Origin of Copper Resistant Cells
Studies on the Adaptation of Yeast to Copper XV*

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The development of resistance to toxic agents in microbes makes an interesting and important problem from various points of view. In this concern, yeast makes an object of special interest, since the method of genetic analysis is available in this organism. The large size of yeast cells makes microscopic observations easy. Although the cluster formation of cells furnishes certain inconveniences in some experiments, a colony grown from a parent-daughter cluster is genetically equivalent to that grown from a single cell.

The results of experiments carried out to disclose the origin of copper resistant cells are reported in this paper.

Material and Method

Saccharomyces ellipsoideus strain K was used as the parent strain. The sub-strain obtained by training the parent strain with the standard medium to which CuSO₄ was added (final concentration: 1 mM) was denoted as Rₙₜ. The Rₙₜ sub-cultured in the medium without copper was designated as Rₙₜₒ. When cells of either Rₙₜ or Rₙₜₒ were plated on the standard agar medium containing 1 to 2 mM of copper, they grew into round and smooth brown colonies. Let this type of colony be called the Rₙₜₒ-type. On the same copper plate, cells of the parent strain grew into colonies with rugged outlines and surfaces.

The composition of the standard medium, called MH, was KH₂PO₄ 5g, MgSO₄·7 aq. 2g, peptone 5g, cane sugar 100g, water 1 l, wort(Bé. 8) 360 ml. For the copper medium, a measured volume of sterilized CuSO₄ solution was mixed with a measured volume of MH at room temperature, or, in the case of the agar medium, at 45° C before solidification.

Results

Observation by plating

Cells were suspended in MH agar at 45° C and a measured volume of this cell suspension was mixed with a small volume of sterilized CuSO₄ solution, and aliquots of the mixture were poured into Petri dishes. Thus a series of plates of graded

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copper concentrations, including those without copper, were prepared from a cell suspension. For plating the parent strain and R_{lb(O)}, 48-hour cultures were used. The culture of about 60 hours of age was used for R_{lb}, since the growth was a little slow in the copper medium.

The survival ratio, namely the ratio of the number of visible colonies appearing in the copper plate, to that in the control plate, was calculated. The survival value of a given type of cells in a given copper-concentration varied rather largely from one experiment to another. Figure 1 was prepared by using the averages of such values, in order to obtain a general picture of copper resistance of the three types of cells.

The comparison of curves for R_{lb(O)} and R_{lb} in the figure shows that the copper-trained substrain, R_{lb}, retained the acquired resistance even after it was subcultured in absence of copper. This usually holds true even for ten or more passages through the normal medium. The survival ratio of R_{lb(O)} was, however, a little lower than that of R_{lb}, when the copper concentration was higher than 3 mM. It is conceivable that the conversion from R_{lb} to R_{lb(O)} and the reverse may be a de-adaptation and a re-adaptation which occur readily. When high copper concentrations are used, some cells of the de-adapted population, R_{lb(O)}, might be injured before they, or their immediate descendants, became re-adapted, the result being a slight lowering of the survival ratio.

The colonial growth was observed by surface plating. When an order of 10^6 cells of the parent strain were plated on 2 mM Cu-MH agar medium, there appeared an order of hundred irregularly shaped colonies composed of white papillae, among which brown and light brown papillae were found. The distribution of the size of colony, as examined after one week's incubation, ranged from the largest to those which were hardly visible to the naked eye. On surveying the plate surface with microscope, there was a continuous gradation of colonial size down to a single cell. Among visible colonies, there were a few large colonies carrying white, light brown or brown sectors. Only in rare cases the R_{lb}-type colony was found. This type of colony may have originated from a cell of R_{lb}-type, or else from a cell of the parent-type if the R_{lb}-type cell was formed in the clone in so early a period that cells less resistant than R_{lb} were overgrown and hence the sector was not formed.

When parent cells were plated on the surface of copper agar, the counting of "visible" or "large" colonies was difficult, since the gradation of colonial size was continuous. However, a rough estimation was made by arbitrarily deciding the visible limit of colonies. According to this result, the survival ratio by the surface
plating method was higher than that by the pour plating method.

