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THE EFFECTS OF COPPER ON CYTOCHROMES BIOSYNTHESIS, CELL DIVISION AND MUTAGENESIS IN *PARACOCCUS DENITRIFICANS*

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Abstract

When *Paracoccus denitrificans* was grown in a newly devised synthetic medium in which copper was extracted by zinc-dibenzyldithiocarbamate, the biosynthesis of cytochrome **c** oxidase was specifically inhibited, while that of cytochromes **b** and **c** remained unchanged. Copper-deficient cells exhibited alterations in cytokinesis leading to the formation of polylobed cells. These alterations were reversibe when copper was restored to the culture medium. When copper was added to the medium, the biosynthesis of cytochromes **b**, **c** and cytochrome **c** oxidase was stimulated for concentrations of copper up to 50 μg per liter. Above this concentration, the biosynthesis of cytochrome **c** oxidase decreased, reaching zero for 1 mg of copper per liter, while that of **b** and **c** cytochromes remained roughly unchanged. In contrast, when cells were grown in an enriched medium supplemented with copper, after an initial stimulation of cytochromes biosynthesis, the level of cytochromes remained unchanged until the concentration of added copper reached 1 mg per liter. Over this concentration, the biosynthesis of cytochromes became inhibited. Prolonged growth in high copper concentrations (100 mg/l) produced two types of mutants: 1) cytochrome **c** deficient cells and 2) cells in which the usual enzymes of the respiratory chain were replaced by **b** and **d** type cytochromes.

Introduction

The importance of trace metals in the regulation of cellular concentrations of hemo- and other metalloproteins is now well recognized. For example, the increased concentrations of iron and protoheme stimulate the synthesis of apohemoglobin and cytochrome **c** respectively. Iron also induces the synthesis of apoferritin [1-4]. On the other hand cytochrome **c** oxidase, ceruloplasmin and other copper containing proteins undergo rapid metabolic changes in response to copper deficiency [5, 6]. However, although the addition of copper stimulates the synthesis of these proteins, the presence of an excess of copper is toxic and cells possess mechanisms to obviate the ill effect of the presence of too many metals when their concentrations do not go beyond certain levels.

Keywords: Copper; Respiratory chain

If the control of hemo- and other metalloproteins biosynthesis is a general function of trace metals in biological systems, it should be found at one or more levels of apoprotein synthesis, heme synthesis or heme turnover [5]. Indeed, for example, copper deficiency in yeast specifically produces a disappearance of cytochrome **c** oxidase levels whereas the amounts of other cytochromes remain unaffected [7]. It has been shown that a precursor of heme **a**, identified as porphyrin **a** , is synthesized and integrated into the apooxidase and it has been suggested that copper controls the biosynthesis of functional cytochrome **c** oxidase at the level of the transformation of porphyrin **a** into heme **a** [5, 8].

Copper is present in a number of proteins in all strata of organisms and has a catalytic or structural function or both. Cytochrome **c** oxidase contains two hemes **a** and two atoms of copper. Because of the universal importance of this enzyme in aerobic cells and its key role as oxygen acceptor and in energy conservation, a tremendous amount of research has been focused on the structure, function and biosynthesis of this protein.

Paracoccus denitrificans is a gram-negative, coccoid, hydrogen-oxidizing bacterium exhibiting many features of the mitochondria in eucaryotic cells. It is even suggested to be a possible ancestor of mitochondria of eucaryotic cells [9, 12]. For this reason, it is a very popular organism for use in the study of electron transfer and energy conservation. This organism, as well as possessing normal electron transport as seen in mitochondria, possesses multiple oxidasic systems such as cytochromes **o**, **co**, **d** and **cd**. Under anaerobic conditions in *Paracoccus denitrificans*, a number of nitrogenous oxidases can act as electron acceptors [12 - 16]. The effect of copper deficiency on cytochrome **c** oxidase biosynthesis in eucaryotic cells is well documented [5-8]. But there is no research on the effect of copper deficiency in prokaryotes.

In this paper, we show the effect of copper deficiency and of the presence of an excess of copper on *Paracoccus denitrificans*. In addition we show that copper used at high concentrations has a mutagenic effect on these cells.

Materials and Methods

Cells

Paracoccus denitrificans (ATCC 17741) was grown at 33°C. For liquid cultures, the cells were placed in Erlenmeyer flasks (2-liter flasks for 500 ml cultures, or 1-liter flasks for 250 ml cultures) and aerated in a New Brunswick shaker at 180 rpm.

