Genetic Relatedness of Yeast Strains Studied by the DNA-DNA Hybridization Method

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Genetic relatedness of 14 yeast strains and 2 mold strains was studied by the DNA-DNA hybridization method. The hybridization was performed between mitochondrial-DNA-free, ^{32}P -labeled DNA of *Saccharomyces cerevisiae* IAM 4009 and cold DNA of other strains. The DNA homology indices deviated considerably even among *S. cerevisiae* strains having similar GC contents, but, in general, yeast strains known to be able to mate with *S. cerevisiae*, showed high homology indices (35~70%). Other species of *Saccharomycetaceae* and 6 asporogenous yeast strains exhibited values of 10~20%. The relatedness suggested from these results was confirmed by the competition experiments and also by the hybridization with ^{32}P -DNA of *Candida pulcherrima* IFO 0561. DNA's of *Aspergillus oryzae* I and *Neurospora crassa* IFO 6067 also exhibited low but appreciable homology indices (5~7%). These results were discussed from the aspects of phylogenetics and also of gene conservation in microorganisms.

Taxonomy of yeasts is mainly based on morphological and physiological observations.¹⁾ These properties are generally unstable and do not fit the purpose of phylogenetic studies. Tsuchiya *et al.*²⁾ proposed an immunological method based on species-specificities of somatic antigens. Another proposal is the use of guanine-cytosine (GC) content in DNA.^{3~6)} This criterion has been reported to be useful for classification of *Candida* species^{4,6)} in which GC contents are divergent.

Currently, the species-specificity in micro-

organisms is discussed in terms of DNA homology. As far as fungal strains are concerned, studies from this aspect are still scarce.⁷⁾ The purpose of the present study is to investigate interrelationships between the DNA homology in yeast strains and their systematic taxonomy.

MATERIALS AND METHODS

Microorganisms. Strains used in this study are listed in Table I. Most of the strains were supplied from the Division of Type Culture Collection of this Institute. Cells of *Aspergillus oryzae* and DNA of *Escherichia coli* were gift from Drs. H. Ishizuka and H. Takahashi of this Institute.

Media. Strains, except for *Saccharomyces rouxii*, were grown in a medium containing, in 1 liter of tap water, glucose, 20 g; yeast extract, 2 g; polypeptone, 5 g; KH_2PO_4 , 1 g and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g. *S. rouxii* was incubated in the medium supplemented with 10%

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1) J. Lodder and N. J. W. Kreger-van Rij, "The Yeasts, A Taxonomic study," North Holland Publishing Co., Amsterdam, (1952).

2) T. Tsuchiya, Y. Fukazawa and S. Kawakita, *Mycopathol. et Mycol. Appl.*, **26**, 1 (1964).

3) R. Storck, *J. Bacteriol.*, **91**, 227 (1966).

4) T. Nakase and K. Komagata, *J. Gen. Appl. Microbiol.*, **14**, 345 (1968).

5) S. A. Meyer and H. J. Phaff, *J. Bacteriol.*, **97**, 52 (1969).

6) A. Stenderup and A. L. Bak, *J. Gen. Microbiol.*, **52**, 231 (1968).

7) S. K. Dutta, N. Richman, V. W. Woodward and M. Mandel, *Genetics*, **57**, 719 (1967).

NaCl. The incubation was carried out at 27°C for 16 to 20 hr on a rotary shaker.

A phosphate-limiting medium used for labeling of DNA contained, in 1 liter of 0.01 M citrate buffer (pH 5.8), glucose, 20 g; L-asparagine, 2 g; casamino acid, 2 g; $(\text{NH}_4)_2\text{SO}_4$, 2 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; KCl, 0.6 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.33 g; KH_2PO_4 , 68 mg; Wickerhan's trace elements and vitamin mixture.⁸⁾

Neurospora crassa was grown under the similar conditions for 24 hr in a medium (pH 5.5) containing, in 1 liter of tap water, glucose, 10 g; yeast extract, 4 g; polypeptone, 5 g; $\text{Na}_3\text{-citrate} \cdot 2\text{H}_2\text{O}$, 2.5 g; KH_2PO_4 , 5.0 g; NH_4NO_3 , 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g and streptomycin-sulfate, 50 $\mu\text{g}/\text{ml}$. A young culture was inoculated to the main medium in a size of 2.5%. Before inoculation, the mycelia were homogenized in a Waring blender for 2 min.

