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Copper Uptake by *Pseudomonas aeruginosa* Isolated from Infected Burn Patients

Muayad M. Abboud · Humodi A. Saeed · Khaled A. Tarawneh · Khaled M. Khleifat · Amjad Al Tarawneh

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Abstract *Pseudomonas aeruginosa* was isolated from infected burn patients and characterized by standard biochemical tests. The in vitro copper uptake was compared between this isolated pathogenic strain and two non-pathogenic control strains of Gram positive bacteria *Bacillus thuringiensi*s strain *Israelis* as well as Gram negative bacteria *Enterobacter aerogenes*. Maximum copper uptake of 470 ppm/g biomass was obtained by *P. aeruginosa* strain, while the control strains *B. thuringiensis* and *Enterobacter aerogenes* had copper uptake of 350 and 383 ppm/g biomass, respectively. However, the lowest copper uptake (60 ppm/g biomass) was observed with another control the saprophytic strain *Pseudomonas* (*Shewanella*) *putrefaciens.* A further investigation regarding the effect of copper toxicity on bacterial growth, gave an MIC score of 600 ppm for *P. aeruginosa* strain compared to 460 and 300 ppm for the two Gram positive and Gram negative control strains, respectively. In tandem with these in vitro findings, blood analysis on burn patients infected with *P. aeruginosa* has indicated a selective decrease of copper (hypocupremia) and ceruloplasmin plasma levels. The iron metabolism was also affected by this copper deprivation leading to a similar decrease in plasma levels of PCV, iron, total iron binding capacity, and transferrin. All these hematological changes were significantly different ($P < 0.05$) from the matched group of non-infected burn patients. The observed hypocupremia in infected burn patients was attributed to demanding scavenger ability by *P. aeruginosa* strain for the copper of plasma.

M. M. Abboud (✉)
Department of Pharmacology, Faculty of Medicine, Mutah University, Karak, Jordan
e-mail: muayadabboud@yahoo.com

K. A. Tarawneh · K. M. Khleifat · A. Al Tarawneh
Department of Biology, Faculty of Science, Mutah University, Karak, Jordan

H. A. Saeed
College of Medical Laboratory Sciences, Sudan University of Science and Technology, Khartoum, Sudan

Introduction

Alterations of heavy metals metabolism in patients with thermal injury have been documented as early as 1970 [21]. Since then many works has linked the thermal injury with a decrease in circulatory levels of heavy metals [3, 4, 15], which was attributed primarily to the loss of these metals through the routes of inflammatory wound exudates and urinary excretion [3, 4, 43]. Other works, however, reported no significant changes in serum concentration of heavy metals like copper and zinc after burn injury [39]. In fact, there was a selective increase of Cu plasma levels being depicted after 1 day of severe burns, which subsequently remained elevated for 9 days [1]. In these severely burned patients, anemia has been frequently observed and ascribed to a multifactorial etiology including hemorrhage, hemolysis, in addition to the suppression of erythropoiesis rate [37, 44].

Pseudomonas aeruginosa is an opportunistic bacterium causing serious infections in burn wounds, respiratory diseases, cystic fibrosis, and leukemia [5, 6]. Despite advancements in medical care, *P. aeruginosa* is still considered as a life threatening pathogen in infected burn patients [25, 40]. Such bacterial infection causes common complications, which contribute substantially to burn morbidity and mortality.

The copper is an essential trace element for living organisms, which shares with iron a unique ability to maintain distinct redox states for proteins that carry out significant biological functions as well as needed for growth and development [22, 31]. Apart from the genetic diseases of Menkes and Wilson's, several human disorders are associated with acquired copper deficiency such as: osteoporosis, rheumatoid arthritis, and cardiovascular disease [29]. These disorders are characterized by low levels of copper ions in serum [8, 20]. Also, copper ion can be potent cytotoxin when accumulated in excess of cellular requirement [23]. Therefore, maintenance of the appropriate Cu homeostasis requires certain balance between its cellular uptake and detoxification [24].

