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Genic Relation between Brown Coloration and Copper Resistance in *Saccharomyces cerevisiae*

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When a copper-containing medium is inoculated with cells of *Saccharomyces ellipsoideus*, the culture becomes predominated by copper resistant variant cells which possess a high ability of producing hydrogen sulfide and become brown-colored owing chiefly to precipitation of copper sulfide (5). Since, however, there is indication that the high hydrogen sulfide production is not the main copper resistance mechanism of the strain (9, 10), it is hoped to study whether the hydrogen sulfide producing ability is genetically distinct from the copper resistance. The present work was undertaken to examine this point.

Materials and Methods

The same strains of *Saccharomyces cerevisiae* were used as in the preceding study (8). Their genetic markers are represented in Table 1. The diploid strain *c* was a spontaneous mutant of *a*. A resistant clone named N714R was obtained by culturing N714 on the standard (LS) agar medium to which $CuSO_4$ was added to give a concentration of 1 mM. It did not differ from N714 as to

Table 1. Genetic markers of the strains used.

Strains	Genetic markers			
N686	α	G	pa	con
N714	a	g	PA	ver
S742	α	g	pa	con
S751	a	g	pa	con
a (S742×S751)		gg	papa	con
c (mutant of *a*)		Gg	PApa	con

a/α : Mating type; G/g : galactose fermentation; PA/pa : pantothenate requirement; con/ver : confluent and verrucose growth on copper-media (see Fig. 1B 7 and 8 in SENO (8)).

the observed markers except copper resistance. A diploid strain, *c*-10, having a low level resistance, genotype being R'/r, was isolated from a culture of *c* in 0.8 mM copper-containing PGV liquid medium (8). Among yeast strains which have never been cultured with media containing significant concentration of copper, some may be more copper resistant than others (7). But the copper resistant strains mentioned in the present paper signify exclusively those obtained by culturing more sensitive strains with copper-containing media.

The culture media, LS and PGV (Table 2), were used according to experimental purposes. For solid media agar was added to 1.5 per cent. Sodium acetate agar (1) and sodium lactate medium (3) were used for sporulation and for spore germination, respectively.

Incubation temperature was 30° for culturing and 18° for spore formation. All the strains were stocked at 0-5° on LS agar medium without copper addition.

Table 2. Composition of media.

Components	LS	PGV
Peptone	3.5g/l	2.0g/l
Sodium glutamate	—	2.0g/l
$(NH_4)_2SO_4$	—	1.0g/l
Yeast extract (powder)	1 0g/l	—
KH_2PO_4	2.0g/l	3.0g/l
$MgSO_4 \cdot 7H_2O$	1.0g/l	1.0g/l
$CaCl_2 \cdot 2H_2O$	—	0.25g/l
Glucose	40.0g/l	—
Sucrose	—	50.0g/l
Vitamin mixture*	—	10ml/1

*Inositol, 1 g; biotin, 200 γ; p-aminobenzoic acid, 200 mg; calcium pantothenate, 20 mg; thiamine, 20 mg; riboflavine, 20 mg; nicotinic acid, 20 mg; and deionized water, 1000 ml.

Mass mating method (2) was adopted for mating. A micromanipulator was used for single cell isolation and dissection of ascus. Asci containing four spores were analyzed exclusively.

Copper concentration-gradient agar plate of 10 cm length was streaked with cell suspensions using painting brushes. Range of visible growth on the plate represent copper resistance of the cell population, and intensity of brown coloration of the growth was tentatively assumed to parallel hydrogen sulfide production of cells. A gradient plate was characterized, for example, as 5/0 PGV when the nutrient used was PGV and the upper wedge contained 5 mM of copper and the lower wedge contained no copper. For details refer to SENO (8).

Inorganic sulfur content of cells was determined by methylene blue method

according to NAIKI (5). Copper content of cells was determined by carbamate method (4).

Results and Discussion

1. *Segregation of brown coloration from copper resistance.*

Dissection of thirty-seven asci of the hybrid N714R × N686 gave 2 : 2 segregation of copper resistance and sensitivity, as assayed by the growth on 5/0 PGV copper-gradient plates (8). Color intensities of these segregants, however, were not necessarily correlated with the levels of their copper resistance, and diverged so widely that some were lighter brown than the sensitive parent N686 and some others were darker brown than the resistant parent. The wide spectrum of color intensities was conventionally classified into 5 grades, and the relation of these color grades to other markers was examined. As shown in Fig. 1, dark brown growths were found more frequently among resistant segregants than among sensitive ones, and among segregants of the