It has become evident from the surface plating experiment that the number of colonies which appear on and in the copper-agar medium does not necessarily represent the number of copper resistant cells which were present in the original culture, but represents in most, if not all, cases the number of cells which could survive and grow to be visible. The colonial growth seems to be accompanied by the appearance of cells endowed with certain degrees of resistance, brown papillae growing when $R_{1b}$ cells were produced.

Training culture

One ml of the parent strain culture in its stationary phase of growth was pipetted into 100 ml of the normal and the 1 mM Cu-MH media, and cells were counted using hematimeter at intervals during incubation. Fink's methylene blue was used to discriminate "dead" cells.

The growth in the normal medium proceeded as shown by the curve A, Figure 2. In the copper medium, on the other hand, "living" cells increased a little at first, and then decreased slightly until the secondary growth took place after 30 hours or so, as represented by the curve C in the figure. Yanagishima et al. reported a similar course of growth which occurred on the copper plate, and described the first logarithmic, the second lag, and the second logarithmic phases. The first logarithmic growth is usually smaller in the liquid medium than on the surface of the plate, when the copper concentration is the same.

The second logarithmic phase is due chiefly to the growth of resistant cells, because cells sampled from the culture in this phase can give rise to the $R_{1b}$-type colonies on the 2 mM Cu-MH plate. Naiki et al. found that the copper content of cells increased during the secondary growth, and Minagawa reported that the $R_{1b}$-specific ribonucleic acid became detectable when the second logarithmic phase began. All these findings show that resistant cells are predominating in the second logarithmic phase.

The secondary growth in copper medium ceases at a lower cell concentration than that attainable by the growth in the normal medium. The medium may have changed so much as to stop the rapid growth, since many surviving cells of the parent type, too, must have continued their metabolism. When cells at the final stationary phase are inoculated in a fresh 1 mM Cu medium, they reach the stationary population almost as high as in the curve A, although the time needed to reach
that level is usually a little longer than in the non-toxic medium.

When the parent strain was inoculated in 0.2 mM Cu liquid medium, the growth was monophasic. The growth rate and the stationary phase level were a little lower than in the normal medium. When the cells grown in this way were seeded on the 1.2 mM copper plate, the result differed from that by the parent strain only in that the relative number of very small colonies was smaller. However, no difference was found between the two when they were plated on media of copper concentrations higher than 1.5 mM. The content of 0.2 mM Cu in the medium may have effected only in eliminating very weak cells.

With the 0.7 mM copper medium, the decrease of cell number in the second lag phase was less conspicuous than with 1.0 mM. The strain trained in 0.7 mM copper medium showed lower resistance than \( R_{ib} \), only when the test plate contained copper more concentrated than 2 mM.

It is important to see how the nature of cells and the populational make-up change before the second logarithmic phase begins. One ml of the stationary phase culture of the parent strain was inoculated in the liquid MH medium containing 1.2 mM of copper. And samples were taken from this culture at intervals to be seeded on 2 and 1.2 mM copper plates as well as on the control plates.

If many of the parent cells inoculated in the copper medium should change gradually to \( R_{ib} \)-type in the second lag period, the cells sampled at a later part of the period would grow into \( R_{ib} \)-type colonies on the copper plate, with less irregularities in the colonial form than those sampled earlier. If, on the other hand, the second logarithmic growth was a continuation of the growth of a small number of resistant cells, a number of regular \( R_{ib} \)-type colonies would grow even from samples taken at early stages in the second lag phase. As mentioned in the foregoing section, the appearance of an \( R_{ib} \)-type colony may not necessarily mean that there had been an \( R_{ib} \)-type cell among the cells plated. However, since the chance of the parent-type cell producing an \( R_{ib} \)-type colony is very small, the plating method is reasonably adoptable for the present purpose.