Preparation of DNA. DNA of yeasts was prepared by the method described by Smith and Halvorson⁹⁾ with slight modifications. Cells were washed twice with SSC (0.15 M NaCl and 0.015 M $\text{Na}_3\text{-citrate}$), once with saline-EDTA-SLS (0.15 M NaCl, 0.1 M EDTA, 2% sodium lauryl sulfate, pH 8.0) and frozen at -20°C. After being thawed at room temperature, a volume of cells was suspended in an equal volume of saline-EDTA-SLS and homogenized for 2 min in a mechanical-cell homogenizer (Braun Co., West Germany). The homogenate was incubated first at 37°C for 4 hr and then at 60°C for 10 min after the addition of NaClO_4 to the final concentration of 1 M. DNA was extracted from the homogenate by shaking for 30 min with an equal volume of chloroform-isoamylalcohol (24:1). The mixture was centrifuged at 4000 rpm for 10 min and the supernatant was treated again with the mixture of chloroform and isoamylalcohol. The DNA was precipitated by the addition of two volumes of ethyl alcohol, spooled by a glass rod, and suspended in SSC. To the SSC solution, NaCl was added to the final concentration of 1 M and the suspension was treated first with 1 mg/ml of Pronase (Kaken Kagaku Co. Tokyo) at 60°C for 4 hr, and then with 100 $\mu\text{g}/\text{ml}$ of RNase I (Worthington Biochem. Co., U.S.A.) at 37°C for 30

min. The treatment with Pronase was repeated once again. To remove proteinous contaminants, the DNA was shaken with chloroform-isoamyl alcohol 3 to 4 times, and precipitated with 2 volumes of ethyl alcohol.

The crude DNA was purified on a methylated albumin-Kieselguhr (MAK) column which had been washed with 100 ml of 0.55 M NaCl dissolved in 0.5 M phosphate buffer, pH 6.8. Elution was made by an exponential gradient of NaCl from 0.55 M to 1.5 M. Peak fractions were collected and dialyzed against SSC. The DNA was precipitated by the addition of ethanol and dissolved again in a small volume of SSC so that the final concentration became 250~400 $\mu\text{g}/\text{ml}$. The solution was stored at -20°C. Purity of the DNA was above 95%.

DNA's of *N. crassa* and *Asp. oryzae* were prepared by the following method: Cells were dried in cold acetone and disrupted in a mortar. DNA was extracted from the dried cells by incubation at 37°C for 4 hr with 6 volumes of saline-EDTA-SLS. The DNA was purified by the procedures described above.

When ^{32}P -labeled DNA was to be prepared, cells were cultivated first in 5 ml of the phosphate-limiting medium at 30°C for 12 hr and then in 50 ml of the same medium containing 10 $\mu\text{Ci}/\text{ml}$ of ^{32}P (carrier free) for another 12 hr at 30°C. The labeled cells were treated in 10 ml of 0.05 M McIlvaine's buffer (pH 5.8) containing 0.6 M sucrose with 1 mg/ml of the snail enzyme (supplied from Dr. S. Iguchi, Ibaragi Univ.) and resulted spheroplasts were suspended in 10 ml of saline-EDTA-SLS. The cells were disrupted in a sonic oscillator at 9 kc for 10 min, and then the homogenate was heated at 60°C for 10 min. ^{32}P -DNA was extracted by the phenol method which was followed by the RNase digestion. The DNA was further purified on the MAK column and peak fractions were subjected to the dialysis against SSC.