In bacteria, the efflux systems are more common resistance mechanisms for handling heavy metals such as copper. One of these mechanisms is the *cop* system that containing the structural genes *cop ABCD* [11, 41]. The *cop B* and *cop D* genes are involved in the transport of copper across the membrane, while the products of *cop A* and *cop C* genes are outer membrane proteins that bind to Cu in the periplasm to protect the cell from copper toxicity. Other types of efflux systems, such as the P-type ATPase, simply operate through pumping toxic Cu ions out of the cell. Interestingly, the P-type ATPases defective in human hereditary diseases of copper metabolism were found to have more similarity to bacterial than other eukaryotes ATPases [41]. Several *Pseudomonas* strains have accessibility for in vitro copper uptake [10, 16, 17]. In particular, the strain *P. aeruginosa* has unique ability to resist high levels of some xenobiotics like antimicrobial agents, solvents, and heavy metals [18]. This resistance is attributed to a combination of decreased outer membrane permeability and the presence of multiple efflux pumps [26, 27].

The present work was undertaken to compare the efficiency of in vitro copper uptake by *P. aeruginosa* with selective control of non-pathogenic bacterial strains. Also, an attempt was made to link the copper scavenger ability with the decrease in serum copper levels observed during the opportunistic infection of thermal injury patients by *P. aeruginosa*.

Materials and Methods

Bacterial Isolation and Growth

The strains *P. aeruginosa*, *P. putrefaciens*, *Bacillus thuringiensis*, and *Enterobacter aerogenes* were grown on Luria–Bertani (LB) medium [19]. This medium is composed of 10 g trypton, 5 g of yeast extract, and 10 g of NaCl per liter of distilled water. The pH was adjusted to 7.0 with 0.1 N NaOH or 0.1 N HCl. The LB agar plates containing 15 g/l of agar in addition to the above mixture. An initial inoculum of 1.5 × 108 viable cells was used in all cultures. Bacterial growth was maintained in 50 ml LB-broth media using agitation rate of 150 rpm and 37°C. The pathogenic strain *P. aeruginosa* was isolated from the blood and scar tissues of hospitalized burn patients. The isolated strain was characterized morphologically and analyzed by the Api 20 NE Kit testing system (Biomeriex, France) as described before [13].

Burn Patients

Forty-two patients (males and females ranged between 19 and 42 years of age) were admitted to hospital at different intervals suffering second or third degree of burning. On first day of admission, blood samples were collected from these patients and considered as pre-infected burn control. Five days after hospitalization, the burn patients usually became more susceptible for infection with *P. aeruginosa.* This bacterial infection was confirmed later by conducting the above mentioned Api 20 NE testing system on corresponding samples from burn patients. To carry out the hematological analysis, blood samples were collected from 42 infected burn patients as well as from similar number of controls including non-infected burn patients and healthy subjects. All blood samples were processed in duplicate for the determination of PCV, transferrin, ceruloplasmin, iron, total iron binding capacity (TIBC), and Copper.

In Vitro Bacterial Copper Uptake

Copper solutions of either cupric chloride dehydrate or cupric nitrate 3 hydrate or cupric sulfate were prepared in distilled water at different concentrations range of 20, 40, 60, 80, 100, 200, 300, 400, 500, 600, and 700 part per million (ppm), respectively. These solutions were sterilized in the autoclave. Meanwhile, 50 mg of bacterial biomass was harvested from exponentially growing bacteria in LB medium and washed twice with 5 ml Ringer's solution (NaCl 0.85%, $CaCl_2$ 0.03%, KCl 0.025%, $NaHCO_3$ 0.02%) using refrigerated centrifuge.

To perform an in vitro copper uptake experiment, the method described before [42] was applied with slight modifications. The harvested bacteria were incubated for 1 h with the corresponding copper compound concentration at 37°C and 150 rpm agitation rate. At the end of incubation period, the bacterial biomass was harvested and washed with 0.1 M ammonium acetate solution. The washed biomass was decomposed by 1% nitric acid for 24 h, and the biomass copper contents were quantified by atomic absorption spectroscopy.

Data Analysis

The results were expressed as mean ± SD and analyzed statistically by student's *t*-test. The correlation between the data was tested by simple linear regression, employing SPSS computer program. *P*-value of less than 0.05 was considered as the lowest limit of significance.

