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Production of Respiratory-deficient Mutants of *Saccharomyces cerevisiae* by (−)Luteoskyrin and (+)Rugulosin

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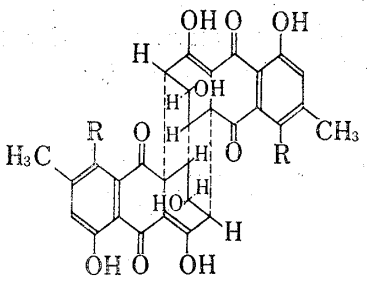
(−)Luteoskyrin and (+)rugulosin, anthraquinoid pigments produced by *Penicillium islandicum*, *P. rugulosum* and other species are hepatotoxic and carcinogenic in mice and rats. The activity of these mycotoxins was investigated in a respiratory-deficient mutation system of yeast *Saccharomyces cerevisiae*. The yeast cells were treated with several concentration of mycotoxins and the colonies produced after treatment were tested for their ability to reduce 2,3,5-triphenyltetrazolium chloride to its red formazan or to reduce a staining by trypanblue. Yeast cells in exponential phase of growth were more sensitive to the cytotoxic and mutagenic effects of rugulosin than cells in stationary phase of growth. Luteoskyrin in concentration from 10^{-4}M to 10^{-5}M and rugulosin in concentration from 10^{-3}M to 10^{-4}M induced a high frequency of mutation and the exposure of mycotoxins to ultraviolet or magnesium ion reduced their ability for mutagenicity.

(−)Luteoskyrin, one of anthraquinones produced by *Penicillium islandicum* Sopp., is hepatotoxic to animals, and a long-term feeding of mice induces hepatoma and cirrhosis.[2] Recent experiment in our laboratory have revealed that (+)rugulosin, anthraquinoid pigment of *P. rugulosum* and others and chemically very closed to luteoskyrin (Fig. 1), also induces hepatic injury[3] and tumorous changes in the liver of mice (unpublished).



R=H (+)rugulosin
R=OH (−)luteoskyrin

Fig. 1. Structures of (−)Luteoskyrin and (+)Rugulosin

Recent numerous reports are documenting mutagenic property of chemicals which exhibits carcinogenic potency, and a number of usefull systems are now available including studies on desoxyribonucleic acid (DNA) damage and repair,[4] bacterial genetic systems[5] and cytogenetic examinations in cultured cells.[6] On this line, the authors attempted to demonstrate a mutagenic property of luteoskyrin and rugulosin to the yeast *Saccharomyces cerevisiae*. The genetic material under investigation resides not in the nucleus of the yeast cell but in the cytoplasm, probably in the mitochondria, and this extrachromosomal hereditary system controls the aerobic metabolism in mitochondria.[7,8] Therefore, mutation of this genetic system modifies a content of cytochrome in a yeast cell to cause an inability to reduce dyes such as 2,3,5-triphenyltetrazolium chloride (TTC). The cellular growth becomes slow.

1) Location: *12 Ichigaya-funagawara-machi, Shinjuku-ku, Tokyo, 162, Japan.*
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In this communication, the authors examined and compared the ability of luteoskyrin and rugulosin to induce the respiratory-deficient (RD) mutant using the color plate method as well as the TTC overlay method.

Materials and Methods

Mycotoxins——(−)Luteoskyrin and (+)rugulosin were isolated from the fungal mats of *P. islandicum* and *P. rugulosum* THOM according to the methods previously reported.[3,9] Aqueous solutions of the mycotoxins were prepared by dissolving the crystals into an equivalent amount of 0.1N NaOH, and pH of the solutions was adjusted to 7.0 by adding a diluted HCl solution.

Cell Cultures——The strain IFO 0233 of *S. cerevisiae* with spontaneous mutation frequency of approximately 1% was kindly gifted from Osaka Fermentation Research Institute. Cultures were maintained on nutrient agar with 2% glucose. For experiments, the method of Nagai[10] was employed with a slight modification, as follows; cells were grown in a standard medium (glucose, 40 g; peptone, 3.5 g; yeast extract, 4.0 g; $(NH_4)_2SO_4$, 3.0 g; KH_2PO_4, 2.0 g; $MgSO_4 \cdot 7H_2O$, 1.0 g, and deionized water per 1 liter) at 30° on a rotary shaker. For experiments with cells of the stationary phase of growth, a 18-hour culture was centrifuged and the cells collected were cultured for additional 4 hours in a flesh medium. After each cultivation, the cell suspensions were centrifuged at 3000 rpm for 10 min, washed twice with 0.85% NaCl solution and resuspended in the saline to give a final cell concentration of 1×10^9 cells per ml.

