Al-Hayat: Journal of Biology and Applied Biology
Volume 8, No 2 (2025): 95-110
DOI. 10.21580/ah.v8i2.28874.

Isolation and Molecular Identification of Cadmium-Resistant Bacteria from Paddy Soil in the Tallo Watershed, Makassar

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Abstract

Cadmium (Cd) contamination in paddy soils is a global concern because it can accumulate in rice through nutrient uptake, posing significant food safety risks. More than one-quarter of the Tallo watershed area in Makassar consists of rice fields that are vulnerable to pollution from industrial activities and urban runoff. This study aimed to isolate and identify Cd-resistant bacteria from paddy soils in the Tallo watershed to determine potential candidates for bioremediation. Soil samples were collected from the rhizosphere of rice fields located at varying distances from the Tallo Main River. Bacterial isolation was conducted using Nutrient Agar, followed by Cd resistance testing on Nutrient Agar supplemented with $CdCl_2$ at concentrations ranging from 10 mg L^{-1} to 110 mg L^{-1}. Molecular identification of the most Cd-tolerant isolates was performed using the 16S rRNA gene with primers 27F and 1492R. A total of 22 isolates were obtained, 19 of which were resistant to 10 mg L^{-1} $CdCl_2$. Only five isolates were able to grow at 100 mg L^{-1}, and just one isolate, L1(4), showed growth at 110 mg L^{-1}. This highly tolerant isolate was sequenced using the 16S rRNA gene and identified as belonging to the genus *Aeromonas*, specifically *A. veronii*. A limitation of this study is the exclusive use of 16S rRNA sequencing, which provides limited resolution for differentiating closely related species. Despite this limitation, the findings indicate that the identified strain represents a promising candidate for the bioremediation of Cd-contaminated soils in the Tallo watershed.

Keywords: 16S rRNA, *Aeromonas veronii*, Bioremediation, Cadmium-Resistant Bacteria, Paddy Soil, Tallo Watershed

Introduction

Cadmium (Cd) is a toxic heavy metal (Charkiewicz et al., 2023; Genchi et al., 2020; Khan et al., 2022; Rahimzadeh et al., 2017) that adversely affects plant health and productivity by disrupting cellular structures and metabolic processes (Rashid et al., 2023). Its potential impact on human health is a major concern, particularly in paddy fields where Cd uptake by rice plants poses risks to food safety (Jiang et al., 2021; F. Wang et al., 2021). If elevated Cd levels are not mitigated during rice growth, the metal can be transferred to developing shoots and ultimately accumulate in rice grains (Feng et al., 2019). This accumulation is driven by

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nutrient-uptake transport mechanisms (Jing et al., 2023), highlighting the urgent need to reduce Cd bioavailability in soils and limit its agricultural impact (Danso et al., 2023).

Globally, Cd contamination in agricultural soils has become an increasing concern (Jiang et al., 2021; Lv et al., 2022; Wang et al., 2016). Rapid industrialization has substantially contributed to Cd pollution in rice-producing regions, often exceeding national food safety thresholds for Cd in rice grains (Wang et al., 2019). In the Yangtze River watershed in China, for example, average Cd concentrations in rice and wheat grains exceeded recommended limits by 62% and 81%, respectively, largely due to industrial emissions and phosphate fertilizer use (Gao et al., 2022). Similarly, Cd levels in paddy soils in Central Hunan were found to be 1.6 to 2.8 times higher than national soil contamination standards (Jiang et al., 2021). These data reinforce the urgency of addressing Cd pollution, especially in regions where rice serves as a primary food source.

In the Makassar region, several rivers and tributaries drain into the Makassar Strait, including the Tallo River. The diverse and intensive activities along the coastal waters of Makassar contribute to environmental pollution. The Tallo Watershed spans an area in which 28.97% consists of rice fields (Wahyuni et al., 2022), placing agricultural lands at heightened risk of Cd contamination. Cd concentrations in the estuarine waters of the Tallo River have been reported at 0.729 ppm, exceeding established environmental quality standards (Setiawan, 2014). This elevated level is likely affected by waste discharge from the Makassar Industrial Estate, which empties directly into the river (Mahluddin et al., 2022; Setiawan, 2014). Cd contamination has also been detected in fish from the Tallo Watershed, surpassing permissible Cd limits for aquatic organisms (Jais et al., 2020), indicating bioaccumulation within local biota. Despite these indications of Cd exposure in the Tallo Watershed, no available studies have isolated, characterized, or molecularly identified Cd-resistant bacteria from paddy soils in this area. The absence of such data leaves a critical gap in understanding the microbial potential for Cd bioremediation within agricultural ecosystems.