A representative result is presented in Figure 3. The viable count on the control plate decreased up to 30 hours of incubation (curve A). The cell count by hematimeter showed that cells had already begun increasing by this time, as was shown in Figure 2. The viable count was, however, much smaller than the total cell number at this period, because many of the new-born cells were still attached to their mother cells. The mean cell number per cell-cluster was not less than 2 at this initial period of the (second) logarithmic phase.

The viable count on the 1.2 mM plate was always lower than that on the control plate (curve B, Figure 3). Notwithstanding that the cells had been in the 1.2 mM liquid medium, some of the "viable" cells failed to grow on the plate containing the same concentration of copper. It should be mentioned here that the copper toxicity becomes less when agar is added to the medium, perhaps because of interac-
tion between copper and agar.

The curve C, Figure 3, represents the change of the number of all visible colonies growing on the 2 mM plate, and the curve D the Rlh-type colonies among them. These were less than one per 10^6 plated cells at the start of the training culture, and increased roughly at a rate of one doubling every 2 to 4 hours. On the other hand, the increase of the total colonies occurred after 15 hours. This was chiefly due to the increase in number of large irregular-shaped colonies, presumably originating from cells with a little higher resistance than the majority of the parent population.

Thus it is evident that cells which are more resistant than the majority of the parent type survived and grew selectively during the second lag phase of the training culture. However, it can not be decided whether the rate of increase of resistant cells is higher than what would be expected from the simple multiplication, because the colony counting method only gives the number of cell-clusters, instead of that of individual cells. No definite conclusion could be drawn from the above result as to whether resistant cells are produced from sensitive ones during the second lag period.

Resistant cells in the parent strain

An attempt was made to determine whether the parent population contained cells with resistance heritable to their clonal offsprings.

The fluctuation test by Luria and Delbrück could not be used, because the variance of the colony number was too large, even among the copper plates which were inoculated with samples from a suspension of parent cells.

The results obtained with Lederberg's replica plating method were not so clear-cut as in his own example, since the sensitive cells had a very high chance of producing visible colonies in the present case. Calculations were made to see if the number of colonies developing on identical sites on each replicate plate was larger than that expected on the hypothesis that every colony on the master plate had an equal chance of growing on the replicate copper plates. Actually it was a little larger than the latter. However, there might have been some colonies of
which more cells were printed on every replicate plate than the others, by chance or because their size was large. Those colonies may have had a higher chance of growing on all of the replicate plates. So it can not necessarily be inferred by the above result that the parent population involved resistant clones in it.

The indirect selection method was tried, for the purpose of selecting R<sub>ib</sub>-type cells from the parent culture. But repeated trials resulted in a failure. When, on the other hand, a number of R<sub>ib(O)</sub> cells had been introduced in the parent culture, resistant cells could be selected by the same procedure. However, the indirect selection became more and more difficult as the proportion of R<sub>ib(O)</sub> cells mixed in the parent cells was lowered. This may be due to the fact that R<sub>ib</sub>-type cells are overgrown by parent cells in the normal medium. Thus the negative result did not necessarily mean that the parent population did not involve cells which carried heritable resistance.

Newcomb's spreading experiment was modified as follows. Filter paper was impregnated with MH-agar medium. The parent cells were spread on it. After a suitable period of incubation one group of the filter paper was transferred on 2 mM Cu–MH plates, the paper being inverted so as to place the micro-colonies in direct contact with the copper medium (the unspread plate). For preparing the spread plate, the micro-colonies on the surface of agar paper were redistributed by glass rod before they were brought into contact with the copper plate. After incubation, large brown colonies were counted, observed through the agar plate. Average counts per plate are given in Table 1.