Media

A copper-deficient medium was designed to obtain copper-depleted cells and was prepared as follows. A minimum medium was prepared from three stock solutions labeled A, B, and C. Solution A contained 1 g KH_2PO_4, 6 g $H_2(NH_4)PO_4$, 1 g $(NH_4)_2SO_4$ and 0.1 g NaCl per liter. Solution B contained 0.1 g $ZnSO_4$, 0.1 g $MnSO_4$ and 0.4 g $FeSO_4$, 0.005 g KI per 250 ml. Solution C contained 20 g $MgCl_2$, 2.5 g $CaCl_2$, 5 g Na_2MoO_4 and 10 mg H_3BO_3 per 250 ml.

The medium was prepared by mixing 1 liter of solution A with 1 ml of solution B and 10 ml of solution C. It was then extracted with zinc dibenzyldithiocarbamate according to reference 7. The copper concentration in the medium after extraction was less than 1 µg/l as determined by atomic absorption [5]. The medium was supplemented with 1% glucose as a carbon source. To this medium, copper was added as $CuSO_4$ at concentrations from 0 to 1.25 mg/l.

Enriched medium was composed of peptone at 4 g/l, yeast extract at 2 g/l and KH_2PO_4 at 10 g/l. The pH was adjusted at 6.8 with H_2SO_4 and 1% glucose was added as a carbon source. To this medium, copper was added as $CuSO_4$ at concentrations from 0 to 100 mg/l.

Chemicals

All chemicals were of the best grade available and purchased from the Merck and from the Sigma chemical companies.

Cultures

Copper-deficient cultures were usually grown in 500 ml of copper-deficient medium for 48 h. The inoculum consisted of 0.5 g cells grown in a copper-deficient preculture. Cultures in enriched medium were grown in 250 ml of enriched medium.

Mutagenesis

Cells were grown for 46 h in enriched medium supplemented with copper at 100 mg/l. An aliquot of the culture was utilized to inoculate fresh medium containing the same supplement in copper. This was done 10 times successively. After that, the cells were transferred once more, but this time to enriched medium which was no longer supplemented in copper. After this last culture, the cells were collected by centrifugation at 2,000 g for 20 min; the pellet obtained was washed twice with 0.01 M phosphate buffer at pH 7.5 and centrifuged each time at 2,000 g. These cells were then plated on high copper concentrations and mutants resistant to high copper concentrations were selected.

Spectrophotometry

Dithionite-reduced-minus-H_2O_2-oxidized spectra of whole cell suspensions were obtained with a DW-2 Aminco spectrophotometer. Each time, 2 g cells were suspended in 10 ml and aliquots of the suspension were placed into two cuvettes. A few grains of dithionite were added to the sample cuvette and 6 µl of 30% H_2O_2 were added to the reference cuvette. The spectrum was then recorded immediately.

The cytochrome content of the cells was calculated using the following values for extinction coefficients: for cytochrome **c**, oxidase, 24, for cytochrome **b**, 18, and for cytochrome **c**, 19, as described in reference 7.

Protein Determination

The protein content in the suspensions was determined according to the method of Lowry [17].

Results

Effect of Copper Deficiency on the Cells Difference Spectra

Figure 1 shows the difference spectra obtained from copper-deficient cells (Fig. 1a) and from cells grown in enriched medium (Fig. 1b). For cells grown in enriched medium, typical absorption bands at 608 nm, 560 nm and 552 nm due to cytochromes **c** oxidase, **b** and **c**, respectively, were observed. When the cells were grown in copper deficient medium, the absorption band at 608 nm was missing, but a broad band in the 594 nm region appeared. Cytochrome **c** oxidase was thus no longer detectable in these cells while cytochrome b_{594} became detectable (around 594 nm).

When copper-deficient cells were grown in copper-deficient medium supplemented with increasing amounts of copper, the synthesis of cytochrome **c** oxidase was resumed as indicated by the reappearance of the absorption band at 608 nm. The amount of cytochrome **c** oxidase per mg protein thus detected increased with the amount of copper added to the medium to reach a maximum for copper concentrations around 25 µg/l. When the copper concentration reached 1 mg/l, the concentration of cytochrome **c** oxidase detectable returned to zero (Fig. 2).

