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Molecular analysis of copper resistance determinant (*cop*A) in copper oxide nanoparticles resistant *Pseudomonas fluorescens* CuO-2 isolated from soil

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Abstract

The pollution of the environment with toxic heavy metals is spreading and to survive under metal-stressed conditions, bacteria have evolved several types of mechanisms. In this study, copper resistance determinant (*copA* gene) in *Pseudomonas fluorescens* CuO-2 was investigated. The strain of *P. fluorescens* used in this study was previously isolated from soil. Genomic DNA was extracted from pure culture and the PCR method was used to investigate the *copA* gene. The *copA* gene that is responsible for resistance to copper was shown to be present in *P. fluorescens* CuO-2 by PCR. Nucleotide sequence of PCR fragment, demonstrated the presence of *copA* gene covering about approximately 300 bp involved in Cu resistance. The comparison of *copA* with other sequences in the genbank database demonstrated significant similarities to *copA* gene from other bacteria. Comparative analysis of CopA protein from different species of *Pseudomonas* revealed that the *copA* gene product may plays a major role in the copper resistance mechanism.

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Introduction

The extensive production and use of nanoparticulate metal oxides are growing rapidly therefore, these nanoparticles can release into the environment and cause undesirable effects. Copper oxide (CuO) NPs are one of the most important nano metals, which are commercially manufactured on a large scale for both industrial and household applications (Alahdadi *et al.*, 2011). CuO NPs exhibits a range of potential physical properties, such as high temperature superconductivity, electron correlation effects, and spin dynamics (Cava, 1990; Tranquada *et al.*, 1995) and have been applied in different areas, including gas sensors (Chowdhuri *et al.*, 2004), catalysis (Jammi *et al.,* 2009), batteries (Zhang *et al.,* 2005) and high temperature superconductors (Dar *et al.*, 2008). CuO NPs can be used as an additive in lubricants, oil, plastics/ polymers, metallic coating and ink (Hernández *et al.*, 2010; Bouvy *et al.*, 2007; Lin and Xing, 2007).

Extensive production and consumption of CuO NPs have increased their release and disposal into the environment. *Pseudomonas* is a genus of Gram-negative aerobic bacteria with functions of ecological, economic, and health-related importance. These bacteria demonstrate a great deal of metabolic diversity and consequently are able to colonize a wide range of niches (Barnali *et al*, 2010). Many studies have shown the potential of *Pseudomonas* species to degrade a variety of compounds (Choi *et al*, 2013).

Pseudomonas fluorescens is a ubiquitous bacterium endowed with a remarkable adaptability to diverse environment. This soil microorganism has the ability to metabolize a variety of divers materials (Tanase *et al.*, 2009). In order to survive in the environment, microorganisms need to develop different mechanisms to confer resistance to heavy metals and nanoparticles. Copper resistance has been observed in several bacteria isolated from environments (Brown *et al.*, 1992). Uptill now, strong Cu resistance has been linked with plasmids in *Pseudomonas syringae* pv. *tomato* and *E. coli* and with the chromosome in *Xanthomonas* (Brown *et al.*, 1992). The genetic determinants, *cop* from *Pseudomonas syringea, pco* from *E. coli.* and *cop* from *Xanthomonas campestris* show strong similarities with each other (Brown *et al.*, 1992). At the molecular level, the *cop* operon of *Pseudomonas yringae* pv. *tomato,* was the first characterized Cu resistant determinant (Cooksey, 1987; Cooksey *et al.*, 1990). Sequence analysis showed that *cop* consists of four structu1al genes designated *copA, copB, copC* and *copD,* which form one cluster and which are oriented in the same direction (Mellano and Cooksey, 1988). Many studies have focused on the antimicrobial activity of nanoparticles but limited information on the resistance of bacteria to CuO NPs is available. The principal objective of this study was to investigate CuO NPs and copper resistance properties of *P. fluorescens* CuO-2 that was isolated from soil. The study also included an analysis of *copA* gene implicated in copper resistance.

