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# Effects of Copper on Oxidative Stress and Growth of *Pseudomonas putida*

Tiffany Marshall
*Utah State University*

# EFFECTS OF COPPER ON OXIDATIVE STRESS AND GROWTH OF *PSEUDOMONAS PUTIDA*

by

**Tiffany Marshall**

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## INTRODUCTION

### *Active Oxygen Species*

Oxygen is an essential compound in all aerobic systems; however, it can also take part in toxic reactions involving active oxygen species (AOS) and transition metals. Most of the time, antioxidant enzymes act as defense mechanisms and keep the production of AOS to a minimum. However, if the defense mechanisms are not adequate, damage may occur and possibly lead to the breakdown of biomolecules such as DNA, lipids, and proteins that are vital to both simple and complex organisms. Oxidative damage could also be a contributing factor to many human pathological conditions including, aging, atherosclerosis, carcinogenesis, diabetes mellitus, lung cancer, and stroke (1, 2, 4, 5, 6, 8, 11).

Active oxygen species such as superoxide anion ($\cdot O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($\cdot OH$) are generated through normal cellular processes, (9). More specifically, superoxide anion is produced from a one electron reduction of oxygen [eqn. 1], and may be generated by enzymes such as xanthine oxidase and NADPH oxidase (11).

$$O_2 + e^- \Rightarrow \cdot O_2^- \qquad [1]$$

Superoxide anion, once generated, can lead to the formation of other AOS, including hydroxyl radical and hydrogen peroxide. For example, it can recombine producing hydrogen peroxide and oxygen [eqn. 2] (4, 12, 14) or it can lead to the production of highly reactive $\cdot OH$ radicals by reacting with transition metals, most commonly iron and copper, via the Fenton reactions [eqns. 3 & 4], (4, 9, 12, 14).

$$\cdot O_2^- + \cdot O_2^- + 2H^+ \Rightarrow H_2O_2 + O_2 \qquad [2]$$

$$\cdot O_2^- + M^{2+} \Rightarrow M^+ + O_2 \qquad [3]$$

$$M^+ + H_2O_2 \Rightarrow M^{2+} + \cdot OH + OH^- \qquad [4]$$

M signifies a transition metal.

Superoxide, under normal conditions, does not always cause oxidative damage. In fact, $\cdot O_2^-$ is not always deleterious to biological systems. Leukocytes often use it to kill bacteria and tumor cells. Therefore, the increase in free radical attacks after formation of $\cdot O_2^-$ and $H_2O_2$ may be due to their reaction with metals such as iron and copper, which would produce more reactive species such as $\cdot OH$ (2, 4, 14) The hydroxyl radical is an extremely reactive oxidant which readily reacts with all known biomolecules by removing or adding a hydrogen atom to the molecule. It is hypothesized that oxidative damage leading to severe pathological conditions is mediated by $\cdot OH$ and its products.

***The Role of Copper***

Copper is a necessary trace element. Copper deficiency can lead to retarded growth, impaired reproduction, and severe problems with the heart (11). On the other hand, high concentrations of copper have been associated with Wilson's disease, Parkinson's disease, and Menke's syndrome (10, 13, 6,). It is thought that the toxicity of copper is associated with its ability to be a catalyst for reactions involving active oxygen species. As shown earlier, copper is involved in the generation of ·OH through redox cycling reactions involving superoxide radical and hydrogen peroxide, [eqns 3 & 4].

Sagripanti and Kraemer (10) have found that Cu(II) in the presence of hydrogen peroxide can damage the transforming ability of DNA and produce strand lesions through a mechanism which involves ·OH radicals but not superoxide. Tkeshelashvili et al. present evidence that Cu(I) or Cu (II) reduce the biological activity of DNA and cause mutations (13).

***Oxidative Damage to Biological Systems and Mammalian Disease***

Oxidative damage may occur where an excessive amount of active oxygen species are generated or where antioxidant defenses are inadequate or impaired. This damage appears to play a role in the breakdown of biomolecules, which in turn can contribute to pathological conditions such as aging, carcinogenesis, stroke, atherosclerosis, lung cancer, and diabetes mellitus. The following pages discuss the mechanisms by which AOS are involved in the development of such conditions.