If the resistant colonies were of the clonal origin, the ratio “spread: unspread” should become larger as the growth of micro-colonies proceeds with time. But actually the ratio remained as low as 2. This result makes it very doubtful that the parent strain carries in it clones which have a higher chance of thriving in copper medium than other clones. It, however, was not necessarily disproved, because resistant cells, once produced in a micro-colony, may have failed to proliferate competition with the parent cells. On the other hand, the fact that the ratio

<table>
<thead>
<tr>
<th>Incubation (hr.)</th>
<th>18</th>
<th>22</th>
<th>25.5</th>
</tr>
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<tr>
<td>Cluster plated</td>
<td>369</td>
<td>369</td>
<td>726</td>
</tr>
<tr>
<td>End No. cluster</td>
<td>1.5 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>6.9 × 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>3.7 × 10&lt;sup&gt;8&lt;/sup&gt;</td>
</tr>
<tr>
<td>Factor increase</td>
<td>4.0 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.9 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>5.2 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Resistant colonies on spread plate</td>
<td>12.5</td>
<td>50.5</td>
<td>80</td>
</tr>
<tr>
<td>Unspread plate</td>
<td>8.7</td>
<td>18.7</td>
<td>44</td>
</tr>
<tr>
<td>Ratio Spread : Unspread</td>
<td>1.4</td>
<td>2.7</td>
<td>1.8</td>
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</table>
"spread: unspread" was about 2 may be explained by supposing that the large brown colonies originated from the most proliferative of the plated parent cells, the cellular vigor (not the copper resistance in particular) being transmitted to daughter cells with some probability.

Experiments so far described are concerned with the ability of cells to grow on the copper medium. An experiment was designed to see if the relative susceptibility of cells to copper in non-nutrient medium is more uniform within a clone than among cells of different clones.

Parent cells were suspended in MH-agar, and this was solidified in sheets of 2 mm thickness. When many of the dispersed cells multiplied in the agar sheets, becoming to 4- to 8-celled micro-colonies, the sheets were shaken in M/15 KH₂PO₄ solutions for different periods, to injure the cells to various degrees. After being washed with KH₂PO₄ solution again, the agar sheets were soaked in Lindegren's methylene blue solution. Stained cells were counted as dead.

The proportion of dead cells in each sheet was determined and the number of micro-colonies in which the constituent cells were all dead or all living was separately counted. On the assumption that each cell has an equal chance of being killed by copper, the probable frequencies of the totally dead and the totally alive micro-colonies were calculated. The calculated and the observed values are given in Table 2.

Table 2. Counts of the totally alive and totally dead colonies.

<table>
<thead>
<tr>
<th>No. of cells per colony</th>
<th>No. of colonies observed</th>
<th>Proportion of dead cells</th>
<th>No. of totally dead colonies</th>
<th>No. of totally alive colonies</th>
</tr>
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<tr>
<td>8</td>
<td>32</td>
<td>0.875</td>
<td>11</td>
<td>22</td>
</tr>
<tr>
<td>8</td>
<td>37</td>
<td>0.622</td>
<td>1.4</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>42</td>
<td>0.464</td>
<td>0.09</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>73</td>
<td>0.384</td>
<td>1.6</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>73</td>
<td>0.187</td>
<td>0.09</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>90</td>
<td>0.150</td>
<td>0.04</td>
<td>2</td>
</tr>
</tbody>
</table>

Except when no totally alive colonies were found because of heavy injury, the observed numbers of totally dead and totally alive colonies were a little, but with statistical significances, larger than the respective calculated numbers. Hence there is a tendency that the relative ease with which a cell is killed by copper is transmitted to its clone at least for two or three generations. The tendency became obscure when one more generation was passed, though the observed samples were not many. It should be noted that the number of totally dead and totally alive colonies was not so many as to permit the presumption that the copper-susceptibility is a character transmitted to descendant cells with a high probability.

Although the growth competition between the resistant and the present-type cells does not come into the question in this experiment, the following should still be born in mind. 1) The observed cells were probably not of Rₐ₃-type, because the cells of this type, even if present in the parent population, must be less than
1: 10^6. Hence the experiment has probably dealt with the order of statistical variation of resistance in a simple population. 2) The ability to proliferate in the copper nutrient medium is not necessarily correlated with the ability of surviving in copper medium under the non-growing condition.