Materials and methods

Sources of chemicals and bacterial strain

Copper oxide nanopowder (CuO; CAS No. 1317-38-0, purity >99%, black, with an average size of < 50 nm and a specific surface area of ~29 m^2 g^{-1}) were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). *P. fluorescens* CuO-2 (GenBank accession No. JX441327) was previously isolated from Soil (Soltani Nezhad *et al.*, 2014).

preparation of cuo nps suspension

CuO NPs suspension of the desired concentration was prepared by mixing CuO NPs in sterile double-distilled water. The dispersed NPs were ultrasonicated (TECNA 6, Italia) for 15 min at 30W. The suspension was kept at 4°C.

Effect of the Presence of Cu Ions and CuO NPs on the Growth of Bacterial Strain

P. fluorescens CuO-2 cells were grown to early logarithmic phase ($OD_{600\ nm}$ = 0.1) in minimal medium (Dimkpa *et al.*, 2011). The early log-phase cells of *P. fluorescens* CuO-2 were harvested and resuspended in sterile distilled water to 10^8 cells ml^{-1}

for all treatments. Treatments included exposure to CuO NPs (100, 200, 300 and 400 mg Cu l^{-1}) and Cu ions (0.1, 0.2, 0.3, 0.4 and 0.5 mM) with shaking at 180 rpm at 30 °C. Controls lacked these additions to the cell suspensions. Growth rates from three replicates were checked every 4 h by absorbance at 600 nm using a bio wave (WPA) spectrophotometer.

Determination of Maximum Tolerable Concentration (MTCs) of CuO NPs and Cu Ions

To determine the maximum tolerable concentration (MTC) of CuO NPs, 10 mg ml^{-1} stock solution of CuO NPs was prepared and added to sterilized nutrient agar medium in concentrations varying from 100 to 600 mg l^{-1}. Analytical grade of $CuCl_2$ $2H_2O$ was used to prepare 1 M stock solution. Stock solution was filter-sterilized and added to nutrient agar medium to final concentration of 0.1 to 0.7 mM for determination of MTC of the metal ions for *P. fluorescens*

CuO-2. Bacterial strain was streaked from log phase and incubated at 30 °C for 2 days (Margeay *et al.*, 1985). The highest concentration that allowed growth after 48 h of incubation at 30°C was considered the MTC.

Determination of Release of Soluble Cu Ions from CuO NPs

CuO NPs dispersed in LB broth at 100 and 200 mg l^{-1} were shaken gently for 60 min and then centrifuged for 30 min at 15000 × g. The supernatant was carefully collected and recentrifuged for 30 min. The Cu ion concentrations in this second supernatant were measured by atomic absorption spectroscopy (Varian SpectrAA 220, Australia) (Dimkpa *et al.*, 2011). The whole study was repeated three times with three replicates of sampling each time.

Amplification of copA Partial Sequence

Genomic DNA was extracted from pure culture of *P. fluorescens* CuO-2 using DNA extraction kit (Cinnagen Cat No: DN8115C), according to the manufacturer's instructions and protocols. Oligonucleotide sequences used as primers for the partial amplification of the copper-resistance gene (*copA*) were *copA*-F: 5´-GTGTACGGTCCGCTGGTTAT-3´) and *copA*-R: 5´-CTTGAACACTCCGGTCCAG-3´. The conditions for PCR amplification were: pre-denaturalization at 94 °C for 5 min, then 35 cycles of denaturing at 94 °C for 45s, annealing at 56 °C for 40s, extension at 72 °C for 45s and a final step for extension at 72 °C for 5 min. PCR products were separated by electrophoresis in a 1.5% (w/v) agarose gel (Merck, Germany) and visualized with ultraviolet illumination after staining with 0.5 µg ml^{-1} ethidium bromide.

Nucleotide Sequence Accession Number

The partial nucleotide sequence of *copA* gene of *P. fluorescens* CuO-2 has been deposited in GenBank under Accession No: KC683807.

Results

Growth of P. Fluorescens CuO-2 in the Presence of CuO NPs and Cu Ions

The growth curves *of P. Fluorescens* CuO-2 for different concentrations of CuO NPs and Cu ions are shown in Fig 1. The growth pattern with all studied CuO NPs concentration was similar to that of Cu ions. It is evident that Cu ions and CuO NPs not affect the growth rate of *P. Fluorescens* CuO-2.