Hybridization experiments. DNA-agar was prepared by the Bolton and McCarthy's method according to the procedures described by Takahashi *et al.*¹⁰⁾ Modifications made were: 160 mg of agar-agar were used for 5 g of DNA-agar-gel; 0.3 ml of 2×SSC (0.3 M NaCl and 0.03 M $\text{Na}_3\text{-citrate}$) containing 0.05~1.5 μg ^{32}P -DNA (*ca.* 10⁴ cpm/ μg) was used for 200 mg of DNA agar-gel and the mixture was incubated at 60°C for 16~20 hr. Unhybridized portion was removed by

8) L. J. Wickerhan, "Taxonomy of yeasts," Tech. Bull. U.S. Dep. of Agr., Washington, D.C. No. 1029 (1951).

9) D. Smith and H. O. Halvorson, "Methods in Enzymology," ed. by S. P. Colowick and N. O. Kaplan, Vol. 12, "Nucleic Acids," Part A, Academic Press, New York and London 1967, p. 538.

10) H. Takahashi, H. Saito and Y. Ikeda, *J. Gen. Appl. Microbiol.*, **12**, 113 (1966).

washing with $2\times\text{SSC}$, and radioactivity in the agar-gel was counted in a thin window gas flow counter (Nippon Musen Co., Tokyo).

Chemical and instrumental analysis. RNA and protein were measured with orcinol and phenol reagents, respectively. The concentration of DNA was determined spectrophotometrically by measuring the optical density (O.D.) at $260\text{ m}\mu$; one O.D. unit corresponded to $50\text{ }\mu\text{g}$ of native DNA and $36\text{ }\mu\text{g}$ of denatured DNA.

DNA was heated in SSC and the thermal denaturation curves were made by use of a spectrophotometer equipped with an automatic recorder (Gilford Co., U.S.A.).

CsCl density gradient analysis was made by a Spinco Model E ultracentrifuge using SW 39 rotar. The rotar was run at 33,450 rpm for 66 hr. Six drop fractions were collected from the bottom of the tube and served for the assay of radioactivity.

RESULTS

Homogeneity of the isolated DNA

Figure 1 shows an elution profile of DNA of *S. cerevisiae* IAM 4009 from the MAK column. The profile suggests that the DNA may consist of at least two classes of DNA molecules with different GC contents. Each fraction was dialysed against SSC and served for the thermal denaturation experiment. As shown in Fig. 2, the DNA from the earlier fractions gave a uniphasic curve, but the DNA from the shoulder portion a biphasic curve.

Corneo *et al.*¹¹⁾ have described that yeast cells cultivated under aerobic conditions contain a considerable amount of mitochondrial DNA (10 to 14% of the total DNA),^{11,12)} and Tewari *et al.*¹³⁾ have presented a thermal transition profile similar to that in Fig. 2. It is generally thought that the GC content in mitochondrial DNA of yeast is lower than

FIG. 1. Elution Profile of *S. cerevisiae*-IAM 4009-DNA from MAK Column.

One mg of DNA was loaded on the column equilibrated with 0.05 M phosphate buffer (pH 6.8) plus 0.55 M NaCl. Elution was made with an exponential gradient of NaCl (between 0.55 M and 1.5 M, each 250 ml) dissolved in the phosphate buffer. Five ml fractions were collected.

that in chromosomal DNA.^{11~13)}

The ^{32}P -labeled DNA of *S. cerevisiae* IMA 4009 also gave an elution profile similar to that in Fig. 2. Under the expectation that the DNA from the earlier fractions (*e.g.* tube numbers 12~14) might contain no or a little amount of mitochondrial DNA, the fractions were used for the hybridization experiments.

Homogeneity of the ^{32}P -DNA preparation was confirmed by the CsCl density gradient centrifugation. Figure 3 shows the sedimentation profiles of DNA from *S. cerevisiae* IAM 4009 and *C. pulcherrima* IFO 0561. The GC contents were calculated from the buoyant densities according to the equation presented by Schildkraut *et al.*¹⁴⁾ and the values obtained for *S. cerevisiae*-DNA and *C. pulcherrima*-DNA, 41.8% and 48.0%, respectively, were approximate to those reported by other investigators.^{3,6,11)}

11) G. Corneo, C. Moore, D. R. Sanadi, L. I. Grossman and J. Marmur, *Science*, **151**, 687 (1966).

12) J. C. Mounlou, H. Jakob and P. P. Slonimsky, *Biochem. Biophys. Res. Commun.*, **24**, 218 (1966).