*Activated oxygen species and DNA*

Research efforts have identified approximately 20 types of oxidatively altered DNA molecules (14). Activated oxygen species can damage DNA at a rate of 100,000 per cell per day in rats and 10,000 per cell per day in humans (1). Most lesions produced in the DNA by AOS are repaired by enzymes which excise the lesions. When antioxidant defenses become overwhelmed or when repair enzymes are not sufficient, lesions can accumulate and contribute to pathological conditions such as aging and cancer (1). Loss of repair enzyme activity increases the rate of spontaneous mutation, and lack of repair enzymes in mitochondrial DNA may account for the high level of oxidative damage to mitochondrial DNA (1, 14).

Lesions are grouped into strand breaks and base modifications. The ·OH radical attacks the sugar portion of DNA, which produces strand breaks (4). Superoxide is also capable of producing strand breaks. A break in the DNA strand must be repaired in order for the cell to function properly; however, in the repairing process there is a high probability that the wrong base will be incorporated into the repaired DNA (4). DNA repair mechanisms are also affected negatively by an imbalance in cellular calcium levels caused by depletion of ATP and NAD (3).

Hydroxyl radical damage to DNA primarily results in one of three modified bases, 8-hydroxyguanine, 5-hydroxymethyluracil, and thymine glycol. Base modifications can result in serious problems in terms of mutagenesis and carcinogenesis (4).

Transitions and substitutions within the nucleic acid bases are also prevalent sources of mutation. C ⇒ T transitions as a result of cytosine damage by ·OH radicals account for a high percentage of mutations (9). Reid et al. showed that G ⇒ C transitions are a frequent mutation caused by Fe(II), and that Ni(II) and Cu(II) often induce G ⇒ T and C ⇒ T transitions (9).

*Activated Oxygen Species and Protein*

AOS also have the potential to damage proteins. Even subtle changes may result in serious consequences, possibly contributing to lung emphysema and stroke (4, 14). Werner syndrome and progeria are two diseases which may be linked to oxidative protein damage because researchers have found that oxidized proteins increase at a much higher rate than normal in people afflicted with the diseases (1).

Hydroxyl radical reacts with both amino acids and protein macro molecules (12, 14). Superoxide readily attacks protein and results in one of the following: fragmentation products, cross linked reaction products, aggregation resulting from denaturation and possibly related to cross linking products, and lesions in the protein structure from increased susceptibility to proteolytic digestion (4, 14). Protein oxidation can severely damage biological systems, because it is assumed that if one protein is damaged, the next one will be if the AOS attack continues (4).

*Activated Oxygen Species and Lipids*

Lipid peroxidation resulting from the reaction of AOS with lipids is considered an important part of the oxidative damage brought about by AOS. Lipid peroxidation by one radical may produce multiple damaged sites, unlike DNA oxidation which results in one or two damaged sites. This is a result of either shorter or longer chain reactions (12).

Peroxidation can change and damage the structure of the lipid, this is apparent when oxidative damage occurs to lipids within a membrane (14). Structural changes occur within the membrane causing it to become rigid forcing the protein membranes to change and ultimately alter the pumping rate of protein channels (4). Membranes with carbon-carbon double bonds are most susceptible to lipid peroxidation (4). Linoleic acid, arachidonic acid, docosahexaenoic acid and other polyunsaturated acids are the primary fatty acids which undergo lipid peroxidation (14).

The products of lipid peroxidation (i.e. aldehydes) have been known to react with nucleic acids or to form cross linkages that inactivate cellular membranes and enzymes, thus possibly contributing to carcinogenesis and mutagenesis. Other lipid peroxidation by-products have been shown to react with low density lipoprotein (LDL) and could ultimately lead to foam cell formation in atherogenesis.

