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Copper bioremoval by novel bacterial isolates and their identification by 16S rRNA gene sequence analysis

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Abstract: Copper-tolerant bacteria were isolated from soil samples taken from a region where metal industries are located. After selecting 2 isolates with relatively higher bioremoval efficiency, the effects of increasing copper concentration, pH, and temperature on the bioremoval efficiency of the growing isolates were determined. Strain N1c and strain N5a showed maximal bioremoval efficiency of 82% and 75%, respectively, in 20 mg/L copper-containing medium at pH 6.8 and 30 °C. Although the isolates did not grow well at pH 5, a low amount of copper was removed at pH 5. Slow growth of N5a at pH 5 allowed for 26% copper removal at hour 80 of incubation. Optimal copper bioremoval of the cells occurred at pH 6.8 and 30 °C. When grown at 37 °C under aerated conditions, N1c showed 31.7% bioremoval in the presence of 100 mg/L copper, and N5a was much more resistant to copper compared to N1c and *E. coli*. The isolates were identified by 16S rRNA gene sequence analysis. The 16S rRNA gene sequence of N5a showed 96%-97% similarity to *Pseudomonas stutzeri* and other *Pseudomonas* spp. The 16S rRNA gene sequence of N1c was 96% similar to *Achromobacter* sp., *Alcaligenes* sp., and a novel genus, *Collimonas*.

Key words: Heavy metal resistant bacteria, copper bioremoval, 16S rRNA

Introduction

Industrial, agricultural, and domestic activities result in an increase in the heavy metal content of soil and water (1). When accumulated in soils, heavy metals such as copper, cadmium, lead, zinc, nickel, mercury, and chromium can reach concentrations that are toxic to living organisms (2). Human factors such as agricultural patterns of soil use, use of chemical fertilizers and pesticides, and industrial pollution affect the available copper content in soils (3). The average copper content of unpolluted soil samples was found to range between 1.6 and 7.5 mg/kg soil (4). Although some metals such as copper are essential to organisms, they are toxic to cells at high levels (5).

Conventional methods such as chemical precipitation, filtration, ion exchange, electrochemical treatment, membrane technologies, adsorption on activated carbon, and evaporation are not very effective or economical when treating large amounts of water and wastewater with low concentrations of heavy metals. Therefore, these methods cannot be used on a large scale (6,7). Metal removal by bacteria is generally achieved by chelation and surface adsorption (8). Live or dead microbial cells and their products are very efficient bioaccumulators of both soluble and particulate forms of metals (9-12). Many studies show that soluble metal ions in the environment could be captured by microorganisms due to the negatively charged groups attached within

their cell wall structure (13). Bacteria, algae, and fungi or their separated components have been successfully used as biosorbents for heavy metal removal (14). Metal uptake is a complex process and it depends on factors such as the chemistry of the metal ions and specific surface properties of the organisms. Physiology of the cells and environmental factors such as pH, temperature, and metal concentration also affect metal uptake by the cells (15). Several microbial genera were used for copper removal processes. Among them, *Pseudomonas* has been shown to be relatively efficient in bioaccumulation of copper from polluted effluents in both an immobilized and mobilized state (16-18).

In the present study, 2 different bacterial species were isolated from an industrially polluted region in Kocaeli, Turkey. The isolates were characterized by 16S rRNA sequence analysis, and the effects of copper concentration, pH, and temperature on the copper-removing ability of the isolates were investigated.

Materials and methods

Isolation of copper-resistant bacteria

Soil samples were collected from 5 different locations in the industrially polluted region of Darıca (Kocaeli, Turkey). The samples were inoculated in lysogeny broth (LB) medium containing 200 mg/L Cu^{+2}. After incubation at 30 °C for a week, the cultures were used to inoculate fresh media containing 200 mg/L copper. Copper-tolerant bacteria were enriched by repeating the enrichment procedure 3 times. The mixed cultures obtained at the end of enrichment were analyzed for their copper-removing capacity using an atomic absorption spectrophotometer (AAS). The mixed cultures with relatively higher copper removal capacity were used for isolation of particular copper-resistant bacteria. Mixed culture (200 µL) was spread on Luria agar (LA) plates with 200 mg/L copper, and individual colonies appearing after 2 days of incubation at 30 °C were streaked on fresh LA medium.

The cells were incubated in an anaerobic cabinet under N_2 atmosphere in order to determine whether they were fermentative. Sporulation ability of the cells was checked by heating the stationary phase culture to 90 °C for 15 min and spreading the appropriate amount of culture on LA. The appearance of colonies after incubation indicated regeneration of spores.