Bioremediation of Cd using microbes is an efficient alternative for mitigating environmental Cd contamination (Zulfiqar et al., 2023). The microorganisms commonly utilized are soil microbes capable of tolerating heavy metals (Ma et al., 2023; Song et al., 2024). Several strains of heavy metal-resistant bacteria have been reported to reduce Cd availability in soil (Ma et al., 2023; Zulfiqar et al., 2023) and decrease its accumulation in rice plant tissues (Ayangbenro & Babalola 2017). This microbiological approach offers an economical and environmentally friendly solution, as it is simple to implement and requires minimal land; therefore, it holds strong potential for reducing heavy metal contamination (Fahruddin et al., 2020). However, information remains limited regarding Cd-resistant bacterial isolates from the Tallo Watershed, which may serve as promising local candidates for microbe-assisted remediation.

To address this gap, the present study aimed to isolate bacteria from paddy field soils within the Tallo Watershed, evaluate their tolerance to Cd through resistance assays, and identify the most tolerant isolate using 16S rRNA gene sequencing. This study provides the first report of Cd-resistant bacterial isolates from paddy soils in this watershed and represents a

preliminary step toward developing localized microbial resources for Cd mitigation in agricultural environments.

Research Methods

Soil samples were collected from the rhizosphere layer at a depth of 0–20 cm (Song et al., 2024; Yu et al., 2021) from three rice fields located in the Tallo Watershed, Makassar, South Sulawesi, Indonesia. The sampling sites included Location 1 (L1), Parang Loe, Tamalanrea District (coordinates: 5°06'17.5"S, 119°27'59.5"E); Location 2 (L2), Lakkang, Tallo District (coordinates: 5°07'26.7"S, 119°28'03.3"E); and Location 3 (L3), Pampang, Panakkukang District (coordinates: 5°07'44.5"S, 119°27'27.1"E). L1 and L2 are situated near the main Tallo River, with L1 being particularly close to the Makassar Industrial Estate (KIMA), while L3 lies farther from the river relative to the other two sites. These locations were selected due to their varying proximities to the Tallo River and adjacent industrial zones, which are known potential sources of metal contamination.

Isolation of Bacteria from Paddy Field Soil in the Tallo Watershed

A 10 g subsample of soil was added to 90 mL of physiological NaCl solution (Merck), homogenized, and labeled as the 10^{-1} dilution. Subsequently, 1 mL of this suspension was transferred into a test tube containing 9 mL of physiological NaCl to produce a 10^{-2} dilution. Serial dilutions were prepared up to 10^{-9} to ensure colony counts within an enumerable range. From dilutions 10^{-4} to 10^{-9}, 0.1 mL aliquots were transferred into sterile petri dishes, followed by the addition of 15 mL of Nutrient Agar (HIMEDIA). Plates were gently rotated clockwise and counterclockwise to evenly distribute the inoculum and then incubated at 28 ± 1°C, the standard temperature for culturable soil bacteria.

Emerging colonies were observed morphologically based on color, elevation, margin, and size. Distinct colony morphotypes were recorded as separate isolates, each assigned a unique code. Colonies were picked using an inoculating loop and streaked on fresh agar to obtain pure cultures. Each isolate was subjected to Gram staining (HIMEDIA) following Smith and Hussey (2005), and the stained preparations were examined under a microscope at 1000× magnification to determine Gram reaction and cell morphology.

Cd-Resistance Test

Cd-resistance was assessed following Verdian and Zulaika (2015). Each bacterial isolate was streaked onto Nutrient Agar supplemented with $CdCl_2$ (Sigma Aldrich). Tests began at a concentration of 10 mg L^{-1}, which was increased in increments of 10 mg L^{-1} until the maximum tolerable concentration for each isolate was reached. Plates were incubated for 24 h at 28 ± 1°C. Isolates showing visible growth were considered Cd-resistant. Cd-resistant isolates were subsequently stored as stock cultures on Nutrient Agar at –4°C.