Results

In Vitro Copper Uptake by *P. aeruginosa* and Other Strains

The in vitro copper uptake was investigated in *P. aeruginosa* isolated from patients with burn injury and compared with two control strains, *Enterobacter aerogenes* as Gram negative and *B. thuringiensis* strain *Israelis* as Gram positive bacteria, respectively. The amounts of bacterial copper uptake by bacterial biomass was measured and expressed as ppm copper uptake/g biomass. Variable amounts of copper uptake were achieved by all three bacterial strains when the exponentially growing bacterial culture was supplemented with cupric chloride dihydrate concentrations ranged from 0 to 700 ppm (Fig. 1). The highest amount of copper uptake (470 ppm/g biomass) was obtained by *P. aeruginosa* culture (Table 1). The control strains *B. thuringiensis* and *Enterobacter aerogenes* showed maximum copper uptake of 350 and 383 ppm/g biomass, respectively. Also under similar growth conditions, the saprophytic strain *P. putrefaciens* exhibited extremely lower copper uptake (60 ppm/g biomass) than *P. aeruginosa* (Table 1) and (Fig. 2).

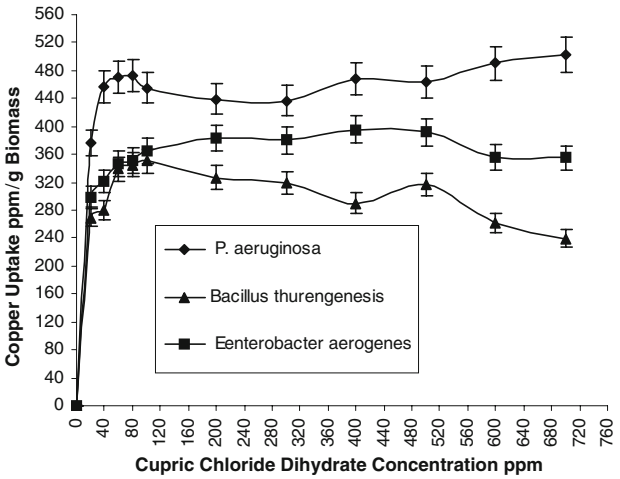

Fig. 1 *In vitro* copper uptake (ppm/g biomass) by *Pseudomonas aeruginosa*, *B. thuringiensis*, and *Enterobacter aerogenes*. Following an exponential growth in LB medium, the harvested bacteria were incubated for 1 h at 37°C and 150 rpm agitation rate with 0–700 ppm concentration ranges of cupric chloride dihydrate as a copper source. The amount of copper content was measured by a flame atomic absorption instrument

Table 1 Maximum copper uptake by different bacterial strains

Bacteria	Maximum copper uptake (ppm/g biomass)
P. aeruginosa	470
P. putrefaciens	60
B. thuringiensis	350
E. aerogenes	383

The peak copper uptake (ppm/g biomass) obtained under similar conditions to that described in Fig. 1 was determined for different bacterial strains, using cupric chloride dihydrate as a copper source

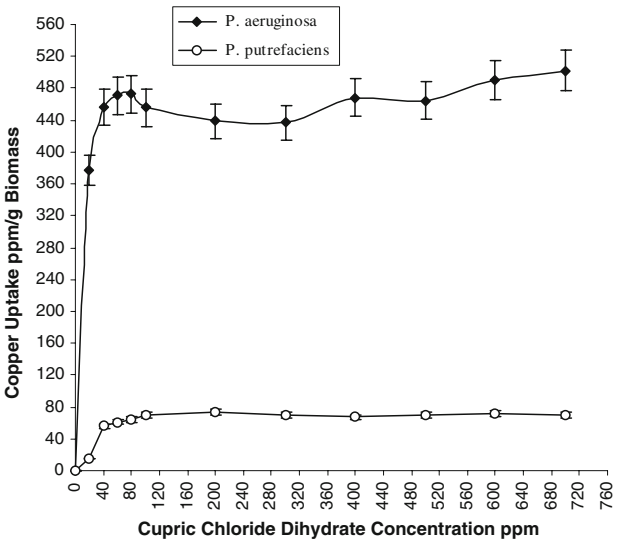

Fig. 2 Comparison of copper uptake (ppm/g biomass) between *Pseudomonas aeruginosa* and *Pseudomonas putrefaciens* using cupric chloride dihydrate as a copper source. The copper uptake was determined under conditions similar to that described in Fig. 1

Bacterial Tolerance of High Cupric Chloride Dihydrate Concentrations

The saturation kinetics of copper uptake observed at high levels of cupric chloride highlighted the need for further investigation regarding the toxic effect of cupric chloride concentration on bacterial growth. Figure 3 indicates that the MIC (minimum concentration of copper supplement that gives 50% growth inhibition) of cupric chloride for *P. aeruginosa* growth was 600 ppm. The same copper supplement produced lower MIC values of 460 and 300 ppm for the controls *Enterobacter aerogenes* and *B. thuringiensis*, respectively.