Effect of temperature, pH, and copper concentration on bioremoval and growth

The effects of 3 different temperatures (25, 30, and 37 °C) and 3 different pH values (5.0, 6.8, and 8.0) on the bioremoval efficiency of the bacteria were investigated by culturing in LB medium containing 20 mg/L copper. The pH adjustment of the medium was done using NaOH or HCl solutions. LB media containing 0, 10, 20, 40, 70, 100, and 150 mg/L copper(II) were inoculated by the bacterial isolates in order to investigate the effect of copper concentration on bioremoval. Cultures were grown for 160 h, and copper concentration in the supernatant was measured at hours 0, 20, 80, and 160 of growth. Growth was monitored by measurement of optical density at 600 nm with a UV-Vis spectrophotometer (GBC-Cintra20). The amount of copper was determined spectrophotometrically on a PerkinElmer AAS (model 1100) at a wavelength of 324.8 nm, and the amount of removed copper was calculated by taking the difference between the initial and final concentrations measured.

Comparison of the resistance and bioremoval capacities of the isolates with *Escherichia coli*

Resistance of the isolates and *E. coli* to 5 different copper concentrations (20, 50, 100, 150, and 200 mg/L) was determined in LB medium at 37 °C. The cultures were aerated by shaking at 130 rpm. The number of living cells at different time intervals was determined by calculating CFU/mL for each strain. The following formula was used to calculate % resistance:

$$\% \text{ Resistance} = \frac{\text{CFU/mL}\,[Cu^{+2}]_n}{\text{CFU/mL}\,[Cu^{+2}]_0} \times 100,$$

($n = Cu^{+2}$ concentration in the medium)

The % removal of copper at 37 °C was determined by measuring copper concentrations in the media

containing 20 and 100 mg/L Cu^{+2}. Medium containing the appropriate copper concentration, but no cells, was used as the control for calculation of % removal. The experiments were performed in duplicate.

Gram staining

The Gram-Hücker staining method (RAL, Martillac, France) was used to stain the cells. The cells were examined under ×100 oil immersion objective with a trinocular phase contrast microscope (Carl Zeiss, Axio Scope model) equipped with an Axiocam Icc3 3.3 Mp FireWire connection digital camera (Carl Zeiss).

RapID biochemical and oxidase test

RapID biochemical test and oxidase test were performed according to the manufacturer's instructions (Remel, Kansas, USA). For the biochemical tests, the diluted bacterial cultures were inoculated into the biochemical reagent-containing wells of a plastic container provided in the kit. For the oxidase test, fresh bacterial cells were smeared on Whatman No. 1 filter paper, and a drop of RAPID oxidase solution was added to the cell smear. An oxidase positive reaction creates a dark purple color on the filter paper.

16S rRNA sequence analysis

N1c and N5a, 2 copper-tolerant isolates, were grown in LB medium overnight. Genomic DNA of the cells was isolated using the Fermentas genomic DNA isolation kit, and the cells were used as a template for amplifying 16S rRNA genes by polymerase chain reaction (PCR). The eubacterial primers fD1, 5′ AGAGTTTGATCCTGGCTCAG 3′ (*E. coli* positions 8 to 27) and rP2, 5′ ACGGCTACCTTGTTACGACTT 3′ (*E. coli* positions 1494 to 1513) (19) were used for amplification. PCR reaction mixtures contained 32 µL of water, 5 µL of 10X PCR Mg^{+2} buffer, 50 pmol of each primer, 5 µL of 2 mM dNTP, 0.5 µg of genomic DNA, and 3 U of Taq DNA polymerase. PCR was carried out in 35 cycles: 1 min at 94 °C, 1 min at 58 °C, and 2 min at 72 °C. The initial denaturation was carried out at 94 °C for 10 min. The final extension was for 10 min at 72 °C. Reaction mixtures were run in a 0.9% agarose gel. PCR products were extracted from the gel with the QIAGEN gel extraction kit and then used for DNA sequencing.

DNA sequencing was carried out at İontek (İstanbul, Turkey) using the chain termination method with the dye-labeled dideoxy terminators of the Thermo Sequenase II Dye Terminator Cycle Sequencing Kit (Amersham). The deduced nucleotide sequence of the data was compared with the National Center for Biotechnology Information (NCBI) database using the BLAST search available through the center's website (http://www.ncbi.nlm.nih.gov/BLAST). The 16S rRNA sequences were submitted to the Gene Bank using the BankIt service. The phylogenetic tree was constructed using the DNASTAR program.

