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Review

Copper toxicity and uptake in microorganisms

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SUMMARY

Copper is a required trace element for growth of microorganisms since it is a cofactor for numerous enzymes. Also, proteins containing copper are important electron transfer carriers. However, at elevated concentrations, copper can be highly toxic to microorganisms. This review examines copper toxicity and uptake in microorganisms, with an emphasis on copper-resistance mechanisms.

INTRODUCTION

Copper (Cu) is in the first transitional period of the periodic table. Like most of the transition metals, Cu (Group IB) forms stable complexes with other elements and can exist in more than one oxidation state [28]. The cuprous state (Cu^+) is highly unstable in aqueous solutions and is readily oxidized to the cupric state (Cu^{2+}) [60]. Copper has a higher ionization potential than metals that precede it in the periodic table, and is therefore not highly reactive. The atomic number of Cu is 29 and the atomic mass is 63.546 [28].

The earth's crust is estimated to contain about 10^{15} metric tons of copper [18]. Copper exists in nature both in its elemental state and in various mineral compounds. When assessing the distribution of copper in the environment, reservoirs resulting from man-made activity must be considered. Copper mining and smelting are important industries, and bioleaching is used to remove copper from low-grade ores [85]. Smelter activities contribute to copper contamination of the environment, as do refining processes and industrial waste effluents. Copper is used as a chemical control agent for microorganisms. The Bordeaux mixture, which contains copper, is used as a fungicide [3] and copper sulfate ($CuSO_4$) has been employed as an effective algicide [24]. Copper sprays are applied to control diseases caused by phytopathogenic bacteria, such as *Pseudomonas syringae* pv. *tomato*, which causes bacterial speck disease of tomato [7]. The widespread use of $CuSO_4$ as a feed supplement for pigs and poultry contributes to metal pollution, due to fecal waste containing high copper levels [19].

Copper is required in trace amounts for the growth and functioning of microorganisms since it is a cofactor for numerous enzymes. Also, proteins containing copper are important electron carriers. Azurin (blue bacterial copper protein) plays an important role in the oxidation of iron (Fe) in *Thiobacillus ferrooxidans* [14]. The blue copper protein of *Thiobacillus versutus* is an electron carrier between methylamine dehydrogenase and cytochrome *c* [78]. Methane mono-oxygenase, the enzyme responsible for the conversion of methane to methanol in *Methylosinus cycloclastes*, has 1 Fe and 1 Cu atom per enzyme molecule [75]. In addition, one of the superoxide dismutases of the marine microorganism, *Photobacterium leiognathi*, also contains a Cu atom [58]. Copper must therefore enter microbial cells in trace levels; however elevated concentrations can exert a toxic lethal effect. The review will examine copper toxicity and uptake in microorganisms.

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TOXICITY OF COPPER TO BACTERIA

The toxic form of copper is generally acknowledged to be Cu^{2+} [69]. Under anaerobic conditions, the conversion of Cu^{2+} to Cu^{+} can be responsible for decreased survival of bacterial species [8]. Toxicity of copper is influenced by factors such as pH, redox potential (Eh), moisture, temperature, copper binding to environmental constituents and interactions with other ions [2,31]. Copper toxicity is not confined to areas of high copper pollution. The presence of 0.006 mg Cu^{2+}/ml distilled water caused decreased viability of *Aerobacter aerogenes* (MacLeod et al., 1967) and *Klebsiella aerogenes'* growth and survival were inhibited at 10^{-8} to 10^{-6} M Cu^{2+} [86].

Mechanisms of Cu^{2+} toxicity include interactions of the ion with proteins, enzymes, nucleic acids and metabolites [29]. As well as decreased viability, an effect of toxic interactions is inhibition of respiration. The addition of 1 to 100 ppm $CuSO_4$ to sewage decreased O_2 consumption over a 5-day period [37]. Similarly, decreased O_2 uptake by sewage microorganisms was observed during exposure to 20 ppm $CuSO_4$ [48] and 40 ppm Cu^{2+} reduced O_2 consumption in an activated sludge system [52]. Aerobic biodegradation of pig waste was shown to be progressively inhibited by up to 500 ppm $CuSO_4$ [61]. Levels of copper ranging from 0.025 to 0.05 ppm caused a 70 to 99.9% decrease in viability of coliforms, coupled with a 73 to 83% decrease in O_2 uptake [24]. In addition, the bioconversion of organic material has been used to assess microbial activity in sewage sludge. Lamb and Tollefson [44] reported a 90% reduction in organic nutrient conversion in the presence of 5 ppm $CuSO_4$.

