Comparative Study of Molecular Size and Structure of Exo- β -Glucanases from *Kluyveromyces* and Other Yeast Genera: Evolutionary and Taxonomic Implications

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A comparative study was made of the molecular weights of the exo- β -glucanases (EC 3.2.1.58) of species belonging to *Kluyveromyces* or to various other yeast genera. Their immunological distances were determined with the exo- β -glucanases from *Kluyveromyces fragilis*, *K. phaseolusporus*, *K. aestuarii*, and *Saccharomyces cerevisiae* as references. The molecular sizes of the enzymes were estimated by polyacrylamide gel exclusion chromatography, and the immunological comparison was done by the microcomplement fixation technique. The molecular weights of the enzymes from ascomycetous species ranged from 24×10^3 to 63×10^3 , but the majority of the species, including most species of *Kluyveromyces*, contained exo- β -glucanases with molecular weights between 35×10^3 and 45×10^3 . Immunological identity was observed among the exo- β -glucanases from the following species: *K. fragilis* (reference species), *K. marxianus*, *K. bulgaricus*, *K. cicerisporus*, and *K. wikenii*; closely related were *K. phaseolusporus* (reference species), *K. delphensis*, *K. lactis*, *K. vanudenii*, and *K. drosophilae*; also closely related were *Saccharomyces cerevisiae* (reference species), *S. italicus*, *S. uvarum*, and *S. chevalieri*. The exo- β -glucanase from *K. aestuarii* showed low degrees of homology with those from all tested species of *Kluyveromyces*. It was concluded from these data that ascospore shape has little significance in the phylogeny of this genus. Other evolutionary implications are discussed.

The genus *Kluyveromyces* was established by van der Walt (17) when he isolated from soil an ascosporogenous yeast, *K. polysporus*, capable of producing up to 60 spores per ascus. Shortly afterward, he described a second species (*K. africanus*) and later emended the generic diagnosis (18), which permitted the transfer to *Kluyveromyces* of several *Saccharomyces* species that formed up to four kidney-shaped or spheroidal spores per ascus. The genus *Kluyveromyces* is presently based on: the ability of its species to release reniform or spheroidal ascospores, soon after their formation, by dehiscence from the ascus; the smooth surface of the ascospores; and vigorous fermentation of glucose (19).

In discussing the phylogeny of *Kluyveromyces*, van der Walt proposed (19) that the genus evolved along two main lines of development. His views were patterned after Wickerham's (24) concept of evolution in the genus *Hansenula*. The two postulated lineages were distinguished on the basis of ascospore morphology (reniform versus spheroidal), each

group containing species that were thought to have evolved toward more advanced states, characterized by the acquisition of the abilities to synthesize α -glucosidase or β -galactosidase or to produce ascospores in numbers greater than four per ascus.

Important contributions were made recently to the study of phylogeny in *Kluyveromyces*; these were based on the nuclear deoxyribonucleic acid (DNA) base composition of its species (10), their DNA/DNA base sequence comparison (2, 8, 9), and a factor analysis of their functional phenotypes (14). The results obtained by these different approaches agree in part with each other and with van der Walt's postulated lineages, but several contradictions are also evident.

This paper reports a comparative study of the exo- β -glucanases (EC 3.2.1.58) extracted from the various species of *Kluyveromyces* and from representatives of other yeast genera. Various properties have been reported of exo- β -glucanases extracted and purified from *K. fragilis*, *Hansenula anomala*, *Saccharomyces cerevisiae* (1), *K. aestuarii* (6), and *K. phaseolusporus* (21). Some properties of the enzymes (e.g., substrate specificity and response to pH) appear

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rather similar, whereas others (e.g., kinetic constants) vary significantly.

The results presented here are based on: (i) a comparison of the molecular weights of these enzymes, and (ii) a study of their immunological relatedness by the microcomplement fixation technique (3). For the latter, *K. aestuarii*, *K. fragilis*, *K. phaseolusporus*, and *Saccharomyces cerevisiae* were used as reference species. It is shown how the results obtained relate to the evolution and classification of the species of *Kluyveromyces*.

MATERIALS AND METHODS

Yeast strains. The yeasts used in this study were obtained from the yeast culture collection of the Department of Food Science and Technology, University of California, Davis. The strain numbers are listed in Tables 1 and 2.