DNA Extraction and Polymerase Chain Reaction (PCR)

A total of 108 bacterial cells from isolates showing the highest Cd-resistance performance were subjected to DNA extraction using the Quick-DNA Magbead Plus Kit (Zymo Research, D4082) (Rante et al., 2024). DNA

quantity and purity were assessed with a NanoDrop spectrophotometer (Thermo Scientific). Samples with concentrations of 5–100 ng/µL and A260/280 purity ratios between 1.8 and 2.0 (Abdel-Latif & Osman, 2017; Srirungruang et al., 2022) were selected for subsequent PCR analysis. DNA integrity was verified by electrophoresis on a 1% agarose gel. Amplification of the 16S rRNA gene was conducted using MyTaq HS Red Mix (2×) (Bioline, BIO-25048) in a final reaction volume of 25 µL. Each reaction contained 12.5 µL of MyTaq HS Red Mix, 0.4 µM primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), 0.4 µM primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3') (Aliyu et al., 2023; Nurhikmayani et al., 2019), 5–100 ng of DNA template, and nuclease-free water. PCR conditions included an initial denaturation at 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 10 s, annealing at 52°C for 15 s, and extension at 72°C for 15 s. PCR products were visualized on a 0.8% agarose gel in TBE buffer and compared against a 1 kb DNA ladder. Amplification was considered successful when bands corresponded to the expected target size of approximately 1,400 bp (Sune et al., 2020). The resulting PCR products were sequenced bidirectionally using the Sanger sequencing method with capillary electrophoresis at 1st BASE.

Phylogenetic Analysis

The 16S rRNA sequences obtained from the isolates were analyzed using the Basic Local Alignment Search Tool (BLAST) at www.ncbi.nlm.nih.gov, comparing the sequences against the National Center for Biotechnology Information (NCBI) GenBank database (Agustiani et al., 2023). For phylogenetic reconstruction, 11 reference sequences representing the closest BLAST hits and relevant type strains (KC210792.1; KX768735.1; NR179579.1; NR043638.1; NR074841.1; NR115351.1; OR214944.1; NR113635.1; ON872224.1; NR112837.1; NR116880.1), along with one outgroup (NR156860.1), were retrieved from GenBank. Phylogenetic analysis was conducted in MEGA 11 using the Neighbor-Joining method with 1,000 bootstrap replications. The Kimura 2-Parameter (K2P) model was applied, consistent with recommendations for bacterial 16S rRNA evolutionary comparison. Positions containing gaps or missing data were removed using the pairwise deletion option. Bootstrap values greater than 50% were displayed on the resulting phylogenetic tree.

Research Results and Discussion

Isolation of Cd-Resistant Bacteria from Paddy Field Soil in the Tallo Watershed

Isolation of soil bacteria from three locations within the Tallo Watershed yielded a total of 22 isolates (see Table 1). Six isolates originated from the rhizosphere of rice fields located near the main Tallo River and the KIMA industrial area (L1). Seven isolates were obtained from rice fields adjacent to the main Tallo River (L2). The remaining nine isolates were collected from rice fields situated farther from the river compared with L1 and L2 (L3). Each isolate exhibited distinct colony morphology, cell shape, and Gram reaction. Isolates were coded based on sampling location, followed by a numerical identifier.

Table 1
Colony morphology and Gram staining of bacterial isolates from paddy field soil in the Tallo Watershed