*Active Oxygen Species and Carbohydrates*

Monosaccharide carbohydrates such as glucose also endure oxidation by AOS. They often undergo autoxidation to form dicarbonyl compounds and hydrogen peroxide. The problem with this is that these products can form new structures by interacting with other molecules (14).

*Aging*

Although AOS have not been proven to be the cause of aging, there is substantial evidence to support the idea that free radical induced damage to biomolecules such as DNA, lipids, proteins and carbohydrates accumulates with age and appears to be an underlying factor responsible for the aging process (1, 11, 14)

*Atherosclerosis*

Modification of low density lipoprotein (LDL) molecules takes place in the presence of metals and can be suppressed by introducing antioxidants (14). This strongly implicates activated oxygen reactions. Products of lipid peroxidation react with LDL extending the amount of time LDL is in the blood circulation and causes macrophage uptake to increase, leading to foam cell formation, arterial fatty streaks, and atherogenesis (4). Oxidatively modified LDL molecules also injure the arterial intima and encourage aggregation of platelets which could lead to clotting (14).

*Carcinogenesis*

Activated oxygen species may play a role in carcinogenesis during the initiation and promotion stages. As with aging, there is no direct causal relationship for oxidative free radial damage in carcinogenesis, but there is a stronger association as more research is completed and information becomes available (4, 9, 10, 14).

Initiation refers to irreversible alterations in the normal genetic material by free radicals, carcinogens or possibly a combination of the two. If initiated cells multiply unabated there is a higher probability that a tumor will develop (14). Promotion refers to a process which builds on processes already present in damaged or altered cells.

Initiation of carcinogenesis by AOS may play an important role in the following five areas: ionizing irradiation, tumor induction by foreign bodies, metal mediated carcinogenesis, tumors by chemical carcinogens, and tumors by dietary manipulations. Evidence points to oxidation of DNA as a possible mechanism by which initiation occurs in carcinogenesis (4).

*Diabetes Mellitus*

Research involving diabetes indicates high levels of oxidative stress in those with diabetic

conditions. Diabetes can be chemically induced with such agents as alloxan, which contributes to the formation of AOS. Addition of SOD and CT suppressed the damaging action of alloxan, providing support for the deleterious role of AOS in diabetes (14).

*Lung Fibrosis and Cancer*

The lungs are highly susceptible to oxidative injury from inhaled free radicals, especially when increased levels of oxidative stress overwhelm antioxidant defense mechanisms present in the fluids lining the respiratory tract. Dusts such as silicates and asbestos can cause lung fibrosis and cancer. This process may be partially mediated by AOS. AOS can also contribute to asthma, cystic fibrosis, and lung HIV infections. The antioxidant enzymes, catalase and SOD give some protection against oxidative stress lending support to the idea that AOS play a role in diseases of the lung (3).

*Stroke*

Stroke, often referred to as ischemia/reperfusion injury results from the interruption of blood flow and consequent reoxygenation.. There is substantial evidence supporting free radicals as a mechanism for injury during reperfusion. Xanthine and xanthine oxidase by-products of ischemia injury and are capable of producing superoxide anion. High levels of iron present during a stroke may react with AOS, resulting in brain injury (4).

***Antioxidant Enzymes***

Aerobic organisms have been equipped with defense mechanisms to limit the levels of active oxygen species and to neutralize their effects. The substances included in this antioxidant defense system include, but are not limited to, such enzymes as superoxide dismutase (SOD), catalase (CT), glutathione peroxidase, proteases, and glycosylases. Under normal conditions there are enough of these enzymes to protect the cell; however, when the intensity of the oxidative stress reaches a sufficient level in a short period of time, antioxidant capacity can be overwhelmed and the cell will be forced to either call on additional adaptive responses or suffer serious consequences from oxidative damage (4). This paper will focus on the role of two major antioxidant enzymes - superoxide dismutase and catalase.