Results and discussion

From the 5 different soil samples taken from heavy metal-contaminated areas of the Darıca district of Kocaeli, 9 different copper-tolerant microbial strains were isolated. Among the 9 isolates, 2 strains, N1c and N5a, formed healthier colonies on solid medium and were found to be more efficient at copper bioremoval than the other isolates.

The influence of different cultural conditions on the copper bioremoval efficiency of N1c and N5a was investigated. In order to decrease the energy expenditure of the process, the isolates were grown at 30 °C without shaking. The growth rate of the isolates was low under these conditions, especially due to the low aeration. The effect of copper concentration on the bioremoval capacity of N1c and N5a is shown in the Table. Bioremoval efficiency increased with time, and maximum efficiency was observed at 160 h of growth. Bioremoval efficiency was the highest in 10

Table. Percent bioremoval of copper by N1c and N5a at 160 h of incubation at 30 °C.

Cu^{+2} concentration (mg/L)	N1c	N5a
0	0	0
10	50	50
20	35	50
40	25	15
70	15	10
100	0	0
150	0	0

mg/L copper-containing medium for both isolates. When the concentration increased to 40 mg/L, the bioremoval capacity decreased to 18%. Bioremoval was negligible in 100 and 150 mg/L copper-containing media. High copper concentration is toxic to cells, and their growth is retarded at elevated copper concentrations. In the study by Ong et al. (20), it was observed that when the copper concentration increased above 7 mg/L in activated sludge, the activity of the microorganisms decreased. In general, copper concentration in the activated sludge is kept below 50 mg/L, and bioremoval efficiency decreases at higher concentrations of copper (21-24).

For determination of optimum temperature and pH for copper bioremoval of the isolates, experiments were performed in 20 mg/L copper-containing LB media at 30 °C without shaking the cultures. This concentration was used in order to eliminate the precipitation problem that emerges under high copper concentrations. Although there was no remarkable difference between the growth rates obtained at 30 and 37 °C, the isolates reached the highest OD_{600} when they were grown at 30 °C (data not shown). Decreasing the incubation temperature to 25 °C resulted in a decreased growth rate, especially for N5a. The highest bioremoval efficiency was observed at 30 °C. At 80 h of growth, bioremoval efficiencies were measured as 82% and 89% for N1c and N5a, respectively (Figure 1). Optimum pH for maximum copper removal was 6.8 (Figure 2). Bioremoval efficiency decreased drastically as the pH changed

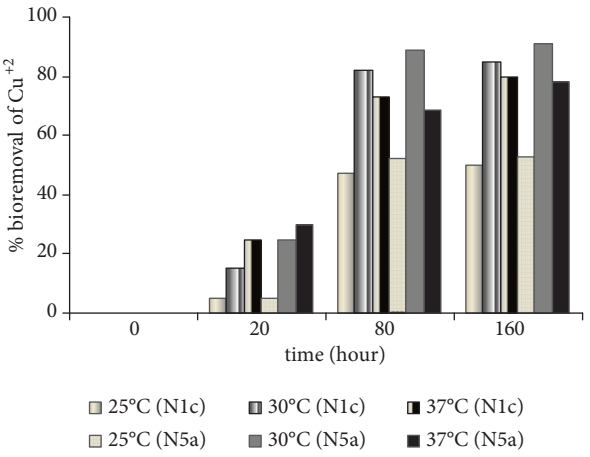

Figure 1. Percent bioremoval of Cu^{+2} at different incubation temperatures.

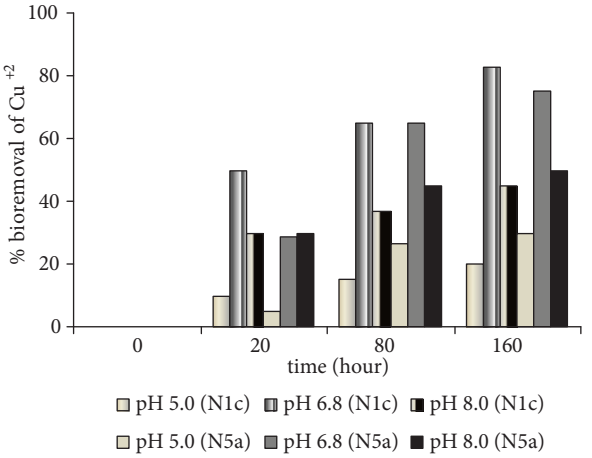

Figure 2. Percent bioremoval of Cu^{+2} at different pH levels.

to 5.0 or 8.0. N1c was affected by pH changes more than N5a. Biomass of the isolates did not show a remarkable increase at pH 5.0. Slow growth of strain N5a at pH 5 allowed 26% copper removal at 80 h of incubation.