13) K. K. Tewari, W. Vötsch and H. R. Mahler, *J. Mol. Biol.*, **20**, 453 (1966).

14) C. L. Schildkraut, J. Marmur and P. Doty, *J. Mol. Biol.*, **4**, 430 (1962).

FIG. 2. Thermal Denaturation Curves of the DNA Fractionated by the MAK Chromatography. Each fraction was dialyzed against SSC and the DNA concentration was adjusted to 0.5 O.D._{260mμ} units. Denaturation curves were made by a spectrophotometry with the Gilford automatic recorder.

▲ No. 19, △ No. 17, ● No. 16, ○ Nos. 14 and 15, □ No. 12.

FIG. 3. CsCl Density Gradient Centrifugation of Purified DNA's of *S. cerevisiae* IAM 4009 (a) and of *C. pulcherrima* IFO 0561 (b).

Twenty μ g of DNA in SSC was mixed with CsCl so that the final density became 1.70 g/cm³. The solution was centrifuged at 33,450 rpm, 20°C for 66 hr in a SW 39 rotor of the Spinco Model E ultracentrifuge. Six drop fractions were collected and served for the assay of radioactivity by a GM counter.

The GC contents of DNA in other strains were calculated according to the equation presented by Marmur *et al.*,¹⁵⁾ from the thermal

denaturation curves (Table II).

DNA homology examined by hybridization method

Control experiments for hybridization were carried out between a definite amount of cold

15) J. Marmur and P. Doty, *J. Mol. Biol.*, **5**, 109 (1962).

FIG. 4. Saturation Level of DNA in Agar-gel.

Two hundred mg of DNA-agar containing 13 μ g of DNA of *S. cerevisiae* IAM 4009 were incubated at 60°C for 20 hr with decreasing amounts of 32 P-labeled DNA (25,000 cpm/ μ g) of the same strain.

DNA of *S. cerevisiae* IAM 4009 and decreasing amounts of 32 P-labeled DNA of the same strain (Fig. 4). Fractions of 32 P-labeled DNA trapped on the agar-gel increased with the increment of cold to hot ratio and the curve reached a plateau at a ratio of 1:100. In later experiments, ratios of 1:100, 1:200 and 1:400 were chosen and the results were expressed as means of three data obtained at the plateau levels.

Interspecies hybridizations were performed between hot DNA of *S. cerevisiae* IAM 4009 and cold DNA of other strains. The term homology index implies relative recovery percentage of the hot DNA from heterologous combination compared to recovery percentage of the hot DNA from isologous combination. Table I shows results of three experiments obtained by use of independently prepared DNA. The homology indices deviated considerably from experiment to experiment, but the deviation was not so large as to disturb the proper evaluation of the species specificity of DNA.

The last column of the table shows results of the hybridization experiment carried out between 32 P-labeled DNA of *C. pulcherrima*

TABLE I. HYBRIDIZATION OF LABELED DNA OF *Saccharomyces cerevisiae* IAM 4009 OR *Candida pulcherrima* IFO 0561 WITH COLD DNA OF VARIOUS MICROORGANISMS

A mixture consisted of 200 mg of DNA-agar, which contained 10~20 μ g cold DNA of each tested strain, and 0.3 ml of 2 $\times$ SSC, which contained 32 P-labeled DNA (15,000~25,000 cpm/ μ g) of *S. cerevisiae* IAM 4009 or *C. pulcherrima* IFO 0561, was incubated at 60°C for 16~20 hr. The agar-gel was washed with 2 $\times$ SSC at 60°C and the radioactivity remained in the gel was counted. The percent of remained DNA to input DNA was 40.4% for Exp. 1, 44.7% for Exp. 2, or 46.9% for Exp. 3 when the hot and cold DNA's were prepared from *S. cerevisiae*, and 28.5% when the hot and cold DNA's were from *C. pulcherrima*.