*Superoxide Dismutase*

SOD is present in almost all aerobic organisms. Its purpose is to prevent further generation of free radicals by catalyzing the reaction listed in [eqn. 5]. SOD is a vital part of the antioxidant defense system. This importance is demonstrated by the fact that organisms lacking SOD have increased levels of oxidative stress (14). There is also some evidence that a decrease in SOD activity might contribute to amyotrophic lateral sclerosis (ALS) by interfering with the mechanism for motor neuron death caused by $\cdot O_2^-$ (1, 7).

$\cdot O_2^- + \cdot O_2^- + 2H^+ \Rightarrow H_2O_2 + O_2$ [5]

SOD is found in large amounts in the liver, adrenal glands, kidney, and spleen. The activity of SOD varies and is regulated through biosynthesis. An increase in SOD activity has been shown to be stimulated by Paraquat, a known source for generation of $\cdot O_2^-$ (14). SOD is classified into three distinct isozymes according to their interaction with metal ions. They are Cu/Zn SOD, Mn SOD, and Fe SOD. Cu/Zn SOD is the primary form found in human cells, whereas Mn SOD and Fe SOD are more abundant in smaller organisms. Cu/Zn SOD is present in the cytosol, and Mn SOD is present in the mitochondrial matrix. *Pseudomonas putida*, expresses two of the three isozymes, Fe SOD (encoded by SOD b) at 80%, and Mn SOD (encoded by SOD a) at 20%. Fe SOD expression is fairly consistent throughout growth; Mn SOD expression is regulated on low iron - medium and is expressed in stationary phase.

*Catalase*

The primary function of catalase is to catalyze the decomposition of $H_2O_2$ to $H_2O$, as illustrated in the following equation.

$2\ H_2O_2 \Rightarrow 2H_2O + O_2$

CT is present at high concentrations in the liver, kidney, and red blood cells. In *P. putida,* like SOD, it exists in most cells as distinct isozymes. The specific role for each isozyme of both SOD and CT has not been determine. There are three separate isozymes for catalase, labeled A, B, and C. Catalase A is the predominant isozyme (85%), followed by catalase B (10%) and catalase C (5%). Catalase A is found in both log and stationary phases, whereas catalase C is only present in the stationary phase. One of the questions we are asking in this experiment is whether or not catalase C activity will show up earlier if the cell is placed under stress. Unfortunately, the specific role for each respective isozyme for both SOD and CT has not been determined.

Gutteridge and Wilkins (13) found that catalase was the most effective protein in reducing hydroxyl radical damage in a copper-dependent system. SOD was reported as being less effective that CT, nevertheless all proteins appeared to reduce oxidative damage. The authors suggest that copper ions which would normally enter into redox cycles to form hydroxyl radicals, instead bind to the amino groups of proteins such as catalase, thus preventing oxidative damage to the cell by decreasing the number of hydroxyl radicals being formed (13). The authors report that copper in the presence of hydrogen peroxide could in fact bring about damage if proteins such as catalase are not available to bind to (5).

The objective of the studies being reported here was to determine if copper increases the expression of specific isozymes of SOD and CT in *Pseudomonas putida.*

## MATERIALS AND METHODS

### *Growth of Bacteria*

The organisms used in this experiment are three strains of Pseudomonas putida, a wild type strain and two mutant strains - Cat C and Sod B. The mutant strains are constructed such that CT and SOD activity can be determined by measuring light emission.

The day prior to running the experiment stock cultures were made up by inoculating three flasks of 100 ml Luria Broth (LB), each with a different strain of *P. putida.* The inoculum was taken from one-day old LB plate cultures made from -80° C freezer stock in 15% glycerol and grown at 26° C. The LB contained no added antibiotics. The stock cultures were grown on a shaker at 140 at 21° C for approximately 12 hours.

The day of the experiment 10 ml of each culture were used to inoculate two 50 ml flasks of sterile LB and allowed to shake at 140 rpm for 30 minutes in order to equilibrate. A stock solution of $CuSO_4$ (10 mg/ml $CuSO_4$ in water) was used to adjust flasks to 50, 100, or 500 $\mu g/ml$ solutions. The copper solution was added throughout a one minute time period, so as not to shock the cells. No additions were made to the control flasks. All six flasks were placed on the shaker, and left there for the duration of the experiment except while taking samples.