COPPER RESISTANCE

The toxic effect of Cu^{2+} on a microbial population is only one ecological aspect of a copper-stressed ecosystem. Equally important is the consideration that the presence of copper may provide selection pressure that causes a microbial population to adapt to the environmental conditions. A major problem in this area is the standardization of terminology. The terms resistance and tolerance are often used interchangeably; while some strains are classified as metal resistant or metal sensitive [7,68,74], others are classified as tolerant and nontolerant [1,25]. In addition there are no universally accepted metal concentrations that distinguish between resistant and sensitive microorganisms. This arises, in part, from the variability of sensivities exhibited by microorganisms. A resistant *E. coli* strain was reported to grow in the presence of up to 20 mM copper while its sensitive derivative was only able to grow at 6 mM copper [74]. In comparison, a *Pseudomonas syringae* pv. *tomato* strain that was inhibited by 1.6 to 2.0 mM copper did not appear to be copper-resistant, but in fact, was more resistant to the metal compared to its sensitive derivative, which was inhibited by 0.4 to 0.6 mM copper [7].

Variations in copper sensitivity are complicated by nonstandardized methods for enumerating metal resistant bacteria. The cation content of commercial microbiological media shows considerable variation; 8 μg Cu^{2+}/g was measured in Oxoid Nutrient Broth while 3 μg/g was present in Difco Nutrient Broth [11]. During the same study, different batches of the same medium had different metal ion contents. These differences demonstrate a potential problem in quantifying the amount of Cu^{2+} that is present in media when selecting for copper-resistant microorganisms.

Another problem is that different media and their components bind metal cations to different extents. Ramamoorthy and Kushner [59] found that when 200 ppm Cu^{2+} was added to Difco Nutrient Broth and the concentration of free Cu^{2+} in solution was measured, most of the Cu^{2+} was bound to the medium; Cu^{2+} was bound to the following media components, in order of decreasing affinity: casamino acids, yeast extract, Bacto tryptone, and peptone. In a similar study, Bird et al. [9] demonstrated that levels of growth medium as low as 1% converted Cu^{2+} to one or more unidentified copper complexes which resulted in changes in its bioavailability. pH can also affect the availability of Cu^{2+}. When 100 ppm Cu^{2+} was added to dilute sewage at pH 4, and the pH adjusted to pH 6, only 43% of the initial Cu^{2+} was detectable [37].

Strains of *Xanthomonas campestris* pv. *vesicatoria* demonstrated differential sensitivity to copper compounds. The number of strains sensitive to 1200 mg/l $CuSO_4 \cdot 5H_2O$, copper hydroxide ($Cu[OH]_2$) and basic copper sulfate were 3, 5 and 2, respectively. Thus, the form of the metal salt may affect the outcome of a resistance assay.

A mathematical solution to the problem of distinguishing metal-tolerant from non-tolerant microorganisms was proposed by Duxbury [25]. A plot of $\log_{10}$ number of colony forming units (CFU) per gram of soil as a function of metal concentration in mM was described by the equation $y = ae^{-bx}$ where y was the number of bacteria growing on metal selective medium, a was the number of bacteria growing on unsupplemented medium, b was a

measure of the toxicity and x was the metal concentration. Using this equation, 1.33 mM copper was determined to be the cut-off concentration distinguishing copper-tolerant and non-tolerant soil bacteria. Although this approach may enable comparison of data between studies, Duxbury [25] cautioned that the derived concentration only applied to the particular medium and pH conditions used in the experiment. An alternate suggestion is to adopt test procedures not involving growth media [9], such as enzyme activity measurements or respiratory activity.

MECHANISMS OF COPPER RESISTANCE

An early report of a copper-resistant organism was by Weed and Longfellow [82] who exposed cultures of *E. coli* to 5×10^{-6} M $CuSO_4$ for 25 h and isolated a variant of the original strain. The colony diameter of the variant was smaller, in comparison to the normal strain, and higher concentrations of copper were required to inhibit its growth.