The concentration of cytochromes **b** and **c** varied also when the copper concentration in the medium changed. The fluctuations were similar for both of these cytochromes. It should also be noted that, for both of them, the concentrations per mg protein decreased to 50% of the value in copper-deficient medium when the copper concentration reached 1 mg/l

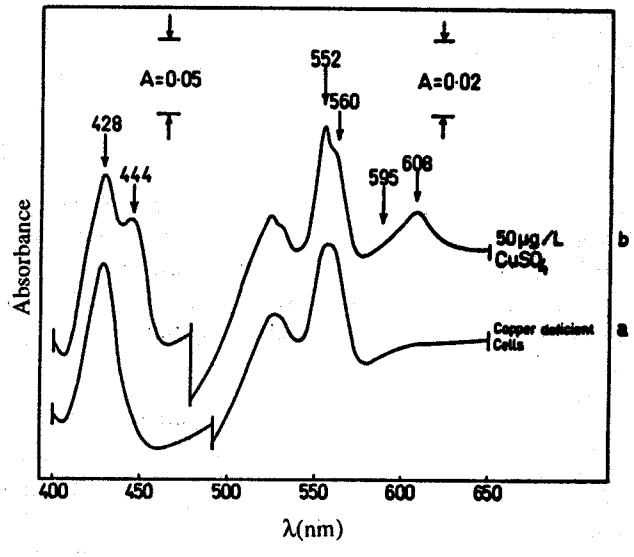

Figure 1. Dithionite-reduced-minus-H_2O_2-oxidized difference spectra of (a) copper-deficient cells and (b) cells grown in enriched medium with no supplemented copper. Cells were collected after 48 h culture in copper-deficient synthetic medium or 24 h culture in enriched medium. 2 g cells were suspended in 10 ml and split between two cuvettes. A few grains of dithionite were added to the sample cuvette and 6 µl H_2O_2 were added to the reference cuvette. Difference spectra of the cells suspensions were recorded on a DW-2 Aminco spectrophotometer.

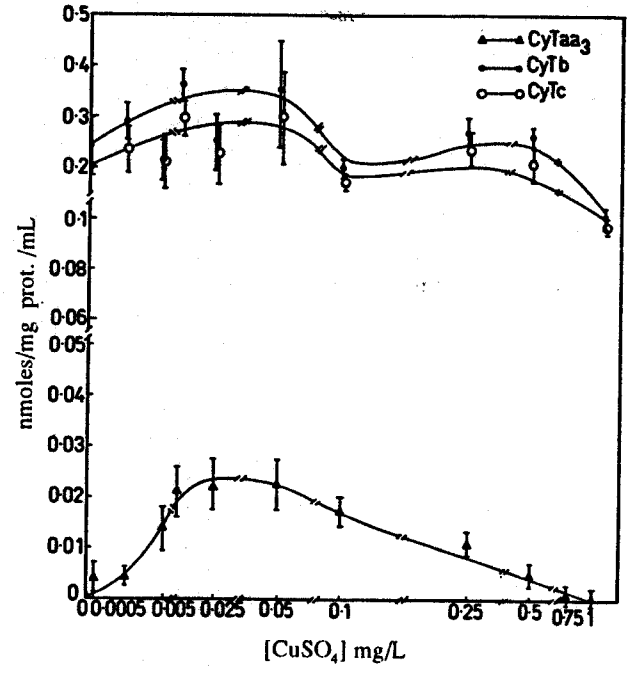

Figure 2. Effect of copper on the biosynthesis of cytochromes in copper-deficient cells grown in synthetic medium supplemented with increasing amounts of copper. (See text).

Fig. 2).

Effect of Copper Concentration on Cell Growth

The yield in cells obtained when copper-deficient cells are grown in copper-deficient medium supplemented with increasing concentrations of copper is recorded in Figure 3. It was observed that the yield increased progressively up to a concentration of 12.5 µg copper/l in the medium where the yield was 136% of that in copper-deficient medium. It returned to 100% at 50 µg copper/l and remained there until the copper concentration was 100 µg/l. Then the yield decreased progressively to reach zero when the copper concentration in the medium reached 1 mg/l.

When cells grown in enriched medium were inoculated in enriched medium supplemented with copper (Fig. 4), the yield remained roughly unchanged until the added copper concentration in the medium reached 2 mg/l. It then decreased progressively and reached 60% of the initial value (Fig. 4), in a medium supplemented with copper at 20 mg/l. This was followed by a sharp decrease of the yield to 13% when copper was added at 50 mg/l. Virtually no growth was observed in a medium supplemented with copper at 100 mg/l.