Location	Code	Colony Morphology					Cell Shape – Gram Type
		Shape	Color	Elevation	Margin	Size	
L1 - Parang Loe	L1(1)	Circular	White	Flat	Entire	Small	Coccus-Positive
	L1(2)	Circular	Creamy white	Convex	Entire	Medium	Coccus-Positive
	L1(3)	Circular	White	Flat	Entire	Small	Coccus-Negative
	L1(4)	Circular	Creamy white	Flat	Entire	Large	Bacillus-Negative
	L1(5)	Circular	White	Flat	Entire	Small	Bacillus-Negative
	L1(6)	Circular	Orange	Umbonate	Entire	Medium	Bacillus-Negative
L2 - Lakkang	L2(1)	Punctiform	Orange	Flat	Entire	Small	Coccus-Positive
	L2(2)	Circular	White	Flat	Entire	Medium	Coccus-Positive
	L2(3)	Circular	Orange	Flat	Entire	Small	Coccus-Negative
	L2(4)	Circular	Yellow	Flat	Entire	Small	Coccus-Positive
	L2(5)	Circular	Creamy white	Flat	Entire	Large	Coccus-Positive
	L2(6)	Circular	White	Flat	Entire	Small	Coccus-Positive
	L2(7)	Circular	Orange	Flat	Entire	Medium	Coccus-Positive
L3 - Pampang	L3(1)	Circular	Orange	Convex	Entire	Medium	Coccus-Positive
	L3(2)	Circular	White	Flat	Entire	Medium	Coccus-Positive
	L3(3)	Circular	Creamy white	Flat	Entire	Medium	Coccus-Positive
	L3(4)	Circular	White	Flat	Undulate	Large	Bacillus-Positive
	L3(5)	Circular	White	Convex	Entire	Medium	Bacillus-Positive
	L3(6)	Circular	Creamy white	Raised	Entire	Large	Bacillus-Positive
	L3(7)	Circular	White	Flat	Entire	Small	Coccus-Positive
	L3(8)	Circular	White	Convex	Entire	Small	Coccus-Positive
	L3(9)	Circular	Yellow	Convex	Entire	Small	Coccus-Positive

Note: *Colony size classification: small (<1 mm); medium (1–2 mm); large (>2 mm).*

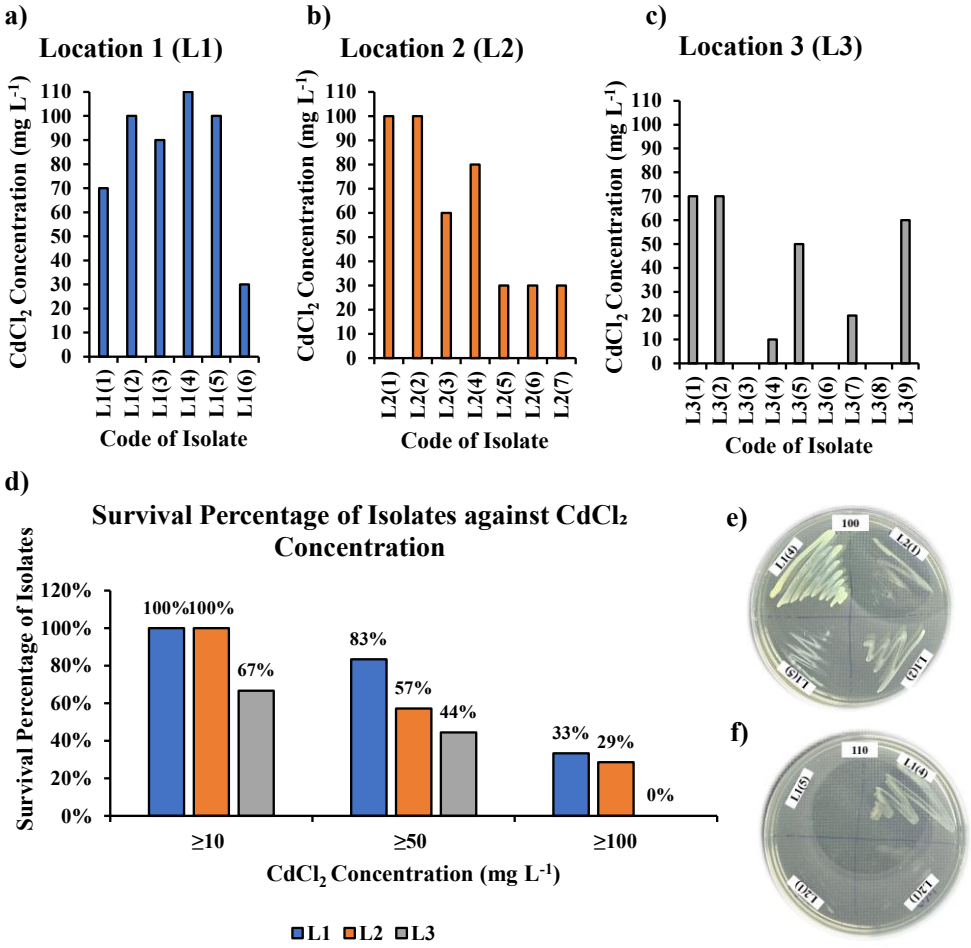
The isolates obtained were subsequently tested for Cd resistance using nutrient agar supplemented with $CdCl_2$ at concentrations ranging from 10 mg L^{-1} to 110 mg L^{-1}. Several studies have employed $CdCl_2$ as a source of Cd^{2+} ions (Feria-Cáceres et al., 2022; Makki et al., 2019; Wu et al., 2016) to evaluate the resistance of soil bacteria to Cd contamination. The addition of $CdCl_2$ to the growth medium functions as a selective agent, allowing only bacteria with Cd-resistance mechanisms to survive.