The use of antimicrobial chemicals as a method of typing strains led to the observation that approximately 28% of the *E. coli* strains tested were resistant to 0.7% (w/v) $CuSO_4$ [26]. A study of sewage sludge revealed that some coliforms were resistant to antibiotics and heavy metals, including 10^{-1} M $CuSO_4$ [42]. A lead-resistant *Achromobacter* spp. isolated from raw sewage showed a simultaneous resistance to $CuSO_4$ [79]. A strain of *Thiobacillus ferrooxidans* was resistant to 50 g/l $CuSO_4$ [43] and strains of *X. campestris* pv. *vesicatoria* were isolated that differed in their sensitivity to different copper compounds [1,50].

Little information is available on the actual mechanisms of these copper resistant phenotypes. Mechanisms of metal resistance include transformation of metals to less toxic forms [70] and decreased accumulation due to efflux or exclusion mechanisms [77]. To date, neither enzymatic transformation nor efflux have been reported as the basis of copper resistance in microorganisms; exclusion of copper from the microbial cell appears to be the main mode of resistance. Since information on this aspect of bacterial copper resistance is limited, the discussion will include mechanisms of copper resistance observed in numerous organisms.

Copper-resistant *Scenedesmus* strains harboured dense intranuclear inclusions that contained copper and acted as a mechanism to protect cells from high internal copper concentrations [67]. Capsular polysaccharides of bacteria may also play a role in protecting the cells from metallic ions. Encapsulated *Klebsiella aerogenes* strains had a two-fold higher survival rate than noncapsulated strains in the presence of 10 ppm copper chloride ($CuCl_2$) [10]. Production of hydrogen sulfide (H_2S) and subsequent precipitation of Cu^{2+} as CuS appears to be a non-specific mechanism of resistance. Protection of *Desulfovibrio* sp. from copper toxicity was correlated with H_2S production [71] and copper-resistant strains of *Saccharomyces cerevisiae* produced more H_2S than non-tolerant strains [53]. More recently, a copper-resistant *Mycobacterium scrofulaceum* strain was found to remove Cu^{2+} from the growth medium by formation of CuS [27].

The presence, absence or expression level of a particular protein may alter sensitivity to Cu^{2+}. Metallothioneins and other copper-binding proteins have been implicated in mediating copper-resistance in *Neurospora crassa* [45], the cyanobacterium *Synechococcus* [57] and selected yeast strains [54]. The absence of outer membrane proteins (Omp) b and c in *E. coli* K12 and Omp b in *E. coli* B/r conferred resistance by presumably preventing the entry of Cu^{2+} into cells [46]. Similarly, a reduction in the amount of Omp F resulted in increased resistance to Cu^{2+} in *E. coli* strains [35,63].

Reduced uptake of copper was associated with copper-resistant phenotypes. *Penicillium ochro-chloron* cultures were resistant to $CuSO_4$ over the pH range 3.0 to 5.0, but were sensitive at pH 6.0; uptake of copper was approximately ten times greater at the higher pH [33]. Strains of *S. cerevisiae* that tolerated 200 mM $CuSO_4$ exhibited decreased ^{64}Cu (half-life, 12.9 h) uptake [83] and copper-resistant chlamydospores of *Aureobasidium pullulans* did not exhibit energy-dependent uptake of Cu^{2+} [32]. Further studies confirmed the importance of the cell wall in preventing entry of metal ions into cells [34]. When grown in the presence of 0.8 mM $CuSO_4$, copper-resistant *E. coli* cells accumulated less ^{64}Cu than copper-sensitive strains [64]. The mechanism of decreased uptake was not investigated in any of these studies.

GENETIC BASIS OF COPPER RESISTANCE

Copper resistance can be plasmid-encoded. Plasmids are extrachromosomal, self-replicating, covalently closed circular (CCC) pieces of DNA and are approximately 1 to 200 kilobase pairs (kbp) in size [49], but may be larger in some microorganisms.

A temperature-sensitive, conjugative plasmid, Rts1, was associated with Cu^{2+} resistance in an *E. coli* host

[39]. Strains without the plasmid grew in the presence of 0.06 mM $CuSO_4$ while strains harbouring the plasmid grew in the presence of 10 mM $CuSO_4$. The plasmid size was estimated to be 140 megadaltons (MDa) in size. A conjugative, 78 MDa plasmid, designated pRJ1004, controlled copper-resistance in a strain of *E. coli* [74]. In this study, an *E. coli* recipient strain was unable to grow on nutrient agar containing 4 mM $CuSO_4$ but transconjugants harbouring the plasmid grew in the presence of 20 mM $CuSO_4$. The trait was inducible and the level of resistance was proportional to the inducing concentration of copper [64].