Procedures for Induction of Mutation——A sterile 20 ml-flask containing 0.5 ml standard medium, 0.3 ml of deionized water, 0.1 ml of the cell suspension and 0.1 ml of the mycotoxin solution, in total volume of 1 ml, was shaken on a shaker for 3 hours at 30°. For experiments on the effect of Mg^{2+} ion, 0.5 ml of the above standard medium was replaced by 0.45 ml of the standard medium free of $MgSO_4$. The cells were preincubated in the presence or absence of mycotoxins for 10 min, then 0.05 ml of $MgSO_4$ solution ($MgSO_4 \cdot 7H_2O$ 1 g/1 liter) was added and the incubation was continued for 3 hours. For experiments on the effect of ultraviolet (UV) irradiation, the flask was illuminated by Manasule UV light (3560 Å) from a distance of 20 cm height during the preincubation for 10 min as described above.

Detection of Mutants——After 3 hours-incubation as above, the RD-mutants induced were detected by the following two systems: TTC-overlay method and color plate method as described by Nagai.[10] Aliquots (0.1 ml) were spread on plates (9 cm diameter) containing spread agar medium (glucose, 20 g; peptone, 1 g; yeast extract, 1 g; $(NH_4)_2SO_4$, 1.5 g; KH_2PO_4, 1.5 g; $MgSO_4$–$7H_2O$, 1 g; agar, 15 g and deionized water per 1 liter). After 3 to 5 days of incubation at 27°, plates were overlayed with TTC-agar (2,3,5-triphenyltetrazolium chloride 0.2 g, glucose 2 g, agar 2.5 g and water 400 ml), and after 2 to 3 hours of additional incubation, colonies were scored on their ability to reduce the dye to form a red formazan. RD-mutants are unable to reduce the dye and remain white. Number of mutants per surviving population (mutation frequency) was calculated for each treatment. In case of color plate method, aliquots (0.1 ml) were spread on spread agar medium supplemented with 6 ml of 2 mg/ml of eosin Y solution and 16 ml of 3 mg/ml of trypan blue solution. After 3 to 5 days of incubation at 27°, normal colonies were large in size and violet in color with faint pinc, whereas mutants were small in size and brilliant violet in color.

In order to minimize the experimental error, three plates were used for each treatment. The results were expressed as an average of three plates.

In some experiment, the mutation frequency was detected 4 days after treatment and incubation of the cells at 27°.

Results and Discussion

Inhibition of Cellular Multiplication by Anthraquinones

The yeast cells were incubated in the presence or absence of anthraquinones. As shown in Fig. 2, a growth curve indicated that, in the absence of mycotoxins, stationary phase was reached in 6 hours and that exponential phase occurred after 2 to 4 hours of incubation. Thus, as described in the Method, a 18-hour culture and a 4-hour culture were used as sources of cells of stationary and exponential phases of growth, respectively. In the presence of mycotoxins in dose from 1×10^{-5}M to 5×10^{-3}M, the growth rate was markedly depressed in parallel to the concentration of anthraquinones added. (−)Luteoskyrin was found to be several

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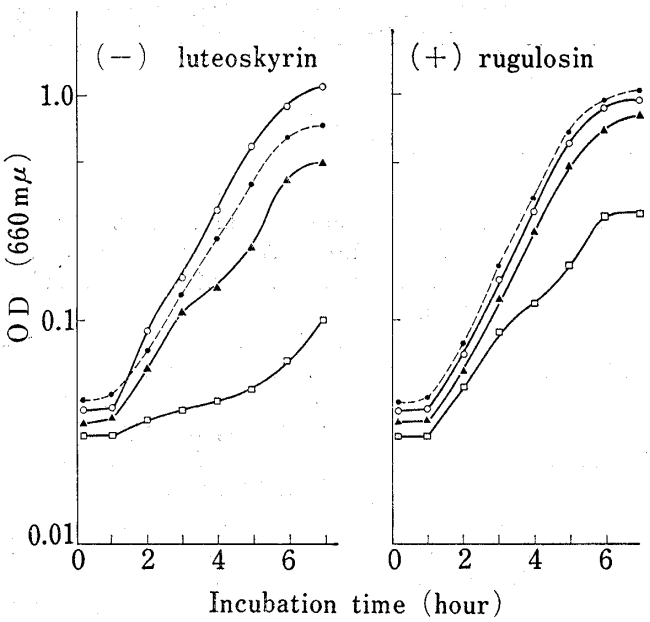

Fig. 2. Inhibitory Effects of (−)Luteoskyrin and (+)Rugulosin on the Cellular Multiplication of Yeast