Light microscopy of copper deficient cells (Fig. 5) showed cells with abnormal morphology. In particular, cell division seemed to be impaired and a number of multinucleated cells were observed. In some cases, the chromosomes did not separate upon duplication. Furthermore, besides the cocci form, the bacterium exhibited various other forms, including a clover-like configuration. These alterations were reversible upon growth of the copper-deficient cells in a copper-supplemented medium.

In contrast to copper-deficient cells grown in minimum medium supplemented with increasing concentrations of copper, bacteria grown in the enriched medium supplemented with increasing concentrations of copper showed a greater tolerance towards high copper concentrations. However, whereas copper toxicity started at 0.1 mg/l in synthetic medium, it did not start until 2 mg of added copper/l in enriched medium.

Effect of Copper Concentration on Cytochromes Biosynthesis

The biosynthesis of cytochromes in cells grown in enriched medium supplemented with increasing concentrations of copper showed a pattern different from that observed in cells grown in synthetic medium supplemented with increasing concentrations of copper. In enriched medium, after an initial

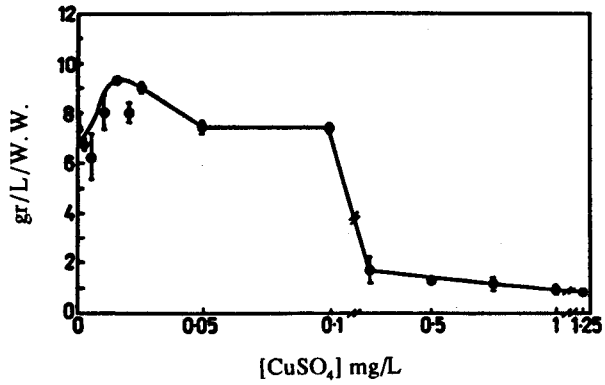

Figure 3. Effect of copper on the yield after 48 h culture in synthetic medium supplemented with various amounts of copper. (See text).

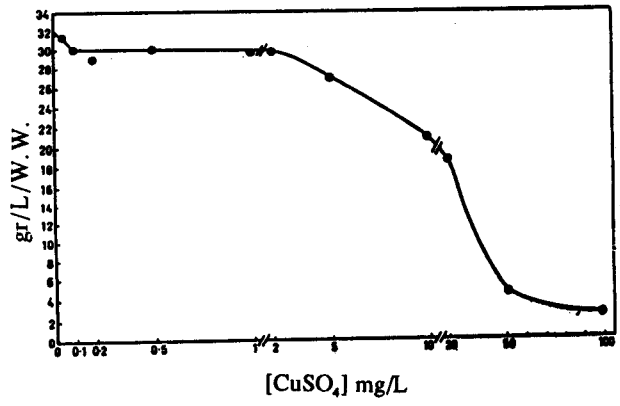

Figure 4. Effect of copper on the yield after 48 h culture in enriched medium supplemented with various amounts of copper. (See text).

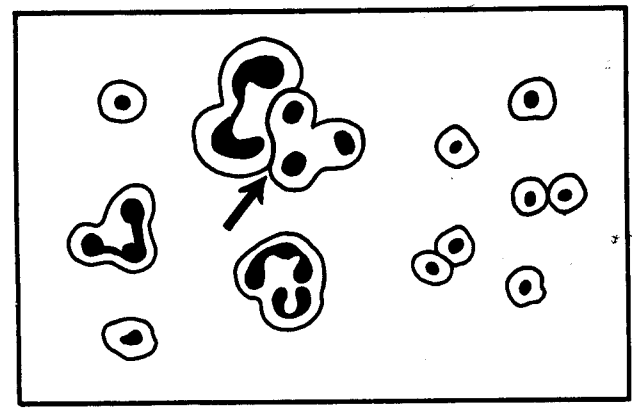

Figure 5. Light microscopy of copper-deficient cells. The arrow indicates abnormal cells in division.

stimulation of cytochromes **c** oxidase, **b** and **c** biosynthesis, a plateau was reached for concentrations of added copper of 0.2 to 1 mg/l, where the amount of cytochromes in nmol/mg protein was the same as in the control with no added copper (Fig. 6).

The biosynthesis of cytochrome $\mathbf{b}_{594}$ was stimulated in the presence of copper, with a maximum of stimulation for copper concentrations of 5 and 20 mg/l. At higher copper concentrations, the biosynthesis of cytochrome $\mathbf{b}_{594}$ was totally inhibited (Fig. 7).