Aeration was shown to be an important parameter for the growth of N1c and N5a. When the cells were grown by shaking at 130 rpm, their growth rates increased remarkably. The resistance of the isolates to copper was compared with that of *E. coli* at 37 °C under aerated conditions. The decrease in cell numbers in N1c, N5a, and *E. coli* cultures under increasing copper concentrations is seen in Figures 3A, 3B, and 3C, respectively. The % resistance of the cells was calculated for 72 h of growth (Figure 3D). It was observed that the % resistance of N5a was higher than that of N1c and *E. coli* at high concentrations of copper. N5a was about 875 and 100 times more resistant than *E. coli* to 150 mg/L and 200 mg/L copper, respectively. At 20 mg/L copper-containing medium, 1.32% of N1c and 8.5% of *E. coli* cells survived after 72 h of incubation, while more than half of N5a cells survived at that concentration. *E. coli* was found to be as resistant to copper as N1c. The resistance of *E. coli* to high copper concentrations is not a surprising finding. In a study by Ibrahim et al. (25), growth inhibition for *E. coli* O157:H7 was negligible in the presence of 50 mg/L copper. *E. coli* is actually known to handle copper toxicity with its multiple systems under varying environmental conditions (26). One of these systems is the membrane-bound cupric-reductase of *E. coli*, which has an important function