Enzyme preparations. The purification of exo- β -glucanases to antigenic homogeneity was done by a combination of ion-exchange and gel exclusion chromatographic procedures, as described previously (1, 6, 21). Protein homogeneity was based on reaching a constant specific activity during chromatography on molecular exclusion and ion-exchange gels (eluted isoionically) and on the formation of a single precipitin band when the preparations were allowed to react with antisera prepared against them. In the case of *K. aestuarii*, additional evidence for homogeneity was obtained by immunoelectrophoresis (6).

Preparations for which homogeneity was not required (those used as heterologous antigens) were obtained from cultures grown in two 1,500-ml batches of yeast autolysate (0.5%)–glucose (5%) in shaken, 2,800-ml Fernbach flasks. The cultures were grown at room temperature until the stationary phase was reached. The cells were harvested by centrifugation and suspended in sodium succinate buffer (0.05 M, pH 5.5) as a thick suspension. They were then disrupted in a Braun homogenizer (Bronwill Scientific, Rochester, N.Y.) and centrifuged to remove cell walls and debris. The supernatants were dialyzed to equilibrium against sodium succinate buffer (0.005 M, pH 6.2), centrifuged at $45,000 \times g$ for 30 min, and applied to columns of diethylaminoethyl-agarose (diethylaminoethyl-Bio-Gel A, Bio-Rad Laboratories, Richmond, Calif.) previously equilibrated with sodium succinate buffer (0.01 M, pH 6.2). After the columns were washed with equilibration buffer until no further protein was eluted, the enzymes were eluted with sodium succinate buffer (0.05 M, pH 5.0) containing 0.45 M sodium chloride. The activity peaks were located in the elution fractions by a rapid method described elsewhere (5). The concentrated exo- β -glucanase solutions (3 to 5 ml) obtained in this manner contained from 0.1 to 10 U of glucanase activity. One unit is defined as the amount of enzyme that will release 1 μ mol of glucose per min, at 30°C and pH 5.2, from 0.5% laminaran (Nutritional Biochemicals Corp., Cleveland, Ohio).

Determination of molecular weights. The molecular weights of exo- β -glucanases were estimated by

gel exclusion chromatography on Bio-Gel P-100 or P-150, according to the method of Whitaker (23). The columns were calibrated with proteins of known molecular weights: ribonuclease A, 13,680; chymotrypsinogen A, 25,650; and chicken ovalbumin, 46,000 (all from Worthington Biochemicals Corp., Freehold, N.J.); pepsin, 35,500 (Sigma Chemical Co., St. Louis, Mo.); and bovine serum albumin, 66,210 (Miles Research Products, Inc., Elkhart, Ind.). Their elution peaks were determined by measuring the absorbance (280 nm) of the elution fractions. The activity peaks of exo- β -glucanases were located by assaying the column fractions for their hydrolytic activity on laminaran and *p*-nitrophenyl- β -D-glucoside (Calbiochem, San Diego, Calif.).

A computer algorithm was used to determine the centers of the elution peaks accurately by a combination of two-curve fitting methods. Each elution quotient was calculated as the ratio of elution volume over void volume. The latter was determined for each run as the elution volume of blue dextran 2000 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), and the standard curves relating elution quotient to the logarithm of molecular weight were interpolated to obtain the molecular weight values of the unknowns.

Immunological methods. Specific anti-exo- β -glucanase sera were prepared by immunizing rabbits against purified exo- β -glucanases, following an immunization schedule recommended in *Methods in Immunology and Immunochemistry* (27). The animals were handled in accordance with the recommendations of the National Institutes of Health (United States). Each of four female adult Dutch Belted rabbits received an intradermal injection of 0.6 ml of enzyme solution mixed with Freund complete adjuvant (Difco Laboratories, Detroit, Mich.). Each emulsion contained ca. 0.5 mg of enzyme protein. Two months after the initial immunization, intravenous boosters containing 0.2 mg of purified enzyme protein in physiological saline were administered at 2-week intervals until satisfactory serum titers were obtained. The rabbits were then starved overnight, and 20 to 40 ml of blood was drawn by venous puncture of the ear and collected in petrolatum-coated tubes. These were incubated at 37°C for 1 h to speed up coagulation and were then stored overnight in the cold to allow clot retraction. The plasma was decanted and subsequently treated as described in detail by Champion et al. (3). The immunological distances among the enzyme proteins were measured by the microcomplement fixation technique as described by these authors (3). Each set of complement fixation coordinates (log serum concentration; percent complement fixed at peak) that did not significantly deviate from linearity were individually used to calculate immunological distance values, which were then averaged for each pair of antigens compared. As a guide in evaluating the experimental accuracy of each average value, the product of the slope ratios (SR) and the standard deviations (SD) of the immunological distances was calculated, and it is reported for every test as $SR \times SD$.