Mutagenic Effect of Copper

By growing cells continuously in high copper concentrations, two mutants resistant to high copper concentrations (100 mg/l) were isolated. They were characterized by their cytochromes spectra. It was found that one of them did not synthesize cytochrome **c** in detectable amounts (Fig. 8). The other mutant did not synthesize cytochromes **c** oxidase and **c** in detectable amounts, but it exhibited **b** and **d** type cytochromes with absorption bands at 560 nm and 629 nm, respectively (Fig. 9).

Discussion

A synthetic medium, deficient in copper, was designed for growing *Paracoccus denitrificans*, in order to study the effect of copper on this bacterium.

Copper deficiency induced an inhibition of cytochrome **c** oxidase biosynthesis while the synthesis of cytochromes **b** and **c** remained unchanged, as shown by the reduced-minus-oxidized spectrum of copper deficient cells. Simultaneously, the appearance of a broad absorption band in the 594 nm region suggested that cytochrome $\mathbf{b}_{594}$ was used as an alternate electron acceptor to O_2. This cytochrome was formerly introduced as cytochrome $\mathbf{a}_1$ [18-19]. Cytochrome **c** oxidase biosynthesis was resumed upon addition of copper to the medium.

Growth of copper-deficient cells in the presence of

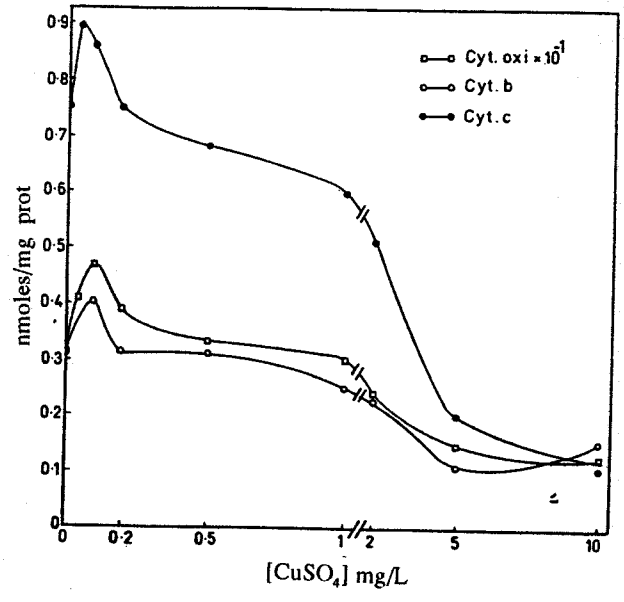

Figure 6. Effect of copper on the biosynthesis of cytochromes in cells grown in enriched medium supplemented with various amounts of copper. (See text)

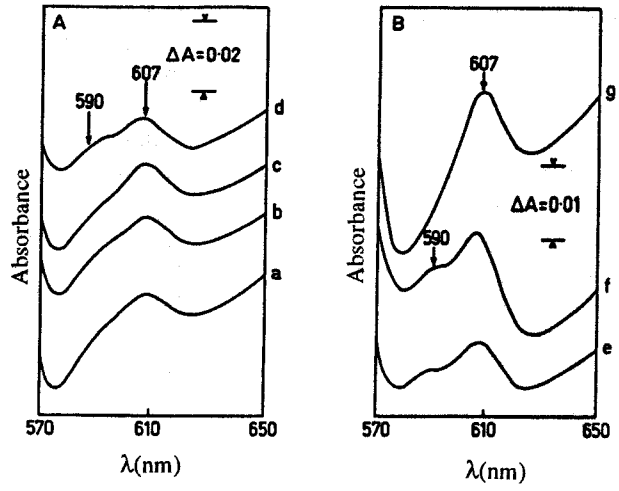

Figure 7. Effect of copper on the biosynthesis of cytochrome $\mathbf{b}_{594}$ in copper-deficient cells grown in synthetic medium supplemented with increasing amounts of copper. (a) No copper supplemented; (b) to (g) copper supplemented at the following concentrations: (b) 50 µg/l, (c) 500 µg/l, (d) 2 mg/l, (e) 5 mg/l, (f) 20 mg/l, (g) 50 mg/l.