The resistance assay across multiple $CdCl_2$ concentrations showed that 19 of the 22 isolates were able to grow at 10 mg L^{-1} $CdCl_2$ (see Figure 1a, 1b, 1c). Three isolates failed to grow at the initial concentration (see Figure 1c), indicating their sensitivity to Cd. Heavy metals such as Cd can inhibit microbial growth and metabolism by causing functional impairment, DNA

damage, protein denaturation, and disruption of cell membranes (Chakravarty & Banerjee, 2008; Khan et al., 2022; Rashid et al., 2023; Shuaib et al., 2021). Microbial sensitivity to Cd varies across taxa, and gram-negative bacteria are generally more tolerant to Cd than gram-positive bacteria (Babich & Stotizky, 1977). Consistent with this, the three isolates that were unable to survive at the lowest Cd concentration, L3(3), L3(6), and L3(8), were gram-positive, making them more susceptible to Cd toxicity.

Figure 1

Graph of the resistance ability of bacterial isolates to $CdCl_2$ concentrations of 0–110 mg L^{-1} based on sampling location: (a) L1; (b) L2; (c) L3; (d) survival percentage of isolates; (e) isolate growth at 100 mg L^{-1}; (f) isolate growth at 110 mg L^{-1}.



All isolates that failed to grow on Cd-supplemented media originated from L3, where only 67% survived at concentrations above 10 mg L^{-1}. In contrast, isolates from L1 and L2 tolerated up to 30 mg L^{-1}, with survival rates of 33% and 29% at ≥100 mg L^{-1}, respectively (see Figure 1d). Only four of the nine isolates from L3 exhibited growth, with maximum resistance observed at 70 mg L^{-1}. This distribution

suggests a decline in Cd tolerance with increasing distance from the Tallo River.

Microbes exhibit strong adaptive capabilities to environmental stressors. Bacterial resistance to heavy metals is associated with metabolic acclimatization, enabling them to transform or mitigate the toxicity of metal ions (Yin et al., 2019). From the NA–$CdCl_2$ plates at 100 mg L^{-1}, five isolates, L1(2), L1(4), L1(5), L2(1), and L2(2), were able to grow. Among them, L1(4) showed the most robust growth, forming denser and thicker colonies than the other isolates (see Figure 1e). L1(4) remained viable at 110 mg L^{-1}, whereas the remaining isolates exhibited no growth at this concentration (see Figure 1f). The diversity and lack of phylogenetic clustering among Cd-tolerant bacteria (Bravo & Braissant, 2022; Ghorui et al., 2023), highlight the need for further taxonomic identification.

PCR amplification of isolate L1(4) produced a clear 1500 bp band corresponding to the 16S rRNA gene (see Figure 2). The 16S rRNA sequence is a widely used marker for bacterial classification, with 98.65% similarity serving as the threshold for species delineation (Kim & Chun, 2014). BLAST analysis of the L1(4) sequence showed its highest identity at 99.36% with 100% query coverage to *A. veronii* strain SA (see Table 2).

Figure 2

Electrophoresis results of 16S rRNA gene amplification of isolate L1(4) (M = Marker, 1 kb DNA ladder; NTC = Non-Template Control).

