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Studies on the Adaptation of Yeast to Copper

XVIII. Copper-Binding Sulfur Substances of the Copper-Resistant Substrain*

By

Nobuo NAIKI

Botanical Institute, College of Science, University of Kyoto

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Fixation of copper in the cell has been presumed to be an important one of resistance mechanisms of the copper-trained substrain (1). Nitrogenous substances of the cell combine with copper (2). However, the copper-containing fraction characteristic of the resistant substrain is the brown colored matter, which shows some indication of sulfide (2). Hence the sulfur content of cells and the generation of hydrogen sulfide by cells were studied. The experiments reported in this paper have strongly suggested the significance of hydrogen sulfide production in the copper resistance.

Material and Method

The yeast used in the experiments was the same strain of *Saccharomyces ellipsoideus* as in the previous papers (1, 2). The copper resistant substrain was obtaind through repeated subcultures in the liquid MH medium** containing 1 *mM* of $CuSO_4$. Incubation temperature was 30°C. Cells were harvested at the stationary phase of growth, washed with water and acetone, and then dried at 85°C.

Nitrogen and copper were determined by the same method as in the previous report (2).

Sulfur in the cells was determined in five categories: i) total, ii) cystine and cysteine, iii) sulfate, iv) performic oxidizable and v) the rest.

i) Total sulfur.—Dried cells were oxidized after MCCHESNEY and BANKS (3, 4). But as errors in the colorimetry were not small in the present case, the oxidized sample was led to gravimetry as follows. After the oxidation the remaining perchloric acid was precipitated with 6 *N* KOH and filtered off. Heated 5% barium chlorate solution was added, drop by drop, to the filtrate kept at 100°C. The

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** KH_2PO_4 5 *g*, $MgSO_4 \cdot 7H_2O$ 2 *g*, peptone 5 *g*, cane sugar 100 *g*, distilled water 1 *l* and malt extract (Bé 8) 360 *ml*.

precipitate of barium sulfate was filtered the next day, ashed, and the weight was measured.

ii) Cystine and cysteine sulfur.—Dried cells were oxidized by performic acid, and then hydrolyzed with 6 *N* HCl (5). In the case of the resistant strain, copper was removed from the hydrolysate by H_2S. HCl was evaporated *in vacuo*. Dinitrophenilation of the hydrolysate was performed by 50% ethyl alcohol containing 2% (w/v) $NaHCO_3$ and 2% (v/v) dinitrofluoro-benzen (DNFB) (6, 7). After 2 hours' shaking, alcohol was removed by vacuum evaporation, and the alcohol-free sample was adjusted to pH 1 with 1 *N* HCl. When DNP-amino acids were extracted by ether, DNP-histidine, mono-DNP-arginine and DNP-cysteic acids remained in the water layer. A measured volume of this part was poured on a silica gel culumn which was previously wetted by 20% acetic acid. The DNP-amino acids were developed with methyl-ethyl-ketone containing the same volume of ether. DNP-cysteic acid, having the R-value 0.18-0.2, was extracted by 2% $NaHCO_3$. And the extinction at 360 mμ was measured by Shimadzu's spectro-photometer.

The standard curve was prepared using DNP-alanine in 2% $NaHCO_3$. However, no appreciable errors were expected, because DNFB is bound to the amino residue 1 : 1, and the color depends on the molar number of DNFB bound (6).

iii) Sulfate sulfur.—Dried cells were suspended in formic acid at room temperature for 4 hours, and centrifuged. The supernatant was concentrated *in vacuo* and added by the same volume of 20% trichloroacetic acid and centrifuged. The deproteinized supernatant was concentrated *in vacuo* again. By addition of 1 *N* HCl and centrifugation, clear dark yellowish supernatant was obtained. Sulfate in this solution was determined by gravimetry as in measuring the total sulfur. Formic acid was used to extract sulfate because the value determined for this category was to be substracted to know the performic oxidizable sulfur (iv). The value determined did not differ from those using trichloro-acetic acid for extraction (8).

iv) Performic oxidizable sulfur.—The same procedure as in iii was adopted, except the use of performic acid* instead of formic acid. The amount of sulfate thus determined minus that in iii gave the sulfur compounds which were oxidized to sulfate by performic acid. Sulfur-containing amino acids, namely, cystine, cysteine and methionine, do not belong to this category.

v) Other forms of sulfur.—Subtraction of the sum of ii, iii and iv from the total (i) gives "the rest", of which a major part is supposed to be methione sulfur.