**Microscopic tracing of clonal growth**

When cells of the parent strain are spread on the 1.2 mM Cu-MH plate, more than 90% of the seeded viable cells grow into visible colonies, including very small ones. Most of them carry a number of small white papillae, brown papillae also appearing on many colonies sooner or later. The brown papillae are composed of cells which grow into Rb-type colonies when transferred to fresh copper agar plates. Hence the parent cell has a very high probability of producing Rb-type cells in its clone growing on the copper medium as papillae, whereas the probability of producing the Rb-type colony itself is extremely small.

Hence the development of parent cells on the copper plate and the production of papillae therefrom was followed using a microscope. A slide glass with a hole, 1 cm in diameter, was placed on another ordinary one. The well thus formed was half filled with 1.2 mM Cu-MH agar, and parent cells were spread on its surface. A cover slip was placed to cover the hole, and was fixed with dots of paraffin to keep the inside aerobic. In this preparation, each cell and the micro-colony growing from it could be recorded and identified during the whole period of development, by the combined use of mechanical stage and checkered ocular micrometer.

Many of the seeded cells budded at first, but the budding decreased gradually. Few cells produced more than three daughter cells. The newly produced cells also lost the budding activity with time. Perhaps they were injured by copper which was penetrating them. Only the young cells, and even only a limited number of them, produced buds. So the growth rate of micro-colonies became very small*, they taking irregular ramified forms.

Methylene blue disclosed that most of old cells were dead. The number of living cells decreased, as shown in the curve C, Figure 2, the death rate** perhaps surpassing the growth rate as referred to living cells. Colonies stopped growing when none of their young cells produced new buds, thus leaving dead colonies of various microscopic sizes.

Many micro-colonies, however, continued their very slow growth. Then, in one of these micro-colonies, a group of cells was found in which cells did not lose the budding activity so fast as before. This cell group grew rapidly, since most of the cells continued budding. It grew forming a smooth round outline, to develop into a papilla later. It was difficult to determine whether the “generation time”*** of this

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* The growth rate was very small even when it was referred to the living cells, instead of total (living and dead) cells.

** Number of death per unit time, per living cell.

*** Either the period from the birth of a cell to the bud formation by this cell, or the period from the formation of a bud to that of the next bud by a cell.
new type of cells was shorter than that of the original type, but it seemed certain that the larger rate of growth in the new type of cells was mainly due to their longevity.

It was thus observed how papillae originated in a micro-colony growing from a parent-type cell. However, since the plate was thin and the colony density was high, the papillae did not grow large enough to be differentiated in color, white or brown. So, it may not be justified to state that the development of brown papillae was actually observed under microscope among multitudes of white ones.

When cells of white papillae were spread on copper agar medium, percentage occurrence of large colonies was higher than when parent cells were seeded. But the colonies were mostly irregular in shape.

Hence, the cells of white papillae are considered to be not fully but intermediately resistant. However, the resistance of such cells was not stable enough to permit more precise studies. It is not yet known either whether the Rb-type cells are produced solely from cells having an intermediate resistance, or whether the direct change from the parent-type to the Rb-type is possible.

The microscopic observation reported above has shown the clonal basis of the gross phenomena represented by the curve C, Figure 2, and the curves C and D, Figure 3. However, there may be some difference in the growth process according to whether the medium is solid or liquid. One point to be mentioned is that the clones of inoculated cells have more chance of producing independent resistant clone in the solid medium than in the liquid one, because, in the latter, the resistant cells which are formed early may multiply so much as to leave no room for later formation of resistant cells in other clones.

Discussion

Various types of colonies grow from the parent strain plated on 1 mM Cu-MH agar. When cells of these colonies are transferred to fresh copper media, they grow with various degrees of ease, but more easily than the parent cells. Among such variant cells, the Rb-type has a tendency to overgrow the other in 1 mM Cu-MH medium. So the substrain, Rb, is easily established and sustained in the copper medium. It is stable also for a considerable number of subcultures in non-copper media. Hence Rb is chiefly used at present for studies of copper resistance.