L-Type tubes containing 2.5 ml of standard medium, 0.5 ml cell suspension and 2.0 ml of test solutions, in total volume of 5.0 ml, were incubated at 30° under shaking, and the turbidity was measured at 660 mμ.
—○—: control —▲—: 1×10^{-5}M
---●---: 5×10^{-6}M —□—: 5×10^{-5}M

times more toxic to yeast cells than (+)rugulosin, in agreement with the previous findings on their antibacterial[11] and antiprotozoal activities.[12]

Tables I and II show the mutation efficiency of (−)luteoskyrin and (+)rugulosin, employing two different methods of detection. When the cells in exponential phase of growth were incubated for 10 min with the mycotoxins in Mg^{2+}-free medium followed by 3 hours-incubation, luteoskyrin caused the mutation frequencies of 37 and 58% at concentrations of 10^{-5} and 10^{-4}M, respectively, while rugulosin caused the frequency of 10 and 51% under the same condition. At 10^{-3}M, luteoskyrin inhibited the multiplication, while rugulosin allowed a presence of a small number of survivors, among which 44% of the cells were RD-mutant. When the yeast cells were treated by 10^{-4} and 10^{-5}M of luteoskyrin for 4 days (Table I-B), the frequency was increased to 100% and 60%, respectively.

The toxic and mutagenic effects of rugulosin varied with the growth cycle of the yeast cells. As shown in Fig. 3, rugulosin in concentration from 10^{-5}M to 10^{-4}M was more effective on the cells in exponential phase of growth than on those in stationary phase.

Table I. RD-Mutation Efficiency of (−)-Luteoskyrin

	Luteoskyrin (M)	Color plate method		TTC-overlay method		Average efficiency (%)
		Mutant/ Survivors	Efficiency (%)	Mutant/ Survivors	Efficiency (%)	
(A)[a]	0	3/232	1.3	2/233	0.9	1.1
	10^{-3}	0/ 1	—	0/ 0	—	—
	10^{-4}	1/ 2	50	2/ 3	66.6	58.3
	10^{-5}	26/ 61	42.6	20/ 61	32.7	37.6
(B)[b]	0	1/154	0.6	6/134	4.3	2.5
	10^{-3}	2/ 2	100	—	—	100
	10^{-4}	7/ 7	100	10/ 10	100	100
	10^{-5}	23/ 38	60.5	39/ 64	60.9	60.7

a) 0.45 ml of Mg^{2+}-free standard medium, 0.3 ml of sterilized water, 0.1 ml of yeast suspension and 0.1 ml of the mycotoxin solution, in total volume of 0.95 ml, were incubated at 30° for 10 min, and 0.05 ml of $MgSO_4$ solution (1 g/l liter) was added to continue an additional incubation for 3 hours.
b) After an incubation at 30° for 3 hours as above, the cell suspension was then incubated at 27° for additional 4 days.
Each values in the table represents an average of three experiments.

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Table II. RD-Mutation Efficiency of (+)-Rugulosin[a]

Rugulosin (M)	Color plate method		TTC-overlay method		Average efficiency (%)
	Mutant/ Surviors	Efficiency (%)	Mutant/ Survivors	Efficiency (%)	
0	3/232	1.3	2/233	0.9	1.1
10^{-3}	5/ 9	55.5	3/ 8	32.8	44.2
10^{-4}	11/ 21	52.3	8/ 16	50.0	51.2
10^{-5}	17/163	10.4	20/277	9.9	10.2

a) Experimental conditions were the same as Table I-(A) except luteoskyrin was replaced by rugulosin.

To determine whether the mutants formed maintained their RD-deficient character after further growth, colonies on plates were selected from the cultures of mycotoxin-treatment (Table I-A) and replated four times on glucose nutrient agar. Three independent experiments revealed that over 95% of isolates were unable to reduce the dye. Exceptionally, 40% of isolated colonies from one plate of 10^{-5}M luteoskyrin-treatment were found to fail to reduce the dye.

These results strongly demonstrated that the both carcinogenic anthraquinones are a potent mutagen to the yeast cells and the mutagenic activity of luteoskyrin is several times higher than that of rugulosin.

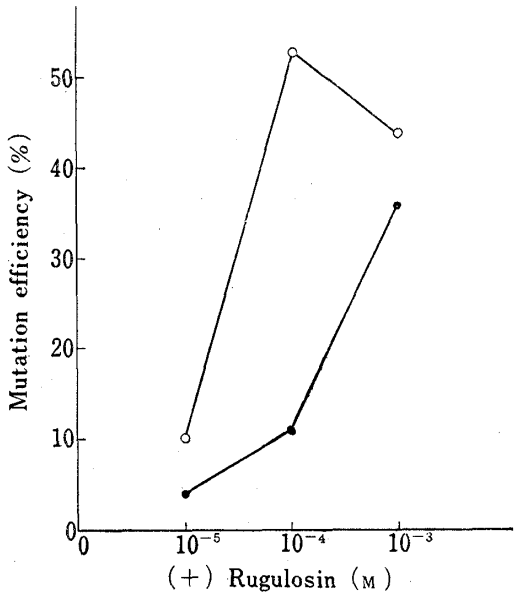

Fig. 3. Sensitivity to Rugulosin of Yeast Cells in Different Phases of Growth[a]

a) Yeast cells were preincubated with (+)rugulosin at 30° for 10 min in Mg^{2+}-free medium.
—○—: cells in exponential phase of growth
—●—: cells in stationary phase of growth