Sulfide content of cells.—Two vials were connected in series by vinyl tubing. The first vial contained a heavy suspension of freshly harvested yeast cells in the main compartment and an equivolume of concentrated HCl in a side arm. The second vial contained zinc acetate solution in the main compartment, and iron alum in H_2SO_4 and dimethyl-*p*-aminoaniline in H_2SO_4 severally in side arms. HCl was mixed with cells in the first vial, and air was bubbled to make the evolving H_2S

* Correction was made for the sulfate introduced by performic acid as impurity originating from hydrogen peroxide.

gas absorbed by the zink acetate in the second vial. After 30 minutes, iron alum and dimethyl-*p*-aminoaniline were tipped in the compartment to turn zinc sulfide into methylene blue (9). No evolution of sulfide was detected when cysteine, cystine and methionine were treated in place of cells in the first vial.

Results

The resistant substrain was cultured in the liquid MH medium in the presence of 1 *mM* of $CuSO_4$ and in its absence. The cultures are designated as R and R_0, respectively. R, R_0 and the parent strain, P, were analyzed for their content in nitrogen, copper and total sulfur.

Table 1. Nitrogen, copper and sulfur content in *mg*/100 *mg* dry cells of the three cultures, and the atomic ratios, Cu : N and S : N.

	Content, *mg*/100 *mg*			Atomic ratio×10^2	
	N	Cu	S	Cu : N	S : N
P	8.45	—	0.241	—	1.2
R	6.40	0.85	0.436	2.9	3.0
R_0	7.76	—	0.260	—	1.4

The results as represented in Table 1 show that the resistant strain cultured in the copper medium contained nearly twice as much sulfur as the parent strain, while the difference was not appreciable when it was cultured in the normal medium. Roughly equal gram atoms of Cu and S were contained in R. But it does not necessarily mean that Cu and S are bound 1 : 1, as the living cell must contain a considerable amount of active thiole group and methionine.

Sulfur compounds were assayed according to the categories as described before. The results are shown in Table 2. R contains more of each type of sulfur than P. However, the difference is especially remarkable with the performic oxidizable form. Sulfide belongs to this category.

Table 2. Amount of sulfur of different forms contained in the parent strain and the copper-cultured resistant substrain, expressed in gram atom S per 10^{-2} gram atom N of the cell.

		i) Total	ii) Cysteic	iii) Sulfate	iv) Perfomic oxidizable	v) The rest
P	S, gr. atom	1.24	0.36	0.05	0.04	0.78
	%	*100*	*29.7*	*4.3*	*3.2*	*62.8*
R	S, gr. atom	3.00	0.71	0.09	0.91	1.29
	%	*100*	*23.7*	*3.0*	*30.3*	*43.0*
R/P		2.4	1.9	1.8	22.8	1.7

In order to estimate the sulfide content of cells, HCl was tipped to the washed cells of R and P. The brown coloration of R bleached soon. And very much methylene blue was formed from the gas obtained from R, but only a little from P.

It has been reported in the previous paper (2) that the brown colored fraction extracted from R contained much copper. Hence it seems most reasonable to suppose that the brown coloration of R cells is chiefly due to copper sulfide. The brown extract from R had roughly the same spectral absorption as the brown colored precipitate prepared by bubbling H_2S gas through a dilute solution of $CuSO_4$.

If the resistant strain produces more hydrogen sulfide gas than the parent strain in the medium which does not contain copper, the increased ability of producing hydrogen sulfide can be regarded as a character of the resistant strain, and the idea will be supported that the resistant strain can alleviate the injurious effect of copper by precipitating it as sulfide. So a preliminary test was made.