The genetical nature of Rb is not yet quite clear. Results of tetrad analyses of hybrids between Rb and its parent strain, and those between Rb of a strain and other untrained strains were more complex than in the case where an untrained resistant strain was crossed with other sensitive strains.

Let Rb be assumed a mutant. Then, copper may appear to be essential in causing the mutation, since the colony grown from a parent cell has a very high chance of producing papillae of Rb cells, in spite of that the presence of Rb
cells can not be proved in the parent population. But the spontaneous mutation and selection hypothesis can also hold: The presence of cells with intermediate degrees of resistance suggests the multistep pattern of the resistance. $R_{ib}$ may be established through repetition of selection of a more resistant mutant, followed by further spontaneous mutation of the selected. In this case, the chance of an $R_{ib}$ cell being found in the parent population should naturally be extremely low.

However, even if the multistep idea is adopted, a possibility still remains that copper is effective, or even essential, in some steps of mutation. A possibility is not rejected either, that the one-step mutation from the parent type to $R_{ib}$ occurs in the copper medium.

If, on the other hand, $R_{ib}$ is assumed to arise solely through phenomic adapta-
tion, the intracellular factor (or condition) which governs the mechanism(s) of copper resistance should be permanently modified during a single passage through the copper medium. The present case differs from those of Oxford authors11) who found that repeated training was necessary to stabilize the acquired resistance.

It was observed under microscope that, in copper media, the growth rate as referred to the number of viable cells increased when cells were produced which did not lose budding ability so soon as sensitive cells. By unpublished experiments it was found that $R_{ib}$ was sensitive to copper, even as sensitive as the parent strain in some cases, when the medium was deficient in any one ingredient of the minimal synthetic medium. Studies on the resistance mechanism, which was partly suggested by Naiki et al.3), will be reported elsewhere.

**Summary**

1. The substrain, $R_{ib}$, obtainable by training the parent strain on 1 mM copper medium, forms round and smooth brown (viz. the $R_{ib}$-type) colonies on 1-2 mM copper agar. On this medium, the parent strain grows irregular colonies carrying white and brown papillae. The latter papillae contain cells of the $R_{ib}$-type.

2. Even after serial subcultures in the normal medium, $R_{ib}$ forms the $R_{ib}$-type colonies on the copper medium. However, a little deadaptation was disclosed when the resistance test was made with higher concentrations of copper.

3. Selective growth of cells of intermediate resistance, as well as of $R_{ib}$-type, as observed when the parent strain was inoculated in a liquid copper medium.

4. The clonal occurrence of resistance could not be observed in the parent strain.

5. Many of the clones of parent cells spread on 1.2 mM copper agar can continue to grow very slowly, older cells losing their budding ability almost as rapidly as new ones are produced. When cells are formed which do not lose the budding ability so soon, the group of such cells grows rapidly and forms a papilla.
Acknowledgments

The authors are grateful to the members of the research group on yeast variation for their cooperation, especially to Prof. T. Minagawa and Prof. Y. Arakatsu for providing many data for the growth curves.

Literature Cited


Dorsiventral Structure of Unifacial Leaves in Several Iris Species

Shun-ichiro IMAMURA* and Michiko HIDA**

今村駿一郎*・肥田美知子**: 二三のアヤメ属植物における単面葉の背腹構造

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In some plants the dorsiventrality of leaves, expressed in the distribution of stomata and assimilating parenchyma, is determined by their situation with respect to the direction of external factors. Some conifers, Thujopsis dolabrata, Chamaecyparis obtusa, Podocarpus imbricata, etc. (3, 4, 5, 6, 7, 9, 11), have assimilating organs, whose dorsiventrality is determined by the direction of incident light. Gravity as a determining factor was reported previously by the senior writer in Iris japonica (8). Dorsiventrality can be readily recognized from the different color of the upper and lower leaf surfaces. It is rarely found in Iris species having equitant leaves, which usually have an isobilateral structure. But such cases of dorsiventrality were later found in 5 species; they are described in the present paper.

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