It is well established that anthraquinoid pigments chelate with divalent cations in neutral solution. In order to examine whether the metal ion-chelated pigments exhibit their mutagenecity, the cells were first suspended in Mg^{2+}-containing medium followed by addition of varied doses of rugulosin. As shown in Table III, the cytotoxic and mutagenic effects of rugulosin decreased markedly as compared with the data in Table II.

According to Shibata, *et al*,[13] luteoskyrin is photo-sensitive, and on exposure to light the mycotoxin gives rise to its photo-product, lumiluteoskyrin. With an aim to disclose whether the exposure of luteoskyrin to UV-light results in changes of its mutagenic

Table III. Effect of Mg^{2+} on RD-Mutation Efficiency of (+)-Rugulosin[a]

Rugulosin (M)	Color plate method		TTC-overlay method		Average efficiency (%)
	Mutant/ Survivors	Efficiency (%)	Mutant/ Survivors	Efficiency (%)	
0	3/96	3.1	1/ 82	1.2	2.1
10^{-3}	2/68	2.9	2/ 93	2.1	2.5
10^{-4}	0/81	0	2/120	1.6	0.8
10^{-5}	3/54	5.5	—	—	5.5

a) Yeast cells were incubated in standard medium with (+)rugulosin.

13) S. Shibata and I. Kitagawa, *Chem. Pharm. Bull.* (Tokyo), 9, 352 (1961).

Table IV. Effect of Ultraviolet Irradiation on Mutation Efficiency of (−)-Luteoskyrin[a)]

	Luteoskyrin (M)	Color plate method		TTC-overlay method		Average efficiency (%)
		Mutant/ Survivors	Efficiency (%)	Mutant/ Survivors	Efficiency (%)	
(A)	0	7/119	1.2	3/107	2.8	2.0
	10^{-3}	—	—	—	—	—
	10^{-4}	4/ 8	50	5/ 13	38.4	44.2
	10^{-5}	25/124	22	24/122	19.6	20.7
(B)	0	1/111	0.9	2/127	1.6	2.2
	10^{-3}	1/ 1	100	—	—	100
	10^{-4}	18/ 18	100	47/ 50	94.0	97.0
	10^{-5}	39/110	35.4	35/105	33.3	34.1

a) Yeast cells in exponential phase of growth were preincubated with luteoskyrin at 30° for 10 min in Mg^{+2}-free standard medium under UV-lamp, and the treated cells were incubated in standard medium at 30° for three hours (A) and followed by further incubation at 27° for 4 days (B).

activity, the cells suspended in Mg^{2+}-free medium were incubated for 10 min in the presence or absence of luteoskyrin under UV-light. As summarized in Table IV, in the absence of luteoskyrin the spontaneous mutation rate was around 1—2%. This indicates no induction of mutation by UV-light under these conditions. While, in the presence of luteoskyrin, its mutation efficiency was smaller under irradiation of UV-light than that under no irradiation (Table I); this reduction in efficiency was remarkable with a luteoskyrin at the concentration of 10^{-5}M.

From these findings, it is highly possible that the carcinogenic anthraquinones reduce their mutagenic activity when chelated with metal ions or changed to photo-product.

As for causes of the RD-deficient mutation by mycotoxins, the two mechanisms are suspected; (a) damage of the genetic material, DNA and (b) inhibition or inactivation of the respiratory enzyme(s). According to our previous experiments, luteoskyrin binds with DNA[14,15)] and nucleohistone,[16)] inhibits the synthesis of ribonucleic acid (RNA) in tumor cells,[17)] and is accumulated specifically in mitochondrial fraction of the liver when administered to mice.[18)] Accumulation of rugulosin into the mitochondria of liver of mice is also clarified (unpublished). These findings support the first hypothesis that luteoskyrin induces a damage of mitochondrial DNA of the yeast cells. According to I. Ueno,[19)] however, luteoskyrin impairs oxidation and phosphorylation of mitochondria derived from animal cells. The second hypothesis that respiratory enzyme(s) is inhibited is not consistent with our data in which the inability of the RD-mutant to reduce the dye persists after removal of the mycotoxin or several passages of mutants on agar plates.

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