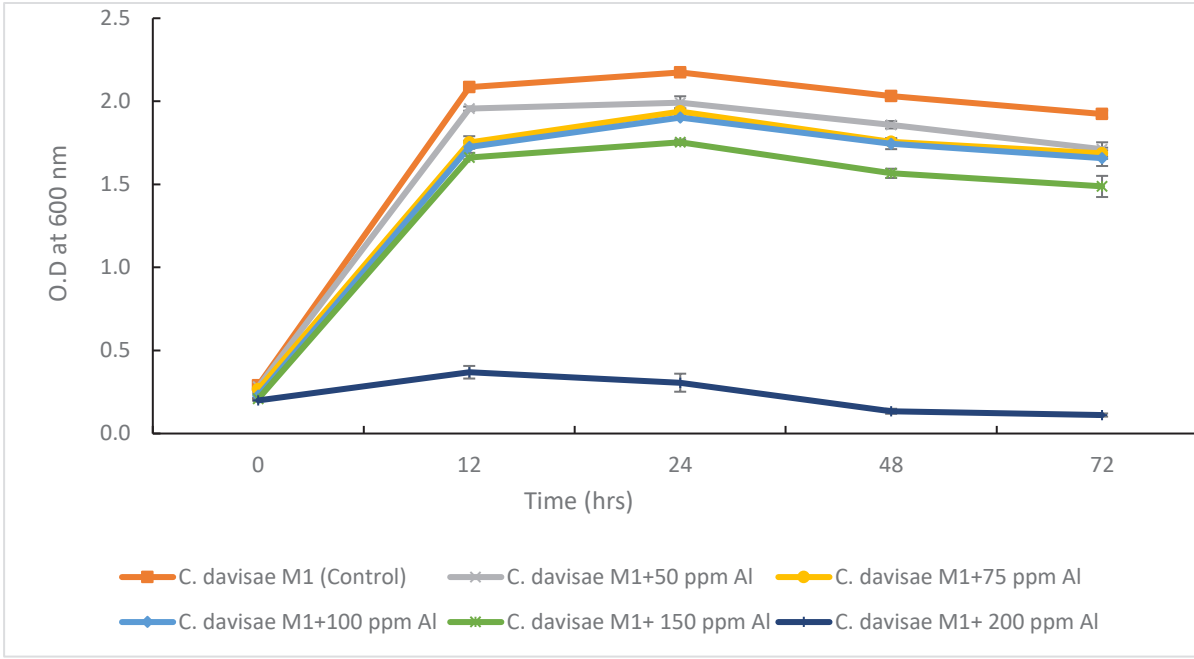



Fig. 2: The growth curve of *C. davisae* M1 in various concentrations of Al monitored for 72 hrs. The graph represents mean results from the value of triplicate cultures with error bars indicating standard deviations (n = 3). The growth was observed at up to 200 ppm Al supplemented in the culture medium.

noticed that the bacterial strain *C. davisae* M1 showed higher Al tolerance, up to 150 ppm for a period of 24 hrs. Metal ion accumulation inside the cell, metal extrusion, metal ion adsorption on the cell surface, membrane or cell wall binding, extracellular or intracellular chelation, producing extracellular compounds, or conversion into a less toxic state could all be the possible factors in the strain's tolerance to high Al concentrations (Piña & Cervantes 1996). Appanna et al. (1994) reported one of the mechanisms for metal tolerance, where an Al-tolerant strain of *Pseudomonas fluorescens* was able to accumulate and detoxify Al by producing an extracellular lipid compound that was rich in phosphorus. However, when we exposed Al to higher concentrations of 200 ppm (higher than MTC), it induced bacterial cell ruptures, resulting in a significant decrease in its biosorption capabilities (Titah et al. 2019). A higher concentration of Al also decreases the growth rate of bacteria by altering or inhibiting some enzymatic reactions (Kurniawan et al. 2018).

## Antibiotic Resistance Analysis

The *C. davisae* M1 bacteria was tested against eight

different antibiotics using the Disk-diffusion method. The bacteria were found to be highly resistant to vancomycin, ampicillin, carbenicillin, and rifampicin while being moderately resistant to kanamycin and streptomycin. However, it also showed high susceptibility to tetracycline and chloramphenicol (Table 3). There have been few reports on antibiotic resistance by *C. davisae* strains, which were published before. In a report by Kanakadandi et al. (2019), *C. davisae* has shown resistance to a variety of antibiotics. The antibiotic resistance profile of *Cedecea* sp. was also published by Grimont et al. (1981). The multidrug resistance of *C. davisae* is caused by a combination of AmpC synthesis and porin deficiency in the cell wall (Ammenouche et al. 2014). Thompson & Sharkady (2020) reported that strains of *C. neteri* harbor multiple chromosomes encoded β-lactamase genes, which help in antibiotic resistance. Some resistance nodulation-cell division (RND) multidrug efflux pumps were also identified, such as AcrB, AcrD, OqxB, and MdtBC.

Previous research has also revealed that different microorganisms have distinct metal tolerance capabilities and antibiotic resistance profiles. Metal tolerance and antibiotic resistance both have been observed in *C. davisae* in a few cases. *C. davisae* GCC 19S1 was isolated and found to be cadmium, copper, lead, iron, and zinc resistant and also showed resistance to a variety of antibiotics (Nath et al. 2020). Four bacterial isolates were isolated in Behrampur. Among these, isolate 3 showed the highest tolerance of 100 ppm to $Al_2O_3$. It also showed resistance to the antibiotic Cloxacillin and was most sensitive to gentamicin (Mohapatra et al. 2018). Therefore, we might conclude that there may be some relationship between Al tolerance and antibiotic resistance that has yet to be discovered and explored.

## CONCLUSION

The present study reported the isolation and identification of the Al- tolerant bacterial strain *C. davisae* M1 based on morphological, biochemical, and 16srRNA gene sequence analyses. It showed tolerance to Al at a concentration of up to 150 ppm. However, with a higher concentration of Al (200 ppm), the growth of bacteria was suddenly inhibited. The bacterial strain also exhibited resistance to many antibiotics, such as vancomycin, carbenicillin, rifampicin, and ampicillin, and moderate resistance to kanamycin and streptomycin. It was also susceptible to tetracycline and chloramphenicol. Different microbes show distinct metal tolerance capacities, antibiotic resistance, and metal resistance systems. Most research has focused on the bioremediation of Al by plants and fungi from contaminated soil and water, whereas limited research has been done on the removal of Al by bacteria, resulting in less study on the bacterial resistance system for Al. In future studies, *C. davisae* M1 could be exploited as a bioremediation agent to remove Al and other toxic metals from metal-contaminated soil and wastewater. In addition, it could also seem to be a good candidate for exploring bacterial resistance systems for Al.

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