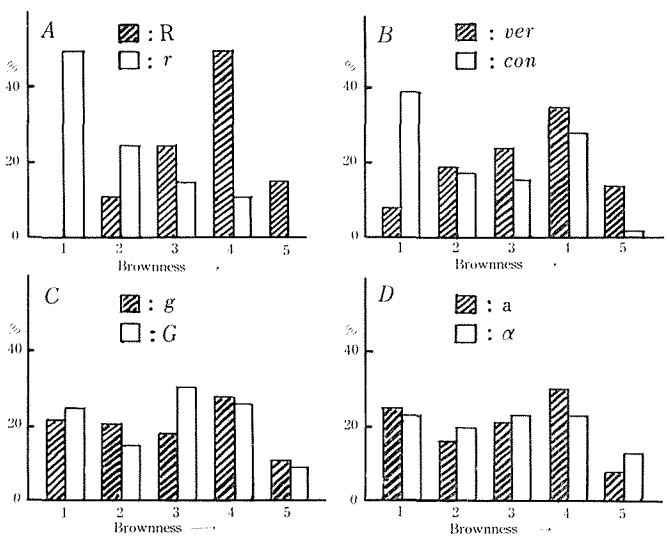

Fig. 1. A–D. Correlation between brown coloration, on the one hand, and copper resistance, growth type, galactose fermentability, and mating type, on the other hand, represented by percentage frequency distribution for the color range (1–5) on 5/0 PGV copper-gradient agar plate, 148 segregants from a hybrid N714R × N686 being observed.

R/r : Copper resistance and sensitivity; con/ver : confluent and verrucose growth on copper-media; G/g : galactose fermentability; a/α : mating type.

verrucose growth type than among those of the confluent growth type. It has been shown, however, that the copper resistance segregates rather independently of the growth type (see Table 4 in SENO (8)). Mating type and galactose fermentability had no correlation with the coloration.

2. *Gene-control of brown coloration.*

In order to exclude complication due to the growth type as encountered in the above experiment, the haploids, S742 and S751, of confluent growth type were used. Both of them showed the growth range of about 2 cm on 3.5/0 LS copper-gradient agar plate, the growth of S751 being, however, darker brown than that of S742 at the stationary phase. Since resistant mutants seemed to have been produced in the culture grown on the gradient plate used, the coloration may have been affected to some extent by them. In order to compare the coloring characters of the original strains, 0.5 mM copper-containing LS agar plate was used, on which sensitive cells could grow before resistant mutant cells grew significantly. And it was confirmed that S751 had a character of coloring more intensely on copper-containing medium than S742.

A sensitive diploid strain, *c* (cf. Table 1), and a strain of the low level (i. e. R′-type) resistance, *c*-10 (genotype R′/r), derived from *c* showed the same grade of light brown color as S742 on the copper-gradient plate. Three asci of *c*-10 were dissected with the result that light brown (typical of S742) and dark brown (typical of S751) segregated 2 : 2 for each tetrad, the segregants of R′-type resistance not being necessarily dark brown colored. Accordingly, the light brown character appears to be controlled by a dominant gene to be designated as LB (light brown), the dark brown character being recessive.

In the previous paper (8), 20 diploid clones of the high level (i. e. R-type) resistance, having genotype R/r, were obtained from *c*-10 by training it with a copper medium. Thirty-three asci from these clones were subjected to tetrad analysis and the relation between brown coloration and copper resistance was investigated by observing the growths after 3-4 days of incubation on the copper-gradient plate. The coloration could be classified distinctly into dark brown and light brown. The continuous spectrum of color intensities as mentioned in section 1 (Fig. 1) might have resulted from some modification of the coloration by two different growth types. The ratios of the two ditypes and the tetratype of tetrads, (LB r : 1b R=2 : 2), (LB R : 1b r=2 : 2), and (LB r : LB R : 1b r : 1b R=1 : 1 : 1 : 1), were 12 : 3 : 18. Although the parental types are unknown in the present case, the large difference between the frequencies of the two ditypes suggests that the loci, LB/1b and R/r, are linked. There is, however, a possibility that some sensitive segregant clones carrying 1b were recorded as LB, since they did not grow thick enough, during the incubation period, on the copper medium to show the color characteristic of them.

3. *Genic relation between brown coloration and copper resistance.*

In order to get tetrads whose parental ditype is known, a resistant haploid was obtained by training a sensitive one and mated by another sensitive haploid. When strain S751 was inoculated in 0.2 mM copper-containing LS liquid medium, two-stepped growth occurred and cells found finally in the culture gave a haploid strain which possessed the R-type resistance and presented dark brown color (only a little darker than its parent, S751) when cultured in presence of copper. The strain was denoted as S751R. The hybrid S751R × S742 was copper resistant. On 3.5/0 LS copper-gradient agar plate, the hybrid and S751R were colored darker brown than the sensitive haploids, S742 and S751, after 2 days of incubation when the former had reached the stationary phase while the latter two, being inhibited by the high copper concentration, had not. Observed after 4-5 days of incubation, on the other hand, both S751 and S751R were darker than S742 and the hybrid, and the resistant strain, S742R, obtained by training S742 showed the same grade of coloration as its parent. Thus the correct recording of the coloration character could be expected better by observing one or two days later than in the preceding section.