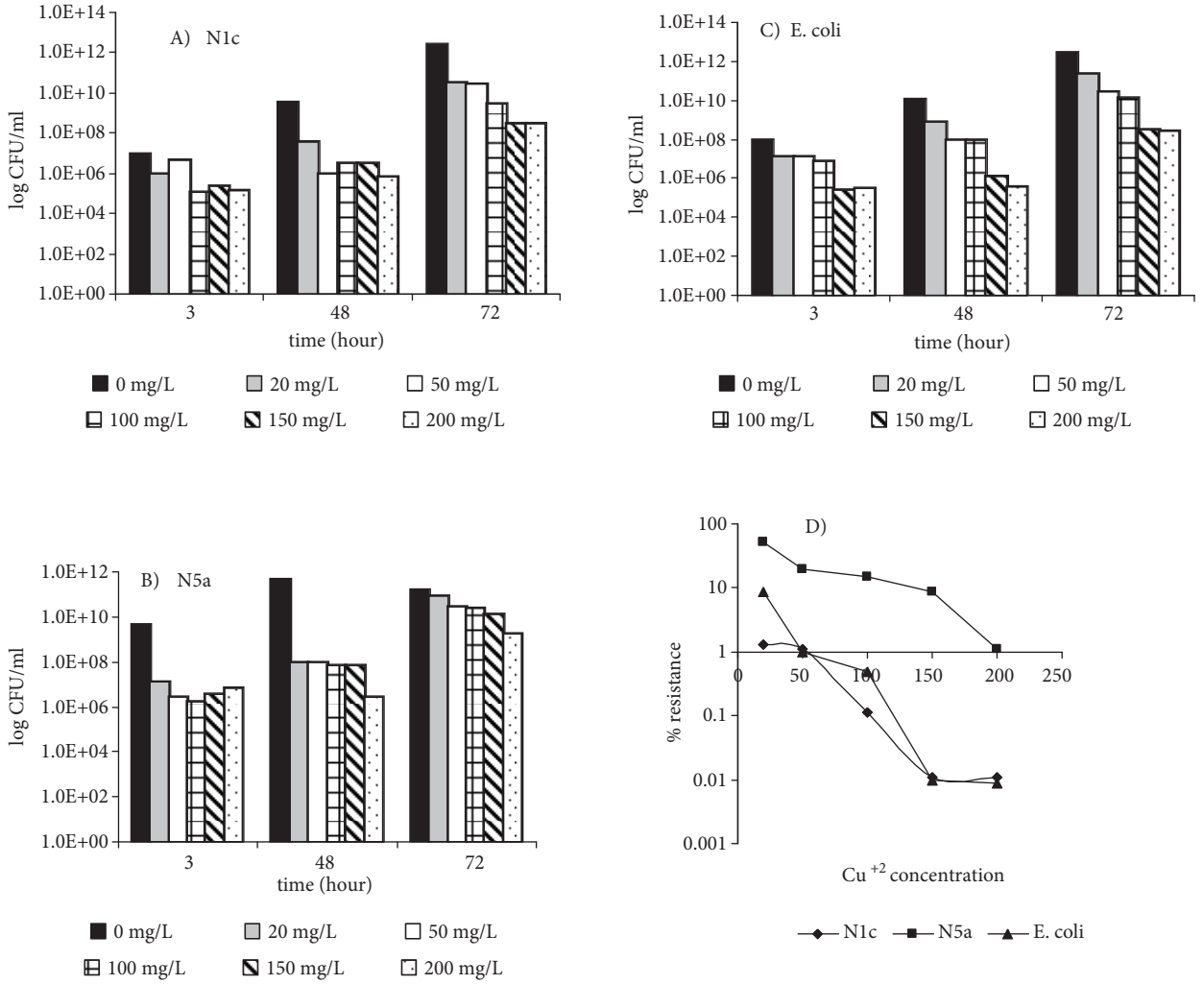

Figure 3. Growth of N1c (A), N5b (B), and *E. coli* (C) and % resistance (D) in LB with different concentrations of Cu^{+2} at 37 °C and 130 rpm.

in mediating tolerance to low or high copper concentrations (27). The genes responsible for copper tolerance of the isolates remain to be determined in future studies.

The copper removal capacities of N5a and N1c were compared with that of *E. coli* at 37 °C and 130 rpm in the presence of 20 mg/L and 100 mg/L Cu^{+2} in LB medium (Figure 4A). In 20 mg/L copper-containing medium, the % bioremoval of N5a was the highest. N1c removed 31.7% of copper in 100 mg/L copper-containing medium, whereas bioremoval of N5a and *E. coli* was negligible at that concentration. Therefore, the resistance level of a bacterial strain may not reflect its bioremoval capacity. Although *E. coli* cells were found to be more resistant than N1c, when we look at the % bioremoval/cell values, individual N1c cells removed the highest amount of copper in 100 mg/L copper-containing LB (Figure 4B). Specific surface properties and the physiological state of the microorganisms might have a role in metal uptake. Copper removal is drastically affected by medium composition and environmental conditions. Because of this, copper removal capacity of a bacterial strain has to be determined for each specific condition under which treatment or bioremoval will be performed. Reaching a high biomass is also important for better bioremoval.

For the phylogenetic analysis of the bacteria 16S rRNA genes were amplified as described in the "materials and methods" section. About 1500-

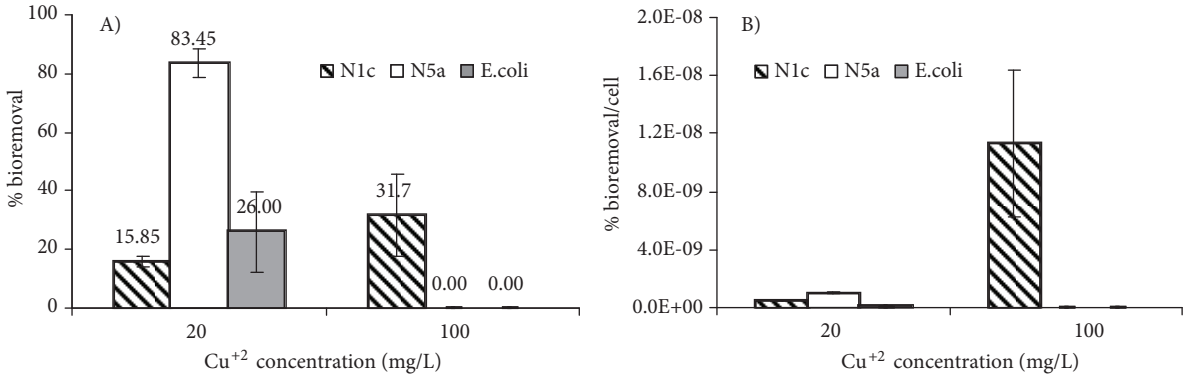

Figure 4. Percent bioremoval (A) and % bioremoval/cell (B) for different bacterial strains (N1c, N5a, and *E. coli*) at 37 °C and 130 rpm.