### *Measurement of Optical Density*

Optical Density (O.D.) was measured with a Spectrophotometer at 600 nm wavelength in a 1 ml cuvette. The absorbance due to Cu(II) in solution was recorded and subtracted from all values.

### *Measurement of Luminescent (Lux) Activity*

The cultures were diluted 1 to 10, or 1 to 100, or 1 to 1000 with fresh LB. One ml samples of each dilution were measured with a Luminometer immediately after addition of 50$\mu l$ of decanol (1 to 10 dilution with ethanol).

## RESULTS

### *Linearity in detection of lux activity*

We determined whether the measured lux activity was related to sample size. We did this by assessing the effect of dilution. We find fairly linear results between lux values of approximately 2,000 and 50,000. In TABLE 1 we see that at 70, 90, and 110 minute time points for a study with sod B cells treated with 50 $\mu$g/ml $CuSO_4$ the results are consistent between the 1 to 10 and 1 to 100 dilutions. There is slight inconsistency found with the 1 to 1000 dilution; however when a value of 100 is subtracted to account for background the results are more linear. The linearity in this experiment and others leads us to the assumption that the lux readings are

reliable and therefore a true indicator of enzyme activity.

**TABLE 1**

| Linearity of lux readings for sod B | | | |
|---|---|---|---|
| **Dilution** | **70 min. ctrl** | **90 min. ctrl** | **110 min + Cu** |
| **10** | 17621 | 26136 | 52200 |
| **100** | 1702 | 2463 | 5314 |
| **1000** | 385 | 372 | 375 |

Data contained in this table taken from 50 $\mu$g/ml experiment with sod B cells

***Consistency in detection of lux activity***

Consistency is another measure for the reliability of this assay. The amount of variability between replicates in identical samples was assessed. The results show up to a two fold variability of lux activity between replicated. The data presented in TABLE 2 illustrate this point.

**TABLE 2**

| Replication of lux readings in cat C | | | |
|---|---|---|---|
| **Time** | **Dilution** | **Cat c - ctrl** | **Cat c - Cu** |
| **180** | 10 | 563 | 678 |
| | 100 | 235 | 160 |
| **190** | 10 | 865 | 757 |
| | 100 | 181 | 329 |

Data contained in table taken from 100 $\mu$g/ml experiment with cat C cells.

***Lux activity and optical density***

Lux activity was measured to assess the expression of the cat C and sod B genes. Optical density (O.D.)was measured to assess the density of the cells in the culture. A lack of increase in O.D. value with time of culture would indicate cell death, possibly due to copper toxicity. Lux readings are also expected to increase because of increases in cell density, but also because of changes in the level of gene expression.

There are differences between the expression of lux activity and culture phase for the cat C and sod B mutants, (see TABLES 3 and 4). In studies where no Cu(II) is added cat C expression measured by lux activity is not apparent until high O.D. values are reached. The expression of sod B occurs in cells of all phases. In experiments where the O.D. values are similar between cat C and sod B at corresponding times, there is a marked difference in the level of expression, (refer to TABLES 3 and 4). Sod B values are much higher than cat C values, and indicate gene expression "turns on" at an earlier time. As stated earlier, significant effects to cat C activity do not appear until late in the culture phase. This might be due to a difference in the promoters between the two mutants. There also seems to be a larger percent of error in cat C due to the low readings which are affected more by subtracting for background.

For each of the experiments, the ratio of lux activity and O.D. in copper-treated cells to control cells was calculated. Ratios greater than 1.0 indicate an increase in enzyme activity and cell growth for treated cells compared to non-treated cells. Ratios less than 1.0 indicate a decrease.