Ten asci of the hybrid were subjected to tetrad analysis. Streaked on 3.5/0 LS copper-gradient agar plate, every tetrad proved to be composed of two clones which grew over the whole range (10 cm) and two which grew only in the range of 2-3 cm. After 4-5 days of incubation, two clones of each tetrad assumed dark coloration just as S751 and the other two assumed light coloration just as S742, irrespective of copper resistance. Hence the regular segregation of LB/lb alleles seems to be recorded. Segregation of mating type alleles was regular.

Table 3. Tetrad distribution.

			I	:	II	:	III
lb R	×	LB r	3		1		6
a R	×	α r	2		2		6
lb a	×	LB α	1		4		5

LB and lb : Light brown and dark brown coloration, respectively, of growths on copper medium ; R/r : copper resistance ; a/α : mating type.

I : Parental ditype ; II : non-parental ditype ; III : tetratype.

Table 3 represents the tetrad distribution obtained by a hybrid, S751R (a lb R) × S742 (α LB r). The ratios, I : II : III, of the tetrad types for the gene-pairs, LB/lb and R/r, were 3 : 1 : 6. It does not differ much from the ratios, 12 : 3 : 18, represented in the preceding section. Hence the latter result seems to be rather correct, although the coloration was observed at incubation period

one or two days earlier than in the present case. Thus, the results represented so far suggest that the genes for copper resistance and for brown coloration are linked together with a significant recombination value.

4. *Copper and sulfur contents of tetrads.*

Finding that not all the copper resistant clones have the character of coloring dark brown on copper-containing media, it was hoped to see the relations among brown coloration, copper resistance, and sulfide content of cells. Copper and inorganic sulfur contents of cells were determined with the three types of tetrads described in the latter part of section 2. One tetrad each was sampled from the three types. The 12 segregants thus obtained and S742, S751 and the diploid *c*-10 were cultured on 0.5 mM copper-containing LS agar medium for 3 days. And the cells harvested were washed with deionized water by centrifugation and analyzed. The results are represented in Table 4.

Table 4. Inorganic sulfur and copper contents of segregants of three tetrad types, grown on 0.5 mM copper-containing LS agar medium.

Tetrad or strain	Genotype		Inorganic sulfur (H_2S μmole/100 mg dry cells)	Copper (μg atom/100mg dry cells)
	Resistance	Brown coloration		
c-10- 2-14	R	1b	0.08	6.6
13	R	1b	0.09	5.6
12	r	LB	0.01	4.5
11	r	LB	0.01	6.4
c-10-14 11	R	LB	0.07	4.9
12	R	LB	0.06	5.5
13	r	1b	0.11	10.1
14	r	1b	0.08	10.1
c-10-12-14	R	LB	0.01	5.3
13	R	1b	0.04	6.7
11	r	LB	0.002	3.6
12	r	1b	0.04	7.4
S742*	r	LB	0.03	4.7
S751*	r	1b	0.11	6.3
*c**	rr	LB1b	0.02	4.7
c-10	R′r	LB1b	0.03	3.8

R, R′, and r : Allelic genes for high level resistance, lower level resistance, and sensitivity, respectively ; LB and 1b : genes for light brown and dark brown coloring, respectively, of cultures on copper-medium.

*See Table 1.

Since the copper content was much higher than the sulfur content, much of copper did not seem to be in the form of sulfide. There was no correlation between copper resistance and copper content of cells. And dark brown growths were not always higher in copper than light brown ones. But the fact that dark brown growths were always higher in sulfide content than light brown ones of the same tetrad is favourable for the view that the brown coloration depends on the copper sulfide content of growth, accordingly on hydrogen sulfide production by cells. It may thus be considered that the gene for high hydrogen sulfide production is distinct from the gene for copper resistance, because the gene for dark brown coloration recombines with the latter as shown in preceding sections.

That copper resistant strains have resistance mechanism(s) other than hydrogen sulfide production has been suggested by the fact that they are copper resistant even in an early growth phase of culture when hydrogen sulfide producing ability is still low (6, 9). Respiratory deficient variants isolated from copper resistant strains retain the resistance (9), in spite of that they have lost the high ability of producing hydrogen sulfide (KIKUCHI, in preparation). This fact also corroborates that cells can be copper resistant without producing much hydrogen sulfide. An investigation on the copper permeability of cells in relation to their copper resistance will be reported in the paper to follow.

Summary

Using a few strains of *Saccharomyces cerevisiae* and copper resistant strains obtained by culturing them in presence of copper, genetic relation of the copper resistance to the brown coloration of growths to be developed on copper-containing media was studied.

When a resistant strain was mated by a sensitive one, resistance and sensitivity segregated 2 : 2 and dark brown coloration accompanied the resistance more frequently than light brown coloration.

Color intensities which sensitive haploids would assume on copper-containing medium were controlled by a gene-pair, light brown being dominant over dark brown. This character recombines with copper resistance.

The coloration of growths seemed to depend on the amount of copper sulfide they contained. But sulfur content of cells was not correlated with their copper resistance. Thus, hydrogen sulfide production does not seem to be a requisite for copper resistance, and copper resistance can segregate independently of it.

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