The synthetic medium* in which sulfate or thiosulfate constituted the sole sulfur source was inoculated by R and P. Both liquid media and agar slants were used. Just above the culture surface small pieces of filter paper were suspended which were infiltrated with the mixture of the saturated solution of lead acetate and glycerin. They would turn blackish if the culture generated some volatile sulfur-containing substances as hydrogen sulfide and mercaptan.

The results as represented in Table 3 tell that volatile sulfur compound seems to be produced more from thiosulfate than from sulfate, more by the slant culture than by the liquid culture, and more by the resistant than by the parent strain.

Table 3. Generation of volatile sulfur compound by cultures of the parent and the resistant strains as detected by lead acetate paper; ⫲, ⫳, + and ±, grades of blacking; —, no blacking.

Sulfur source	Sulfate						Thiosulfate					
Medium	Slant			Liquid			Slant			Liquid		
Incubation, days.	1	2	3	1	2	3	1	2	3	1	2	3
P	—	±	+	—	—	±	+	+	⫲	+	+	⫳
R	±	+	⫳	—	±	+	⫳	⫲	⫲	+	+	⫳
No inoculation	—	—	—	—	—	—	—	—	—	—	—	—

In order to see the generation of hydrogen sulfide from resting cells, cells of R, R_0 and P were harvested at 24 hours of cultures and suspended in the medium composed of $M/100$ Na_2SO_4, $M/15$ KH_2PO_4 and 5% sucrose. The cell suspensions were aerated at 30°C, and the hydrogen sulfide produced was trapped by zinc acetate solution in the second vials. It was shown in an hour of aeration that only

* Sucrose 50 *g*, $(NH_4)_2SO_4$ 2 *g*, KH_2PO_4 3 *g*, $MgSO_4 \cdot 7H_2O$ 1 *g*, $CaCl_2$ 0.25 *g*, inositol 10 *mg*, traces of vitamins and microelements, and distilled water 1 *l*. When $Na_2S_2O_3$ and Na_2SO_4 were used as the sulfur source, $(NH_4)_2SO_4$ and $MgSO_4$ were replaced by respective chlorides.

a trace of hydrogen sulfide was generated by P, while considerable amounts were generated by R_0 and R, and that more by R_0 than by R.

Discussion

The resistant substrain which are colored brown in the copper medium seems to contain copper sulfide as the brown substance. The substrain generates hydrogen sulfide in the medium without copper addition. Hence the fixation of infiltrated copper as sulfide is believed to be one of the resistance mechanisms.

The copper-cultured resistant strain contains more of each category of sulfur compounds than the parent strain. Some sulfur compounds, such as cysteine, may be combined with copper in the cell. Methionine is one of those amino acids that form copper complexes of high stability (10). It cannot be said, however, that all the excess amounts of sulfur compounds in R cells are bound with copper. Sulfur metabolism or R cells may be different from the parent strain. A higher content in thiole compounds may help the metabolism going on under the abnoumal intracellular conditions (11, 12). Some sulfur compounds may be present in the cell as the intermediates on the way to H_2S.

The difference in the total sulfur content between R and P is roughly twice as large as the difference in the performic oxidizable sulfur between the two (Table 2). Hence even if it is assumed that all the excess sulfur compounds are bound with copper, sulfide occupies a position among the copper-trapping sulfur compounds so big as to correspond to the sum of all the other forms.

The generation of hydrogen sulfide has been reported for many kinds of microbes, sulfate reducing bacteria (13, 14), yeast-like fungi (15, 16), fluoresent bacteria (17), *E. coli* (18, 19) and others (20). In each cases, hydrogen sulfide is produced more from thiosulfate than from sulfate. The same result as this has been obtained also with R, R_0 and P used in the present research. The hydrogen sulfide generation by these strains using sulfate, sulfite and others as the sulfur source is being studied.

Summary

1. The resistant strain cultured in the copper medium contained more of various types of sulfur compounds than the parent strain cultured in the normal medium. The difference in the sulfide content between the two cultures was especially remarkable.

2. Even in the medium without copper, hydrogen sulfide gas was produced more rapidly by the resistant strain than by the parent strain.

3. It is inferred that the resistant strain can detoxify copper in the cell by precipitating it as sulfide.

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