***Results of 50 $\mu$g/ml experiment***

We found that the 50 $\mu$g/ml $CuSO_4$ solution had no effect on lux activity for sod B or cat C cells (refer to TABLE 3). Upon repeating the experiment we found similar results. The lack of positive results doesn't appear to be caused by copper toxicity, because increases in O.D. values with time of culture between treated and non-treated cells were similar, (refer to TABLE 4). As the experiment proceeded from 0 to 24 hours, the lux readings showed an overall increase which corresponded to an increase in cell number. The increase in cat C cells was not as great as that of sod B due to differences in their promoters.

## TABLE 3

| Lux activity for *50 μg/ml* treatments 03/04/96 | | | | | | | |
|---|---|---|---|---|---|---|---|
| Time (min) | Dilution | Cat c - ctrl | Cat c + Cu | Cu / ctrl | sod b + ctrl | sod b - Cu | Cu / ctrl |
| **0** | 1 | 354 | 245 | .7 | 1832 | 2625 | 1.4 |
| **5** | 1 | 293 | 243 | .8 | 2340 | 2435 | 1.0 |
| **10** | 1 | 275 | 308 | 1.1 | 2650 | 2728 | 1.0 |
| **15** | 1 | 302 | 313 | 1.0 | 2144 | 2853 | 1.3 |
| **20** | 1 | 375 | 361 | 1.0 | 2623 | 2770 | 1.0 |
| **25** | 1 | 301 | 266 | .9 | 3027 | 2114 | .7 |
| **40** | 1 | 328 | 385 | 1.2 | 2391 | 2271 | .9 |
| **55** | 1 | 294 | 342 | 1.2 | 3030 | 3909 | 1.3 |
| **75** | 1 | 340 | 398 | 1.2 | 1775 | 2147 | 1.2 |
| **85** | 1 | 460 | 426 | 1.1 | 2770 | 1834 | .7 |
| **90** | 1 | 421 | 456 | 1.1 | 3053 | 3304 | 1.1 |
| **110** | 1 | 478 | 377 | .8 | 3221 | 3767 | 1.2 |
| **125** | 1 | 547 | 561 | 1.0 | 4220 | 3681 | .9 |
| **960** | 1 | 13893 | 23962 | 1.7 | 425300* | 599260* | 1.4 |
| **1200** | 1 | 24856 | 28077 | 1.1 | 179210* | 311540* | 1.7 |
| **1440** | 1 | 11935 | 11904 | 1.0 | 110030* | 396680 | 3.6 |

## TABLE 4

| Optical Density for *50μg/ml* treatments 03/04/96 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Time (min) | wt - ctrl | wt - Cu | Cu / ctrl | Cat c - ctrl | Cat c + Cu | Cu / ctrl | sod b - ctrl | sod b + Cu | Cu / ctrl |
| **0** | .0525 | .0708 | 1.4 | .0919 | .0650 | .7 | .0545 | .0517 | .9 |
| **10** | .0849 | .0735 | .9 | .1365 | .1224 | .9 | .0799 | .0622 | .8 |
| **40** | .0888 | .1056 | 1.2 | .1256 | .1307 | 1.0 | .0748 | .0901 | 1.2 |
| **70** | .1104 | .1355 | 1.2 | .2016 | .1739 | .9 | .0896 | .1000 | 1.1 |
| **90** | .1417 | .1657 | 1.2 | .2051 | .2137 | 1.0 | .1025 | .1095 | 1.1 |
| **105** | .1920 | .2060 | 1.0 | .2262 | .2756 | 1.2 | .1211 | .1300 | 1.1 |
| **130** | .2420 | .2981 | 1.2 | .2867 | .2899 | 1.0 | .1311 | .1308 | 1.0 |
| **960** | 1.9365 | 2.0055 | 1.0 | 2.1025 | 2.1172 | 1.0 | 1.7168 | 1.6725 | 1.0 |
| **1440** | 2.3222 | 2.3290 | 1.0 | 2.4227 | 2.6799 | 1.1 | 2.2018 | 2.1495 | 1.0 |

***Results of 100 μg/ml experiment***

The results of the 100 *μg/ml* experiment were similar to those of the 50 *μg/ml* experiment. The copper did not significantly effect lux expression for sod B or cat C expression, (refer to TABLES 5 and 6). There is a slight increase in ratio for lux activities at 420 and 430 minute readings; however, this increase is lower than the two fold variability observed in control studies (refer to section entitled *Consistency in detection of lux activity*). As with the previous experiment the lack of enzyme expression is not due to low or inconsistent O.D. values.