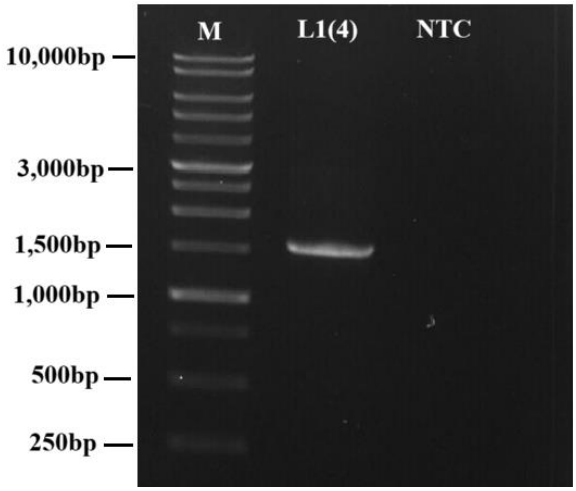


Table 2

Top 10 BLAST hits of 16S rRNA sequences of isolate L1(4) against NCBI (excluding uncultured sample sequences)

Top BLAST Hit	Query Cover	Per. Ident	Accession
Aeromonas veronii strain SA	100%	99.36%	KC210792.1
Aeromonas allosaccharophila strain Aerall01	100%	99.22%	OK513039.1

Aeromonas veronii strain zy01	99%	99.22%	KX768735.1
Aeromonas veronii TS18-B	99%	99.22%	LC487867.1
Aeromonas sobria strain:14H 11	100%	99.15%	AB473004.1
Aeromonas veronii strain LRC7	100%	99.15%	MT226399.1
Aeromonas veronii strain BL1604	100%	99.15%	KY767536.1
Aeromonas veronii strain PCG1	100%	99.15%	MN581681.1
Aeromonas veronii strain CH-GX-NN-SL1-2-2021	100%	99.15%	ON203101.1
Aeromonas veronii strain AV2006	100%	99.15%	OP476498.1

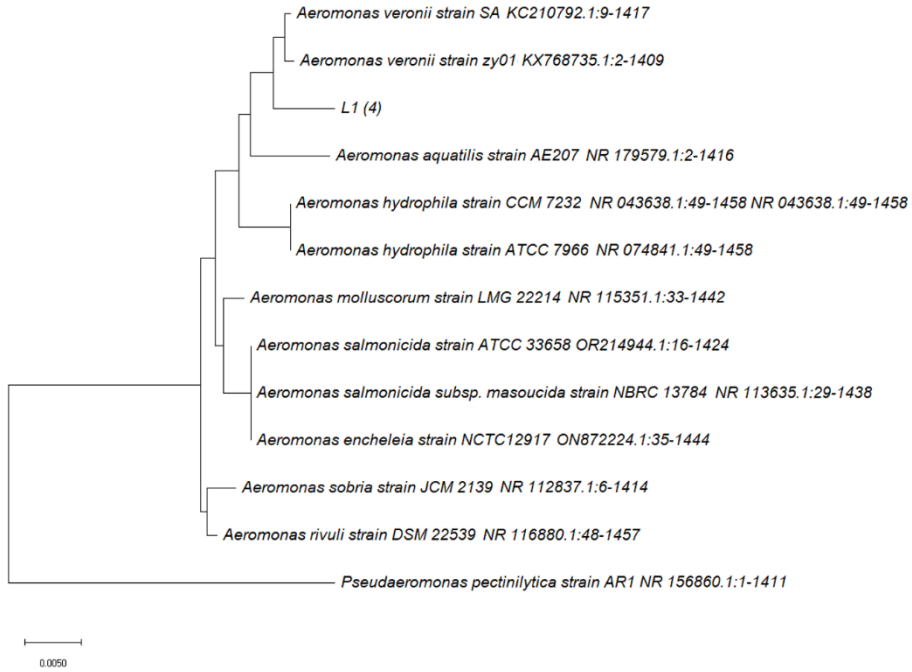
Based on the BLAST analysis of the 16S rRNA sequences, isolate L1(4) was identified as *A. veronii*. This result is supported by the phylogenetic tree constructed using the Neighbor-Joining method (see Figure 3), which shows that isolate L1(4) clusters closely with *A. veronii* compared to other species within the same genus. According to Martínez-Murcia et al. (2016), 16S rRNA gene sequencing, DNA–DNA hybridization, and phylogenetic analysis are effective approaches for determining the taxonomic placement of bacterial isolates within *Aeromonas*. Gram-staining results indicated that isolate L1(4) is a gram-negative bacillus (see Table 1), consistent with characteristics of the *Aeromonas* genus. Members of *Aeromonas* are predominantly aquatic organisms, but they also occur in soil, vegetables, and various food products (Gonçalves Pessoa et al., 2019; Pessoa et al., 2022). Guerra et al. (2022) reported that *Aeromonas* populations are often more abundant in soil than in water or vegetation, suggesting strong adaptation to terrestrial environments. Previous studies also documented the tolerance of *Aeromonas* spp. isolated from mining soil to Cd concentrations of up to 24 ppm (Ibrahim et al., 2020). *Aeromonas sobria* has similarly been isolated from heavy-metal-contaminated soils in the Tanjaro region of Iraq, showing tolerance to Zn, Cu, and Ni (Qurbani et al., 2022).