Conjugational transfer of a 125 MDa plasmid conferred copper resistance on *X. campestris* p.v. *vesicatoria* recipients [68]. Copper resistance in *P. syringae* pv. *tomato* strains was controlled by two conjugative plasmids, which were 101 and 67 kilobases, respectively (kb) [7]. Another 35 kb plasmid found in copper-resistant strains of *P. syringae* pv. *tomato* was further characterized by restriction enzyme analysis and the copper resistance genes were located on a 4.4 kb *Pst*I fragment [15]. A *P. cepacia* strain was resistant to 400 μg/ml $CuSO_4$ and contained four plasmids (42.0, 5.1, 1.3 and 1.1 MDa), but the plasmid functions have not been elucidated [76]. The removal of copper by H_2S precipitation to form CuS was associated with the presence of a 173 kb plasmid in *Mycobacterium scrofulaceum* [27]. The presence of a plasmid may affect other cellular functions and indirectly alter a cell's ability to tolerate copper. The introduction of plasmids R124 and ColV, I-K94 into *E. coli* strains increased resistance to Cu^{2+} [35,63]. In both studies, resistance was not specific for Cu^{2+}; strains harbouring the plasmids were resistant to other low molecular weight, hydrophilic inhibitors. Resistance was associated with decreased levels of Omp F but it was not certain if the plasmids coded for a mechanism that caused decreased Omp F synthesis or if the presence of the plasmids stressed the cells to the extent that synthesis of the Omp F protein was affected.

Microorganisms resistant to both metals and antibiotics have also been isolated. Bacteria isolated from drinking water exhibited multiple antibiotic resistance as well as tolerance to 3200 μg/ml $CuCl_2$, $Pb(NO_3)_2$ and 1600 μg/ml $ZnCl_2$ [13]. *E. coli* strains of bovine and porcine origin demonstrated multiple drug and metal resistances [36] and Varma et al. [79] reported that several sewage-borne isolates were resistant to both inorganic salts and antibiotics. The high frequency of isolates displaying both phenotypes may be explained by the fact that antibiotic resistances, like metals, are frequently determined by plasmids (30). Copper resistance genes may also be chromosomally encoded. Using a variety of plasmid isolation techniques, 39 bacterial isolates resistant to 1000 μg/ml $CuSO_4$ were screened for extrachromosomal plasmids but none were detected [76].

Cu^{2+} UPTAKE

Accumulation of metals by microorganisms is considered to occur in two stages: rapid energy-independent binding to cell walls followed by energy-dependent transport [4,12]. The review by Belliveau et al. [6] is recommended for more information on this subject. Since little information is available on Cu^{2+} uptake in bacteria [17,76], this discussion will include examples of other microorganisms that also exhibit Cu^{2+} transport.

Copper uptake by *Bacillus subtilis* and *P. fluorescens* was passive and uptake depended on nutrients available in the medium [4]. Copper adsorption by *Rhizopus arrhizus*, *Cladosporium resinae* and *Penicillium italicum* was reported by De Rome and Gadd [20]. Copper specific uptake by polymers extracted from activated sludge suggests that retention of copper in this type of system was primarily binding by bacterial extracellular polymers [65]. Weathers et al. [81] reported that up to 95% of copper was removed in a *Penicillium ochro-chloron* suspension via passive, reversible uptake. It was proposed that this organism could be used as a biotrap for heavy metal removal from electroplating wastewaters [81].

There are fewer reports on the energy-dependent phase of copper transport into microbial cells. Following initial adsorption of copper onto the cell surface of *Debaryomyces hansenii*, energy-dependent copper transport was observed [80]. An optimum pH of 8.0 was observed for transport, and the concentration of copper where transport was half the maximal value (K_m) was estimated to be 0.01 mM. *Aureobasidium pullulans* also exhibited a second phase of energy-dependent Cu^{2+} uptake [32]. Copper uptake followed Michaelis-Menten kinetics; a K_m of 0.22 mM copper was observed for yeast-like cells and a K_m of 0.20 mM was observed for mycelium. Gadd and Mowll [32] demonstrated that Cu^{2+} uptake was inhibited at 4°C or in the presence of 50 mM 2-deoxy-D-glucose, 0.2 mM potassium cyanide (KCN) or 500 μmol dinitrophenol (DNP). The use of metabolic inhibitors, such as DNP to demonstrate lack of energy-dependent transport of cadmium has also been reported [5]. Decreased cadmium uptake was due to the ability of DNP to bind to cadmium, rather than because DNP inhibited metabolic