TABLE 5

| Lux activity for *100 μg/ml* treatments 03/14/96 | | |
|---|---|---|
| Time (min) | Cat c Cu / ctrl | Sod b Cu / ctrl |
| 0 | .8 | .9 |
| 10 | 1.1 | 1.1 |
| 180 | 1.2 | .8 |
| 190 | .9 | .9 |
| 315 | 1.2 | 1.0 |
| 325 | 1.0 | 1.0 |
| 420 | 1.5 | 1.2 |
| 430 | 1.1 | 1.2 |

TABLE 6

| Optical Density for *100μg/ml* treatments 03/14/96 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Time (min) | wt - ctrl | wt + Cu | Cu / ctrl | Cat c - ctrl | Cat c + Cu | Cu / ctrl | sod b - ctrl | sod b + Cu | Cu / ctrl |
| 0 | .0452 | .0451 | 1.0 | .0482 | .0341 | .7 | .0414 | .0227 | .5 |
| 10 | .2271 | .2356 | 1.0 | .1500 | .1562 | 1.0 | .1184 | .1123 | .9 |
| 40 | .6246 | .5236 | .8 | .4214 | .3866 | .9 | .2632 | .2249 | .9 |
| 70 | 1.0412 | 1.0013 | 1.0 | .8592 | .8895 | 1.0 | .6063 | .6292 | 1.0 |

***Results of 500 μg/ml experiments***

The data from two replicated experiments reveal an increase in sod B expression in response to the high concentration (500 *μg/ml*) of copper. The increase is manifest early in the experiment, between the 10 and 60 minute time points, (refer to TABLE 7 and TABLE 8). After the sixty

minute time point lux activity decreases in response to a decrease in cell number, perhaps due to the toxic effects of copper, (refer to TABLE 9 and TABLE 10). The toxicity was apparent as a reduced growth rate for mutant and wild type cells. Sod B and cat C cells recovered slightly at the 24 time point.

The cells in the second experiment were slightly more sensitive to copper toxicity than those in the first experiment. This is demonstrated by a decrease in rate of change of O.D. values earlier in the experiment, especially for sod B cells.

There was no significant increase (greater than a factor of 2) in the expression of cat C at the higher concentration of copper. A decrease in lux activity, at approximately the same time point sod B begins to decrease, occurs in response to a decrease in cell number also perhaps due to the toxic effects of copper.

**TABLE 7**

| **Lux Activity for *500 μg/ml* Experiment #1, 04/11/96** | | |
|---|---|---|
| **Time (min)** | **Cat c Cu / ctrl** | **Cu / ctrl** |
| **0** | **2.4** | **1.0** |
| **10** | **5.1** | **4.6** |
| **20** | **2.7** | **4.3** |
| **30** | **2.3** | **4.6** |
| **40** | **1.0** | **7.7** |
| **60** | **.6** | **2.6** |
| **130** | **.3** | **.8** |
| **140** | **.5** | **.8** |
| **270** | **.5** | **.3** |
| **390** | **.4** | **.7** |
| **1355** | **1.5** | **1.4** |
| **1440** | **1.3** | **1.6** |

## TABLE 8

| Lux Activity for *500 μg/ml* Experiment #2, 04/17/96 | | |
|---|---|---|
| Time (min) | Cat c Cu / ctrl | Sod b Cu / ctrl |
| 0 | 1.2 | 1.2 |
| 5 | 5.2 | 1.4 |
| 15 | 1.3 | 2.2 |
| 30 | 1.0 | 1.3 |
| 45 | 1.3 | 2.5 |
| 60 | .7 | 2.6 |
| 75 | .7 | .9 |
| 95 | .8 | 1.3 |
| 120 | .6 | .7 |
| 180 | .5 | 3.7 |
| 240 | .6 | .3 |
| 1200 | 2.2 | 3.1 |