Microorganisms ^{b)}	DNA homology index ^{a)} , %, to			
	<i>S. cerevi.</i> IAM 4009		<i>C. pulcher-</i>	
	Exp. 1	Exp. 2	Exp. 3 ^{c)}	IFO 0561
<i>S. cerevisiae</i> IAM 4009	100	100	100	12
<i>S. cerevisiae</i> L 8256	66	57		11
<i>S. cerevisiae</i> IAM 4518	69	55	64	11
<i>S. carlsbergensis</i> IAM 4206	43		36	9
<i>S. fragilis</i> IAM 4763	20	15		10
<i>S. rouxii</i> IAM 4028	11	11		
<i>S. pastori</i> IFO 1013	16			12
<i>P. membranaefaciens</i> IAM 4025	18	12		9
<i>H. anomala</i> IAM 4213	14		11	10
<i>D. klockeri</i> AHU 3420	13	10	11	10
<i>Sch. octosporus</i> IAM 4842	13	11		
<i>C. pulcherrima</i> IFO 0561	10	11	8	100
<i>C. robusta</i> IFO 0718	55		59	9
<i>T. colliculosa</i> IFO 0381	22		15	10
<i>Neurospora crassa</i> IFO 6067	7		6	9, 10
<i>Aspergillus oryzae</i> I	5			3, 5
<i>Escherichia coli</i> B	1.5		1	0
Calf thymus	3.5		1.5	0
None	0		0	0

^{a)} The relative recovery percentage of hot DNA from heterologous combination compared to that of hot DNA from isologous combination.

^{b)} *S.*: *Saccharomyces*; *P.*: *Pichia*; *H.*: *Hansenula*; *D.*: *Debaryomyces*; *Sch.*: *Schizosaccharomyces*; *C.*: *Candida*; *T.*: *Torulopsis*; IAM: Institute of Applied Microbiology, Univ. of Tokyo; L: Carbondale strain given by Dr. C. C. Lindgren; IFO: Institute for Fermentation, Osaka; AHU: Faculty of Agriculture, Univ. of Hokkaido, Sapporo.

^{c)} 32 P-DNA used for Exp. 3 had been purified by the CsCl density gradient ultracentrifugation (fraction No. 90~98 in Fig. 3a).

IFO 0561 and cold DNA of other strains. *C. pulcherrima* was chosen as a reference because the GC content of the DNA was the highest among the tested strains, and also because DNA of this strain hybridized scarcely with that of another reference, *S. cerevisiae* IAM 4009. None of the tested strains showed high DNA-homology with *C. pulcherrima* IFO 0561.

Confirmation of DNA homology by competition method

The hybrid formation between hot and cold DNA's of *S. cerevisiae* IAM 4009 was tested in the presence of large amounts of homologous and heterologous DNA. As shown in Fig. 5, DNA from closely related strains inhibited the hybrid formation significantly, but the inhibitions by DNA from less-related strains were small.

FIG. 5. Competitive Effects of Isologous and Heterologous Cold DNA's on the Formation of Isologous Hybrid.

An annealing mixture contained 13 µg of cold DNA of *S. cerevisiae* IAM 4009, and 0.7 µg of ³²P-labeled DNA of the same strain. The hybridization was carried out in the presence of increasing amounts of cold-*S. cerevisiae* IAM 4009-DNA (○—○) *S. cerevisiae* L 8256-DNA (●—●), *C. pulcherrima* IFO 0561-DNA (△—△) or *E. coli* B-DNA (▲—▲). The reaction mixture was incubated at 60°C for 20 hr. The radioactivity remained in the DNA-agar was assayed after being washed with 2×SSC and expressed as percent radioactivity of the control, which was 44.2% of input hot DNA.

DISCUSSION

Strains used in the present study, except for *Schizosaccharomyces octosporus*, belong to either tribe Saccharomycetaceae or subfamily Cryptococcoideae.¹⁾ The GC contents of the DNA in these strains range between 36~47% and the DNA homology indices between 10~100%.