activity [5]. It is advisable to conduct uptake assays in the absence of a metabolizable C-source (e.g. 2-deoxyglucose), at 4°C, in the presence of KCN or use killed cell suspensions (irradiated with ultraviolet light to maintain cell morphology).

Energy-dependent copper influx was observed in *Penicillium ochro-chloron* [33]. At pH 3.0, influx showed saturation kinetics with a K_m of 390 μM copper and a maximum influx rate (V_{max}) of 22 nmol/h/10^7 cells. Uptake was inhibited by 0.2 mM KCN and by incubation at 4°C. Copper uptake studies in fungi often involve ^{64}Cu [32–34,83] but recently a Cu^{2+}-selective electrode was used to study copper uptake by *S. cerevisiae* [21]. Metabolism-independent binding to cell surfaces was followed by metabolism-dependent intracellular uptake with a K_m of 1.13 M and a V_{max} of 2.22 nmol Cu_{2+}/mg/min. Adsorption of Cu^{2+} by *E. coli* Rts1 (Cu^{2+}-resistant) and *E. coli* W677 (Cu^{2+}-sensitive) revealed that both strains adsorbed almost identical amounts (6.5–7.4 μmol/mg) in the presence of glucose as an energy source [16]. In addition, killed cells adsorbed Cu^{2+} at rates very similar to viable resting cells [16].

Copper uptake was biphasic in some bacteria but, to date, there is no evidence for a Cu^{2+}-active transport system in bacteria [62,66].

PLASMID RTS1

Plasmid Rts1 (Rts1) was first isolated by Terawaki et al. [73]. A strain of *Proteus vulgaris* resistant to sulfonamide, streptomycin, tetracycline and kanamycin transferred only the kanamycin resistance by conjugation [73]. The size of the kanamycin resistance (R) factor was estimated to 120 MDa [23] but was later reported as 140 MDa [39]. Both values were based on analyses using alkaline sucrose density centrifugation. More recently, the size of the plasmid was estimated to be 127.5 MDa [56]. The conjugative properties of the R factor were temperature-dependent and spontaneous elimination occurred at 42°C [73]. Growth of cells harbouring the R factor were also inhibited at higher temperatures [23,73]. Inhibition of growth was associated with a protein of molecular weight 80000 in the outer membrane of *E. coli* cells harbouring Rts1. The protein was believed to be involved in temperature-sensitive cell replication and/or division and was designated "protein T" [41]. Analysis of the R factor DNA revealed its replication was temperature sensitive [72]. Further studies showed that Rts1 was synthesized at the higher temperatures, but could not be isolated as CCC DNA [23]. When Rts1 was placed in *E. coli* mutants that lacked either adenylate cyclase or cyclic adenosine 3′,5′-monophosphate (cAMP) receptor protein, the temperature-dependent effects were not observed. This suggested that cAMP was required for the inhibition of CCC DNA formation [84].

As well as temperature-dependent functions, other genetic markers were associated with Rts1. *E. coli* strains harbouring Rts1 excreted extracellular DNase [51] and tolerated higher levels of Cu^{2+} [39]. In the latter study, derivatives of Rts1 were obtained that conferred kanamycin and copper resistance, kanamycin resistance only, and copper resistance only. Plasmid Rts1 also restricted T4 phage growth at 32°C [38]. Replication of Rts1 is under stringent control [72,73] and recently, a 1.1 MDa *Eco*RI/*Hind*III fragment was confirmed to contain the replication determinants [40].

To understand Cu toxicity and uptake in bacteria better, additional information is still required on the actual uptake mechanism(s) in microorganisms. When this information is forthcoming, it may provide knowledge that is necessary to determine the mechanism(s) of plasmid-encoded Cu resistance in selected bacterial strains. This is especially important as Cu is a required trace element, yet it can also exert an inhibitory or lethal effect on bacterial growth at relatively low concentrations.

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