## TABLE 9

| Optical Density for *500μg/ml* treatments 04/11/96 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Time (min) | wt - ctrl | wt + Cu | Cu / ctrl | Cat c -/ ctrl | Cat c + Cu | Cu / ctrl | sod b - ctrl | sod b + Cu | Cu / ctrl |
| **10** | | | | **.0587** | **.0448** | **.8** | **.0518** | **.0541** | **1.0** |
| **35** | | | | **.0697** | **.0678** | **1.0** | **.0685** | **.0603** | **.9** |
| **50** | | | | **.0849** | **.0638** | **.8** | **.0727** | **.0669** | **.9** |
| **130** | | | | **.1378** | **.0926** | **.7** | **.1477** | **.0926** | **.6** |
| **280** | | | | **.4601** | **.1447** | **.3** | **.4317** | **.1501** | **.3** |
| **400** | | | | **.9839** | **.2750** | **.3** | **1.0311** | **.3129** | **.3** |
| **1440** | | | | **2.0568** | **1.9671** | **.9** | **2.1501** | **2.0643** | **.9** |

Reference O.D. for Cu soln: .1757

* The wild type cells for this experiment did not grow therefore no data obtained.

## TABLE 10

| Optical Density for *500μg/ml* treatments 04/17/96 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Time (min) | wt - ctrl | wt + Cu | Cu / ctrl | Cat c - ctrl | Cat c + Cu | Cu / ctrl | sod b - ctrl | sod b + Cu | Cu / ctrl |
| **no Cu** | .0435 | .0417 | 1.0 | .0531 | .0475 | .9 | .0373 | .0330 | .9 |
| **20** | .0497 | | | .0578 | .0574 | 1.0 | .0431 | .0388 | .9 |
| **40** | .0620 | .0737 | 1.2 | .0637 | .0628 | 1.0 | .0598 | .0375 | .6 |
| **80** | .0825 | .0956 | 1.2 | .1166 | .0646 | .6 | .0723 | .0561 | .8 |
| **180** | .1432 | .0727 | .5 | .1470 | .0874 | .6 | .1632 | .0591 | .4 |
| **240** | .2328 | .1046 | .4 | .2305 | .1129 | .5 | .1663 | .0621 | .4 |
| **1200** | 2.0668 | .9122 | .4 | 2.0839 | 1.399 | .7 | 2.0843 | 1.537 | .7 |

Reference O.D. for Cu soln: .1757

## DISCUSSION

Lux observations with cat C and sod B were good indicators of different promotor abilities for the genes. Cat C was activated only in stationary phase. Sod B was consistent in its expression. Thus, the assay method is appropriate for the study we conducted.

Copper at 50 and 100$\mu g/ml$ concentrations had little effect on growth or gene expression. At 500$\mu g/ml$, there was increased expression of sod B for a time period of about 60 minutes after copper addition. A more rapid, but short-lived effect of cat C may occur also. However, at this high level, toxicity to the cells by copper was apparent because cells no longer increased at a normal reproductive rate. This toxicity may effect cell metabolism that in turn limits lux expression.

The apparent increase in sod B and cat C expression would be consistent with the response of the cell to protect itself against oxidative stress induced by the copper treatment. Perhaps, looking at the other isozymes of catalase in *P. putida* (cat A and cat B), or the other MnSOD isozyme would elucidate a response of these enzymes against AOS.

The ability of copper to induce such a response has far reaching implications. As we discussed earlier, AOS are a key factor in the development of a diverse number of pathological conditions. If copper is able to increase the production of cat C or sod B, without being toxic itself, it could indirectly help rid the body of AOS and thus stop the breakdown of biomolecules and ultimately the development of diseases associated with aging.

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