Table 1. Location of Conserve domains of copper resistance protein A from *P. fluorescens* A506.

Conserved Domains	Location
54 – 164	Cu-oxidase_3
171 – 345	Cu-oxidase
452 – 569	Cu-oxidase_2
6 – 569	copper_res_A

Determination of MTCs of CuO NPs and Cu Ions

MTCs of *P. fluorescens* CuO-2 against Cu ions and CuO NPs have shown that this strain was capable of growing at high concentration of heavy metals. We have determined that *P. fluorescens* CuO-2 is able to growth at CuO NPs concentrations up to 400 mg ml^{-1} and Cu ions concentrations up to 0.4 mM.

Release of Cu Ions

During dispersion, a substantial amount of Cu ions was quickly released from CuO NPs into the growth medium. The 100 mg l^{-1} of CuO NPs released 3.78 ± 0.003 mg l^{-1} Cu ions and 200 mg l^{-1} of CuO NPs released 7.52 ± 0.003 mg l^{-1} Cu ions into the medium. The release of ions from CuO NPs was mass dependent: less ions were released as CuO NPs concentration declined. Dimkpa *et al* demonstrated that the release of ions by NPs contributed to the toxicity of NPs (Dimkpa *et al*., 2011).

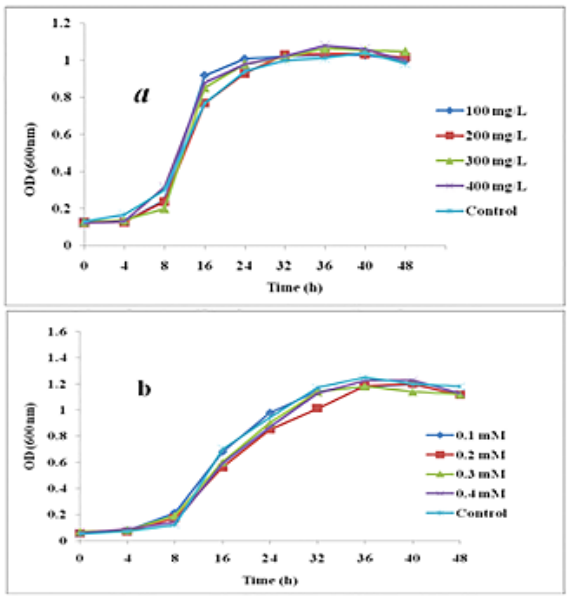

Fig. 1. Representative batch growth profile of *P. fluorescens* CuO-2 in minimal medium dosed with different CuO NPs (a) and Cu ions (b) concentrations and control (without CuO NPs and Cu ions) at 30°C.

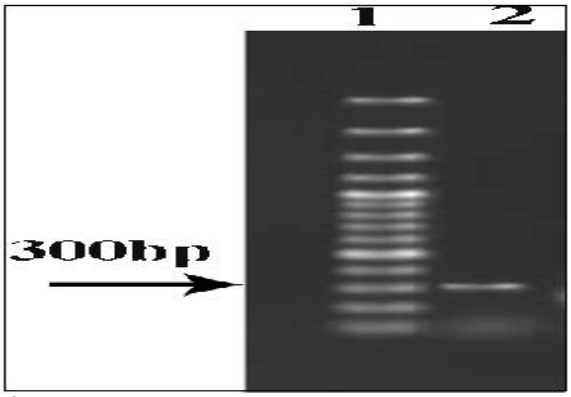

Fig. 2. Agarose gel electrophoresis of *copA* PCR product. Lanes 1: DNA size marker; 2:*cop*A.

Amplification of copA gene

Presumptive evidence for the presence of the locus *cop*A in the genome of *P. Fluorescens* CuO-2 was obtained by using the primer pair *copA*-F and *copA*-R. The specific primers used for the amplification of the *cop*A gene yielded a band of approximately 300 bp (fig 2).