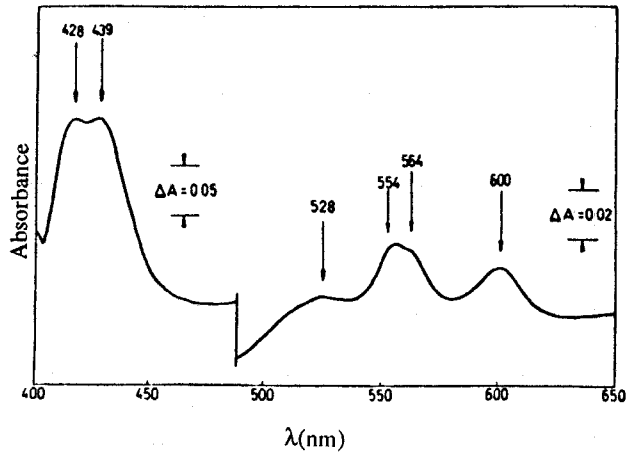

Figure 8. Dithionite-reduced-minus-H_2O_2-oxidized spectrum of the type c mutant. This mutant has no detectable cytochrome c; cytochrome c1 is detectable at 554 nm.

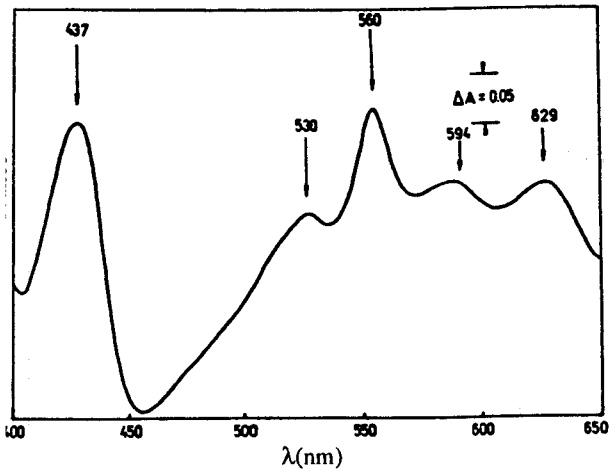

Figure 9. Dithionite-reduced-minus-H_2O_2-oxidized spectrum of the type d mutant. No cytochromes **c** oxidase or **b** are detectable. Instead, a cytochrome **d**, with absorption at 629 nm becomes detectable.

increasing amounts of copper led to an increase in yield for moderate copper concentrations (up to 12.5 µg/l). Growth inhibition started when the copper concentration reached 100 µg/l. No growth was observed for copper concentrations of 1 mg/l and above.

In contrast, when *Paracoccus denitrificans* was grown in enriched medium supplemented with increasing concentrations of copper, the yield remained unchanged for added copper concentrations up to 2 mg/l. The limit of copper toxicity in enriched medium was extended to a concentration in copper equal to 20 times that in minimum medium, since growth inhibition started when the copper concentration reached 2 mg/l (instead of 100 µg/l in synthetic medium). No growth was observed for an added copper concentration of 100 mg/l. Copper-deficient cells grown in a synthetic medium thus exhibited an increased sensitivity towards copper. This may be due to chelating of copper by the yeast extract and the peptone present in enriched medium.

The morphological studies of copper-deficient cells showed that the presence of cytochrome **c** oxidase is necessary for normal cell division. The enzyme is a transmembrane protein and its absence will affect the membrane structure, thereby possibly interfering with proper cell division.

The mutagenic effect of some metals such as Ni, Cd, Co, has been demonstrated [20-25], but the mutagenic effect of copper had not been observed previously.

Our investigations showed that prolonged growth in the presence of high concentrations of copper enabled us to isolate two types of mutants. These mutants, which we labeled "c" and "d", respectively, show abnormal cytochrome patterns.

The mutagenic effect of copper may be due to the Fenton reaction [21, 26, 27]. The mechanism generally invoked for hydroxyl radical production involves the presence of superoxide, hydrogen peroxide and either iron or copper ions. This is shown in the sequence of reactions (1) to (3) hereafter.

$$O^-_2 + M^{n+} \rightarrow O_2 + M^{(n-1)+} \quad (1)$$

$$O^-_2 + M^{(n-1)+} + 2\,H^+ \rightarrow H_2O_2 + M^{n+} \quad (2)$$

$$M^{(n-1)+} + H_2O_2 \rightarrow M^{n+} + \bar{O}H + \dot{O}H \quad (3)$$

The transition metal ion (M^{n+}) is reduced by superoxide (equation (1)) and the reduced ion donates an electron to another molecule of superoxide to give hydrogen peroxide (equation (2)). This then reacts with the reduced metal ions from equation (1) to produce hydroxyl radicals (equation (3)). The hydroxyl radical is known to attack DNA and could be held responsible for the mutagenic effect observed in this research.

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