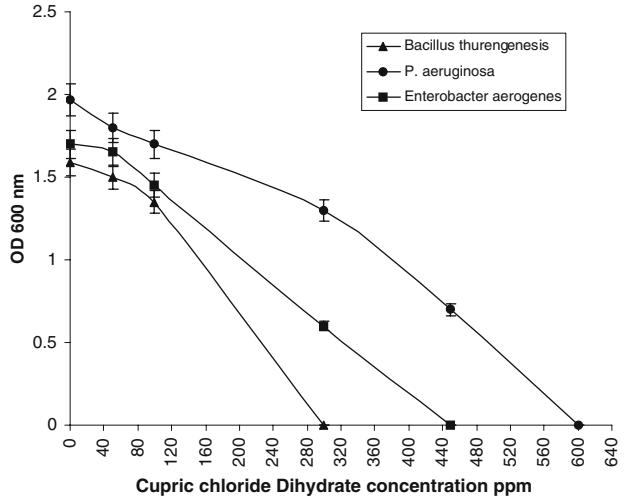

Fig. 3 Effect of different cupric chloride concentrations on growth of bacterial strains. The bacterial strains *Pseudomonas aeruginosa*, *B. thuringiensis*, and *Enterobacter aerogenes* were grown for 24 h in presence of 0–700 ppm concentration ranges of cupric chloride dehydrate as described in Fig. 1. The bacterial growth expressed as 600 nm OD was determined every 2 h

Bacterial Copper Uptake from Supplements of Other Copper Sources

A replacement of cupric chloride dehydrate with other sources of copper supplements like cupric sulfate or cupric nitrate 3 hydrate; slightly lowered the amount of Cu uptake by these bacterial strains (Figs. 4, 5). Irrespective of the copper supplement being used, the overall pattern stressed a powerful ability of *P. aeruginosa* for copper uptake compared to the control bacterial strains.

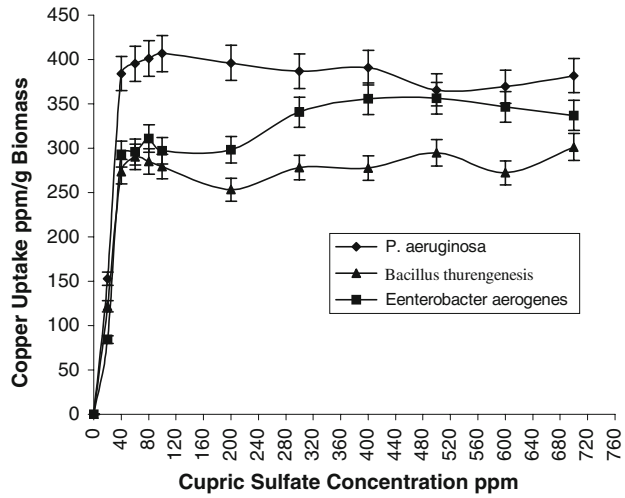

Fig. 4 Amounts of copper uptake (ppm/g biomass) by *Pseudomonas aeruginosa*, *B. thuringiensis*, and *Enterobacter aerogenes* using cupric sulfate as a copper source

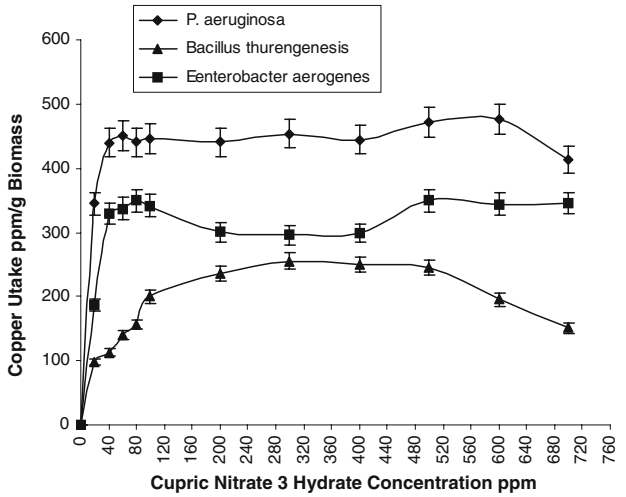

Fig. 5 Cu uptake (ppm/g biomass) by *Pseudomonas aeruginosa*, *B. thuringiensis*, and *Enterobacter aerogenes* in presence of cupric nitrate 3 hydrate as a copper source

Hypocupremia in Burn Patients Infected with *P. aeruginosa*

Blood analysis was made on two groups of hospitalized burn injury patients. The first group had confirmed infection by *P. aeruginosa* while the second group was a control group of pre-infected burn patients.