The resistance assay conducted on isolate L1(4), identified as *A. veronii*, demonstrated its ability to survive at a $CdCl_2$ concentration of 110 mg L^{-1} (equivalent to 110 mg kg^{-1}). This represents a notably high Cd concentration. For comparison, the maximum permissible Cd concentration in soil is 1 mg kg^{-1} (Ji et al., 2012), while the national Cd contamination threshold for paddy soil in China is 0.3 mg kg^{-1} (Lv et al., 2022). The ability of isolate L1(4) to tolerate the highest tested $CdCl_2$ concentration highlights its potential application as a bioremediation agent. Sen et al. (2014) reported that *A. veronii* exhibits substantial resistance to heavy metals, and when combined with *Stenotrophomonas maltophilia* and *Bacillus barbaricus*, the consortium achieves greater metal-reduction efficiency than individual isolates.

Similarly, Matyar et al. (2014) showed that *Aeromonas hydrophila* demonstrated the highest resistance to heavy metals, likely due to prolonged exposure to agrochemical contamination in its native environment.

Figure 3

Phylogenetic tree illustrating the relationship of isolate L1(4) based on 16S rRNA sequences, constructed in MEGA 11 using the Neighbor-Joining method, with Pseudaeromonas pectinilytica *strain AR1 as the outgroup.*



The persistence of *Aeromonas* strains in heavy-metal-contaminated environments is closely associated with their possession of plasmids carrying metal-resistance genes, enabling them to tolerate high toxicity levels (Hossain & Heo, 2022; Tataje-Lavanda et al., 2024)(Hossain & Heo, 2022; Tataje-Lavanda et al., 2024). One major strategy employed by *Aeromonas* is biosorption, in which non-specific metal ions bind to extracellular polysaccharides, surface-associated polymers, and various proteins, serving as a non-enzymatic detoxification mechanism (Qurbani et al., 2022; Valls & de Lorenzo, 2002)(Qurbani et al., 2022; Valls & de Lorenzo, 2002). These structural molecules act as protective barriers that limit metal entry and support cellular survival under stress. Supporting this mechanism, *Aeromonas* sp. Y23 has been reported to form interconnected long-chain cells when exposed to Cd, a morphological adaptation that restricts Cd penetration and enhances resistance (Zhang et al., 2026)(Zhang et al., 2026).

From an ecological perspective, the presence of highly Cd-tolerant *Aeromonas* populations in agricultural soils suggests their potential role in natural attenuation processes, whereby microbial activity reduces Cd mobility and bioavailability in the rhizosphere. Such microbial interactions may lower Cd uptake by rice plants, ultimately mitigating food safety

risks in paddy systems exposed to heavy metal contamination. In this context, the ability of isolate L1(4) to withstand 110 mg L^{-1} $CdCl_2$ highlights its promise as a candidate organism for bioremediation in Cd-contaminated soils.

Conclusion

Isolation of bacteria from rice field soil in the Tallo watershed, Makassar, resulted in 22 isolates, 19 of which exhibited resistance to $CdCl_2$ at 10 mg L^{-1}. Among these, five isolates were capable of growing at concentrations up to 100 mg L^{-1}. Of the five, isolate L1(4) demonstrated the strongest growth performance and remained viable at concentrations up to 110 mg L^{-1}. Molecular characterization using the 16S rRNA gene identified isolate L1(4) as *A. veronii*. These findings suggest that *A. veronii* is a promising candidate for Cd-tolerant bacterial applications. However, resistance observed on nutrient agar does not directly confirm its bioremediation potential in soil systems. Thus, further functional assays are required to assess its effectiveness in Cd-contaminated soil environments.

Acknowledgment

The authors sincerely acknowledge the support of LPPM-UNHAS through the *Penelitian Dosen Pemula Unhas* (PDPU) scheme under contract number 00310/UN4.22/PT.01.03/2024.

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