Rapid Paper

Distribution of Zinc in *Euglena gracilis* Wild and Mutant Cells Cultured in Zinc Enriched Medium as Proved by X-Ray Microanalysis

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A mutant strain of *Euglena gracilis* Z isolated in our laboratory is highly tolerant to excess zinc and was proved by X-ray microanalysis to accumulate the element in micro-spherical bodies in the cytoplasm. On the other hand, the wild strain which is less tolerant to zinc had a uniform distribution of zinc within the cell when cultured in the zinc enriched medium. The production of the zinc-accumulating globular substances in the mutant cells is considered to be the main protective mechanism against the metal toxicity.

A trace of zinc is essential for the normal growth of organisms; it is involved in a variety of biological functions as a cofactor in various enzymatic systems. However, surplus zinc, like other heavy metals, is toxic and in some cases lethal.¹⁾ *Euglena gracilis* has often been used to study the essentiality and toxicity of zinc. So far it has been most suitably used for studies of zinc deficiency,²⁾ antagonism between zinc and cadmium,^{3~6)} and morphological changes of cells by cadmium under zinc deficient conditions.⁷⁾

During our study on the tolerance of *Euglena gracilis* Z strain to heavy metals, we found chlorosis induced by zinc with loss of flagella and motility.⁸⁾ Bleaching of *Euglena* by an excess of metal had not been reported.

In addition, mutant cells of *E. gracilis* that are highly tolerant to zinc were isolated from zinc enriched medium in our laboratory.⁹⁾ They can grow normally in concentrations of zinc up to 1,000 $\mu\text{g/ml}$. While many mutagens such as ultraviolet light, antibiotics, high temperature, etc., cause permanent loss of chloroplasts in *Euglena*,¹⁰⁾ the mutagenic activity of heavy metals has not so far been reported.

The zinc content of the mutant cells cultured in 500 $\mu\text{g Zn/ml}$ medium was found to reach to about 7,000 $\mu\text{g Zn/g}$ dry cells.¹¹⁾ The distribution and forms of zinc accumulated in the mutant cells are our present concern and of utmost importance to elucidate the zinc tolerance in this organism. This paper deals with the presence of zinc-accumulating globular substances in the mutant cells observed by analytical scanning electron microscopy.

MATERIALS AND METHODS

Each of the wild and mutant strains of *Euglena gracilis* Z was cultured in 200 ml of synthetic medium in a 500-ml Erlenmeyer flask with light at 25°C on a rotary shaker. The composition of the medium was based on that of Hutner¹²⁾ and glucose was added as the carbon source. The initial pH of the medium was adjusted to 3.5 with diluted HCl. Zinc enriched medium (500 $\mu\text{g Zn/ml}$) was made by adding ZnCl_2 to the control medium (1 $\mu\text{g Zn/ml}$). After 10 days of cultivation the cells were harvested by centrifugation and washed twice each with deionized water and with phosphate buffer (pH 7.3). They were fixed with a 2.5% glutaraldehyde-2% formaldehyde solution for one hour. The fixed cells were dehydrated with a series of graded ethanol solutions in the usual manner, suspended in isoamyl acetate, and then dried with the aid of a critical point drier. The dried cells were settled on a carbon holder with graphite paste and coated with vaporized carbon to give electrical conductivity.

A scanning electron microscope (JEOL JSM-840) with an energy dispersive (ED) X-ray microanalyzer (KEVEX 7077QJ) was used for local analysis. The accelerating voltages for the secondary electron images (SEI) and the backscattered electron images (BEI) were 5 and 15 kV, respectively.

RESULTS AND DISCUSSION

Figure 1(a) and (b) show the secondary electron images (SEI) of the mutant and wild-

FIG. 1. Secondary Electron Images (SEI) of *E. gracilis* (a) Mutant and (b) Wild-type Cells Cultured in 500 μg Zn/ml Medium.

A white grain on the cell surface in (a) is supposed to be a contaminant since no Zn K_{α} was observed. Bar 1 μm .

FIG. 2. Backscattered Electron Images (BEI) of *E. gracilis* (a) Mutant and (b) Wild-type Cells (Identical to Cells in Fig. 1).

Bar 1 μm .

FIG. 3. ED X-Ray Spectra of *E. gracilis* (a) Mutant and (b) Wild-type Cells.

Arrows in Fig. 2 show the analytical points. Zn L_{α} line and Na K_{α} line were overlapped.

type strains, respectively, cultured in 500 μg Zn/ml medium. A normal flagellum and the beautifully curved pellicular striae on the mutant cell membrane were seen on the graphite paste background (Fig. 1(a)). The surface

was as smooth as that of the wild-type cells cultured in the control medium. It seems that no toxicity of zinc was revealed morphologically on the outer membrane of the mutant cell. On the other hand, manifest effects of zinc

could be observed on the wild-type cell membrane as shown in Fig. 1(b). The cell surface lost smoothness with irregularly meandered striae and grooves. Figure 2(a) and (b) show the backscattered electron images (BEI) of the identical specimens in Fig. 1(a) and (b) respectively. Several tens of small spherical bodies with intense brightness were distributed widely in the mutant cell as shown in Fig. 2(a). These globular substances, 0.5 μm in diameter, could be clearly distinguished from cytoplasmic paramylon, a storage polysaccharide, since paramylon granules were recognized to be some dark spheroids among the light globules on the BEI.

X-Ray microanalysis was done to evaluate the local elemental distribution within the cell. The electron probe was focussed on several points including the bright globules. One of the analytical points is shown by an arrow in Fig. 2(a) and the analyzed area was estimated to be nearly 1 to 2 μm in diameter. The ED X-ray spectrum at the point indicated by the arrow is shown in Fig. 3(a), in which an intense signal due to Zn K_{α} was clearly observed at 8.63 keV. No zinc signal was detected in the areas other than the bright regions. As the detection limit of zinc by this method is 0.1 to 1.0% by weight in an analytical area,¹³⁾ an extremely high content of zinc is present in the area containing bright globules. Incident electrons accelerated at 15 kV penetrate into the organic sample to the depth of 10 μm and both the backscattered electrons and X-rays are emitted from deeper layers than the layer from which the secondary electrons are emitted.^{14,15)} From these observations together with the fact that no zinc was detected on the pellicle and the cell membrane by a transmission electron microscopic X-ray microanalyzer, the globular substances were judged to be intracellular components which have high content of zinc. In contrast, in the BEI of the wild-type cell shown in Fig. 2(b), the bright regions were dispersed formlessly within the cell and the presence of the globular substances could not be confirmed. Figure 3(b) shows the ED X-ray spectrum at one of the

bright regions of the wild-type cell, in which a rather smaller Zn K_{α} signal was observed than expected from the result that the zinc content of the wild cell was about 2,500 $\mu\text{g/g}$ dry cells. From these observations zinc incorporated in the wild-type cells seemed to be distributed widely in the bright regions and not to be concentrated on the globular substances as in the mutant cells.

The major difference between the mutant and wild-type cells in the behavior toward zinc could be ascribed to the localization of the metal as globular substances in the former and its delocalization throughout the cell in the latter. In 500 μg Zn/ml medium, the mutant cell grows normally without morphological changes in contrast to the wild-type cell which was damaged seriously on the cell surface. It is, therefore, plausible that the zinc resistance of the mutant cell could be ascribed to the ability to form globular substances that can bind excess zinc in the cytoplasm. Based on our chromatographic studies, metal-binding substances such as metallothioneins were not found in the mutant cell cultured in the zinc enriched medium.^{11,16,17)} Isolation and characterization of the globules together with their induction for the detoxification of heavy metals are in progress.

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