bp PCR products were cut from the gel and used for DNA sequencing. Sequencing was performed using the forward and reverse primers used for PCR amplification. The 16S rRNA gene sequences of N1c and N5a strains were submitted to GenBank. The accession numbers assigned to N1c and N5a are JN899140 and JN899141, respectively. A phylogenetic tree was prepared by comparing the conserved regions of 16S rRNA from the isolates with 16S rRNA gene sequences from 10 other genera (Figure 5). Comparison of 16S rRNA gene sequences revealed that N1c showed about 96% similarity to *Achromobacter*, *Alcaligenes*, and a novel genus, *Collimonas* (28). A 96% similarity in 16S rRNA gene sequences is a low value for identification at species level. Therefore, N1c may be a member of a novel bacterial species. *Achromobacter* and *Alcaligenes* are closely related genera (29), and both belong to the family *Betaproteobacteria*. *Achromobacter* is also known for its copper-containing nitrate reductase enzyme (30). Similar to N1c, strain R14C4 isolated from the biofilm communities of a reactor by Zilouei et al. (31) was found to be equally related to *Collimonas fungivorans*, *Achromobacter xylosoxidans*, and various *Alcaligenes* spp. (98% similarity). R14C4 was shown to degrade chlorophenols at high degrees.

As a result of a BLAST search, 96% similarity was observed between the 16S rRNA gene sequence of N5a and *P. stutzeri*. Several other *Pseudomonas* spp. previously isolated from different sources, such as metal-contaminated soil samples or vineyard soil, had different bioremoval efficiencies (32,33). *P. putida* CZ1, isolated by Chen et al. (33), was also found to be efficient in zinc bioremoval.

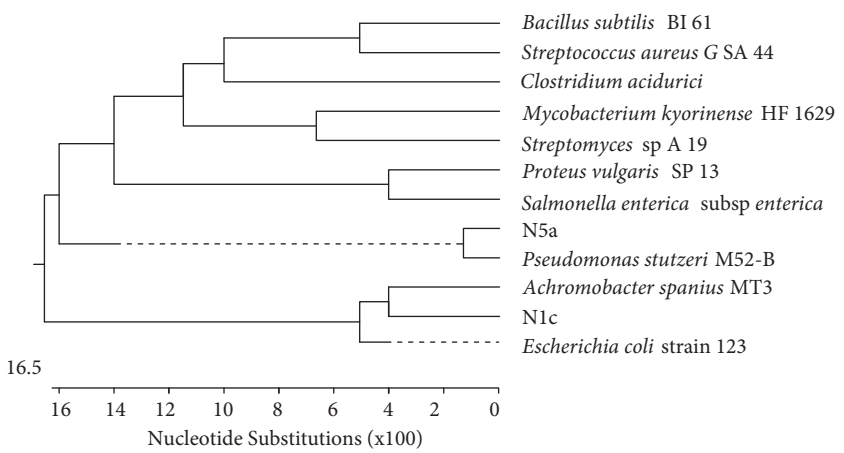

Figure 5. Phylogenetic tree prepared with DNASTAR balanced branches program. The distances between ancestors of the tree are averages. Dotted lines indicate negative branched length.

As with any other *Pseudomonas* species, N5a cells were observed as gram-negative and rod-shaped bacteria under the microscope. It was also observed that the colony morphology of N5a was identical to that of *P. stutzeri*. Although *Alcaligenes* species and *Achromobacter* species are gram-negative bacteria, Gram stains of N1c cells revealed gram-variable reactions in our study. There are some reports of gram-variable reactions in *Alcaligenes* (34) and *Achromobacter* species (35). Cells of N1c were rod-shaped under the microscope, and RAPID oxidase test results indicated that both of the isolates were oxidase-positive. The other RAPID biochemical tests did not help to identify N1c and N5a. It was shown that the isolates were not spore-formers and were not able to grow in anaerobic conditions. These results also supported the 16S rRNA gene sequence identity of the isolates.

The present study reports the copper resistance level and bioremoval efficiency of 2 local isolates that were possible members of *Achromobacter* sp. and *P. stutzeri*, respectively. However, further characterization is necessary in order to provide genus and species names for the isolates investigated in this study. These bacterial genera or species play an important role in waste treatment processes. Heavy metal tolerance is a desired property for a microorganism used in waste treatment processes. In this respect, N5a cells can be used for treatment of copper-rich wastes, and N1c cells are suitable for copper bioremoval in aerated sludges containing high amounts of copper. The capacity of N1c and N5a for denitrification or degradation of hazardous pollutants will be determined in future studies.

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