Homogenous Analysis of copA Gene

The nucleotide sequence of the *copA* gene was aligned with *copA* gene partial sequences of other copper resistant bacteria. Sequence comparisons with our copper resistant PCR fragment [Accession number: KC683807.1] showed a high homology with copper resistance genes from other bacteria (91% with a copper resistance gene from *P. fluorescens* A506 [CP003041.1]. By comparing these two genes, it was determined that the beginning of our PCR fragment was the same as the nucleotide 504 in *P. fluorescens* A506. By comparing our PCR fragment with protein sequence of copper resistance gene from *P. fluorescens* A506, it was found that the second, third and fourth nucleotides of this fragment were related to amino acids 169 of protein sequence. Hence, to find the protein sequence of PCR product, beyond the second nucleotides was translated to protein sequence by using the HCV sequence database. Partial sequence of copper resistance protein A from isolated strain was compared with protein sequences in the NCBI database using protein blast program and blastp algoritm. The blastp results showed that the protein sequence encoded by this DNA fragment (Sequence ID: gb|AGL96592.1) is 95% similar to protein encoded by the *copA* gene of *P. fluorescens* A506 (Sequence ID: ref|YP_006325270.1). By comparing these two protein sequences the following results is obtained (Fig 3). These changes are probably due to mutations that occur in nature. In this protein fragment, five amino acids have changed. As shown in the figure 3 with arrows, the results of three of these changes are amino acids with similar activity that actually their change has no special effect on protein behavior. According to blastp result, phylogenic tree based on partial sequences of copper resistance protein A from isolated strain and its neihborhood refrences proteins was drived by CLC protein Workbench softwar, version 5.2 (Fig 4). In the phylogeny (fig 4) has shown that protein sequence of

P. fluorescens CuO-2 is very similar to copper resistance protein and copper oxidase from other bacteria. It is interesting that the copper resistant protein of *Entamoeba hystolytica* that is belong eukaryotes, is also great similarity to the sequences has been listed. This subject shows the conservation of this sequence among organisms as well.

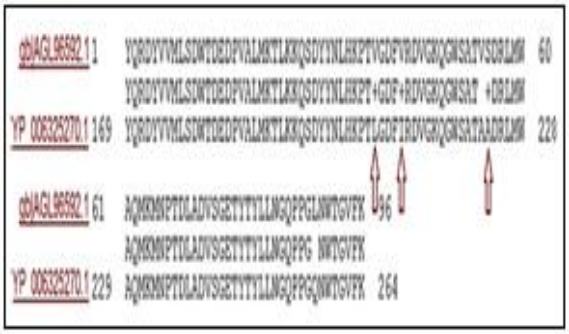

Fig. 3. Amino acid sequences alignment of CopA protein from *P. fluorescens* CuO-2 and *P. fluorescens* A506.

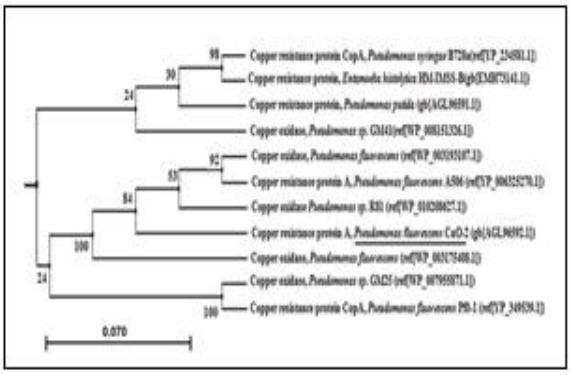

Fig. 4. Neighbor-joining tree based on copper resistance protein A sequences, showing relationships of *P. fluorescens* CuO-2 with closely microorganisms based on Neighbor-joining algorithm by CLC sequence viewer software version 6.1. Bootstrap values are shown at branch points and the scale bar indicates 0.070 estimated sequence divergences.

Conserved Regions of the Gene Fragment Sequenced

For find regions of similarity between mentioned sequences, partial sequences were arranged using protein alignment tool by CLC protein Workbench softwar, version 5.2 (fig 5). The results show, that many sequences in these protein fragments are similar and among 11 organisms are conserved.