Two strains of *S. cerevisiae*, L 8256 and IAM 4518, show homology indices of 55~70%. Strain L 8256 is a Carbondale strain. As this strain has been used for a long period for genetic studies, genetic purity of the strain is doubtful. Strain IAM 4518 and the reference strain IAM 4009 were supplied from the Division of Type Culture Collection of this

TABLE II. GENETIC RELATEDNESS OF YEAST STRAINS VIEWED FROM THREE ASPECTS

Strains	DNA homology index, % to <i>S.c.</i> 4009 ^{a)}	GC % of DNA ^{b)}	Serological group ^{c)}
<i>S. cerevisiae</i> IAM 4009	100	39.5	II
<i>S. cerevisiae</i> L 8256	57~66	39.5	II
<i>S. cerevisiae</i> IAM 4518	55~69	39.5	II
<i>C. robusta</i> IFO 0718	55~59	39.5	II
<i>S. carlsbergensis</i> IAM 4206	36~43	39.5	II
<i>S. fragilis</i> IAM 4763	15~20	40.5	II
<i>S. rouxii</i> IAM 4028	11~11		II
<i>S. pastori</i> IFO 1013	16	42	III
<i>T. colliculosa</i> IFO 0381	15~22	43	VII
<i>P. membranaefaciens</i> IAM 4025	12~18	37	III
<i>H. anomala</i> IAM 4213	11~14	36	IV
<i>D. kloederi</i> AHU 3420	10~13	36	V
<i>C. pulcherrima</i> IFO 0561	8~11	47	VI
<i>Neurospora crassa</i> IFO 6067	7	50	
<i>Aspergillus oryzae</i> I	5		
<i>Escherichia coli</i> B	1~1.5	52	
Calf thymus	1.5~3.5	42	

^{a)} Summary of Exp. 1~3 in Table I.

^{b)} GC % was calculated from the thermal transition profiles.

^{c)} From Tsuchiya *et al.*²⁾

Institute. We do not know which of the above-stated three strains can be regarded as the authentic culture of *S. cerevisiae*. Anyway, the DNA homology indices diversified largely among the three *S. cerevisiae* strains in spite of the similar GC contents.

S. carlsbergensis is a species known to be able to mate with the Carbondale strain of *S. cerevisiae*.¹⁶⁾ Homology index of this strain is 36~43%, and may be corrected to 70~80% by taking the index of strain L 8256 as 100%. The correlation between homology indices and mating compatibilities is an interesting subject worthwhile to be studied further.

Other *Saccharomyces* species show considerably low homology indices. *S. pastori* is a species classified by Phaff¹⁷⁾ into *Pichia*. The homology index of this strain is only 16% of that of *S. cerevisiae* IAM 4009. Tsuchiya *et al.*²⁾ have described that *S. pastori* can be discriminated from other *Saccharomyces* species by the serological method.

Candida robusta exhibits a relatively high homology index. This species may be an imperfect form of *S. cerevisiae* as have been stated by Lodder and Kregar-van-Rij.¹¹⁾ The

serological and genetic studies made by Tsuchiya *et al.*^{2,18)} have supported the view. *Torulopsis colliculosa* has also been reported to be an imperfect form of *S. fermentati*, but according to our study the homology index is 15~22%.

Another interest in the present study is the lower extreme of the homology indices. It is about 10% in the so-called yeasts. Takahashi *et al.* have published an opinion¹⁹⁾ that bacterial DNA may consist of species-specific portion and interspecies-common portion (conserved portion such as ribosomal RNA cistrons). If this view can be extended to fungal DNA, the residual homology, about 10%, observed in the present study may represent the interspecies-common DNA. The DNA's from *N. crassa* and *Asp. oryzae* show homology indices of 5~7%. If these values also represent the conserved portion in fungal DNA, it may be speculated that the DNA conservation is considerably larger in fungi.

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16) C. C. Lindegern, S. Spiegelman and G. Lindegern, *Pro. Nat. Acad., Sci.*, **30**, 346 (1944).

17) H. J. Phaff, *Antonie van Leeuwenhoek*, **22**, 114 (1956).

18) T. Tsuchiya, Y. Fukazawa, I. Sato, S. Kawakita and J. Katsuya, *Japan J. Exp. Med.*, **28**, 413 (1958).

19) H. Takahashi, H. Saito and Y. Ikeda, *Biochim. Biophys. Acta*, **134**, 124 (1967).