Significant decrease ($P < 0.05$) in blood levels of PCV, copper, iron, TIBC, ceruloplasmin, and transferrin were particularly found in the *P. aeruginosa* infected burn group as compared with the control non-infected burn group (Table 2).

Discussion

Present data demonstrated high efficiency exhibited by *P. aeruginosa* for the uptake as well as tolerance of copper ions when compared with two selected controls of non-pathogenic Gram positive and Gram negative bacterial strains, respectively. Moreover, a similar comparison with the weakly pathogenic strain *Pseudomonas* (*Shewanella*) *putrefaciens* provided further support for the characteristic affinity of *P. aeruginosa* strain to copper uptake. In contrast to *P. aeruginosa*, the saprophytic strain *Pseudomonas* (*Shewanella*) *putrefaciens* is rarely implicated in severe clinical syndromes of human [9, 18, 30].

The *P. aeruginosa*, as obligate aerobe, possesses stringent requirement for iron metal ions [12] and such demand may be extended to the copper ions as well. These two ions are utterly needed to facilitate the enzyme activities of bacterial aerobic respiration during multiplication of *P. aeruginosa* in the infected host [28].

Table 2 Comparison of hematological changes between burn injury patients infected with *Pseudomonas aeruginosa* and a control of pre-infected burn patients

Level of blood constituents	Pre-infected burn patients (control)	Burn patients infected with *P. aeruginosa*	*P*-value
PCV	40.44 ± 6.70	36.44 ± 5.14	0.011
Copper (μg/dl)	82.81 ± 27.27	45.94 ± 15.04	0.030
Iron (μg/dl)	119.07 ± 35.1	11.16 ± 5.59	0.010
TIBC (μg/dl)	196.88 ± 15.75	18.98 ± 9.67	0.006
Transferrin (mg/dl)	417.03 ± 13.02	399.88 ± 16.23	0.042
Ceruloplasmin (mg/dl)	110.5 ± 16.1	94.75 ± 13.83	0.048

Blood analysis was carried out on 42 burn patients and similar number of controls. *P*-value of <0.05 was considered significant

TIBC total iron binding capacity

We demonstrated a distinct hypocupremia in *P. aeruginosa* infected burn patients, which was absent in the matched group of non-infected burn patients. The lack of hypocupremia in the later group excludes the possibility of copper loss in the *P. aeruginosa* infected burn patients through the routes of inflammatory wound exudates or urinary excretion, as has been suggested for some non-infected burn patients [3, 4, 43]. A similar decrease in copper contents of the plasma has been linked with the operation of an effective efflux mechanism for the transport of copper by the *Plasmodium* that infects erythrocytes [34].

It is known that the metabolism of copper and iron metals in mammals are mainly intersect through the ferroxidase activity of ceruloplasmin protein, and therefore, a demanding stress affecting the copper metal content is likely to affect also the iron metal concentration [14, 35]. Moreover, copper deficiency can increase the frequency of infections as well as the infection-associated erythropoiesis impairment [38]. These remarks may explain the apparent anemia, which were manifested in present work through the decreased levels of PCV, iron, transferrin, and TIBC. Previous works have documented similar form of anemia in patients with cystic fibrosis as a consequence of the opportunistic infection by *P. aeruginosa* [36].

Copper resistance proved to have important contribution to the competitive survival of some *Pseudomonas* species in their environment [45]. In particular, the resistance to heavy metals during *P. aeruginosa* infection may have special health concern due to its potential for inducing further resistance of this bacterium to antibiotics. Recently, some works [7, 33] have documented a selection of cross-resistance by zinc and copper uptake against the effect of imipenem antibiotic on *P. aeruginosa*. This cross-resistance was attributed to a common co-regulation mechanism between imipenem influx and heavy metals efflux.

Owing to the essential roles played by copper ions in wound healing, immunity, and antioxidant defenses [2], it is not surprising to perceive a significant involvement of hypocupremia in the complications of cardiovascular disease, respiratory, and immune inadequacy [32]. This finding could stimulate similar study in infected burn patients to determine a possible correlation between the severity of *P. aeruginosa* infection and the extents of bacterial copper scavenger ability.

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