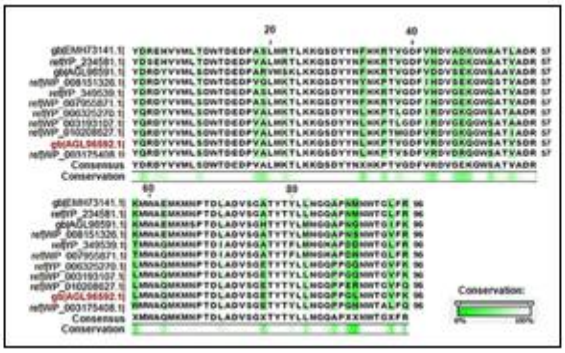

Fig. 5. Conserved regions of copper resistance protein A in *P. fluorescens* CuO-2 and closely related members of the genus *Pseudomonas*.

The Functional Role of Protein Sequence

Because this partial protein sequence is very similar to copper resistance protein A in *P. fluorescens* A506, the role of this protein is described. Conserve domains of copper resistance protein A from *P. fluoresceens* A506 (ref|YP_006325270.1) are shown in fig 6 (Marchler-Bauer and Bryant, 2004; Marchler-Bauer *et al.*, 2009; Marchler-Bauer *et al.*,2011). As shown in the figure 6 the three parts of this protein have formed three super families that act as functionally important regions of protein (specific hit) and with other regions make up the cooper_res_A multi domain. As mentioned in table 1, the PCR product formed from amino acids 169 to 265, which is part of Cu- oxidase super family. This fragment is conserved in many organisms.

Discussion

In order to survive in the environment, bacteria need to develop different mechanisms to confer resistances to heavy metals. There is no general mechanism for resistances to all heavy metals. Bacteria have evolved several types of mechanisms to tolerate the uptake of heavy metals. These mechanisms include the efflux of metal ions outside the cell, accumulation and complexation of the metal ions inside the cell, and reduction of the heavy metal ions to a less toxic state (Nies, 1999). In this paper, the *cop*A gene that is responsible for resistance to copper ions was shown to be present in *P. fluorescens* CuO-2. The chromosomal mediated copper resistance has also been reported in P. *fluorescens* (Yang *et al.*,

1993). Comparative analysis of CopA protein from different species of *Pseudomonas* revealed that the *copA* gene product may plays a major role in the copper resistance mechanism. This is consistent with the results described by Mellano and Cooksey (Mellano and Cooksey, 1988) which showed that the *copA* and *copB* genes from P. syringae pv. tomato are essential for resistance and the *copC* and *copD* are only required for maximum copper resistance. In *Pseudomonas* spp, *copA* is present in the *cop* operon (*copABCDRS*) on chromosome. CopA belongs to multicopper oxidase protein family (Yang *et al*., 1993; Cha and Cooksey, 1991). Copper resistance is mediated by sequestration of copper in the periplasm by the copper binding proteins CopA and CopC and in the outer membrane by CopB (Cha and Cooksey, 1991). CopD appears to be an inner membrane protein involved in copper uptake in conjugation with CopC (Cha and Cooksey, 1991; Cha and Cooksey, 1993), whereas CopR and CopS are involved in activation of copper resistance operon (Mills *et al*., 1994).

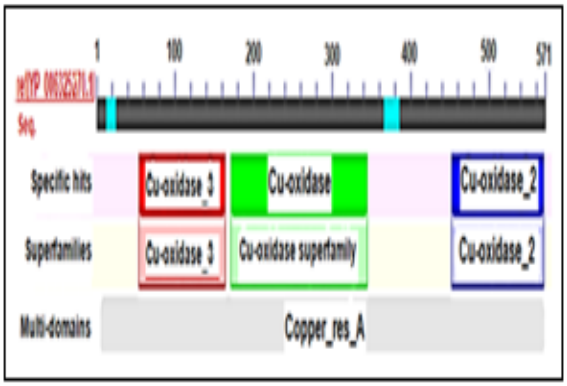

Fig. 6. Conserve domains of copper resistance protein A from *P. fluorescens*.

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