A large difference between the slopes of two complement fixation curves may be an indication that the proteins being compared differ from each other by features other than their amino acid sequences. In

such cases, the validity of immunological distance as a measure of relatedness is uncertain. Since the SD, in this case, is mostly an expression of experimental variation, the product $SR \times SD$ is a better index for the reliability (when it has a low value) of each immunological distance measurement reflecting primary structure differences. Thus, immunological distances for which the $SR \times SD$ values are in excess of about 30 should be considered with circumspection.

RESULTS

Molecular weights of yeast α -glucanases. The strains of the species of *Kluyveromyces* studied are listed in Table 1, together with the molecular weights of their principal α -glucanase components. Most of these enzymes have a molecular weight of approximately 40×10^3 . Those of *K. delphensis*, *K. vanudenii*, and *K. veronae*, however, were significantly lower. The molecular weights of α -glucanases of other ascomycetous yeasts ranged from 24×10^3 to 63×10^3 (Table 2). Surprisingly, these two extreme values were determined for two species of the same genus, *Schizosaccharomyces*.

A preliminary survey of basidiomycetous yeasts, including representatives of the genera *Aessosporon*, *Filobasidium*, *Leucosporidium*, *Phaffia*, and *Rhodospiridium*, was also made. All were characterized by levels of α -glucan-

ase activity which were so low that molecular weight determinations were impractical. It was noted, however, that these yeasts usually produced α -glucanases with higher molecular weights than those found in ascomycetous yeasts, i.e., higher than 50×10^3 .

Immunological comparison of yeast α -glucanases. The reference strains were selected for their ability to produce sufficient amounts of α -glucanase with adequate stability and for their postulated phylogenetic relationships as determined by other methods (8). The results shown in Tables 3 through 5 illustrate data obtained with reference strains of *K. fragilis*, *K. phaseolusporus*, and *K. aestuarii*, respectively. The sera against α -glucanases from these three strains were highly specific, and all had high titers (i.e., 14,000, 9,000, and 17,000, respectively). The data (Table 3) show that the α -glucanases from the type strains of *K. bulgaricus*, *K. cicerisporus*, *K. marxianus*, and *K. wikenii* showed no significant differences from that of *K. fragilis* and (Table 4) that those from the type strains of *K. delphensis*, *K. lactis*, *K. vanudenii*, and *K. drosophilum* were all, to some degree, quite similar to that of the type strain of *K. phaseolusporus*. The data in Table 5 show the absence of strong cross-reactivity between the α -glucanase from *K. aestuarii* UCD-FS&T 61-29 and the other α -glucanases tested.

Additional experiments were performed with the enzyme from *Saccharomyces cerevisiae* UCD-FS&T 74-83 as a reference (Table 6). The α -glucanase from this yeast proved to be a rather poor immunogen, for it produced antisera with titers lower than 1,000; this precluded its use in an extensive, in-depth survey. The α -glucanases from strains of *Saccharomyces chevalieri*, *S. italicus*, and *S. uvarum* were the only ones to react, each giving reactions of near-identity with *S. cerevisiae* UCD-FS&T 74-83.

DISCUSSION

Comparison of molecular weights of yeast α -glucanases. Yeast α -glucanases are somewhat diverse in properties such as their kinetics of hydrolysis (Table 7), but most of those surveyed are generally similar in their substrate specificities and their responses to pH. They hydrolyze laminaran [α -(1 $\rightarrow$ 3)-glucan] and pustulan [α -(1 $\rightarrow$ 6)-glucan] by the successive removal of glucose residues from the non-reducing end. In *K. aestuarii*, it was shown (M. A. Lachance, Ph.D. dissertation, University of California, Davis, 1977) that the hydrolysis product, D-glucose, retains the β configuration, but whether other yeast glucanases cause an anomeric inversion is not known. Their action on

TABLE 1. Molecular weights of the α -glucanases of the type strains of the species of *Kluyveromyces* and as determined by gel exclusion chromatography on Bio-Gel P-100 or P-150

Species	UCD-FS&T ^a strain no.	CBS ^b strain no.	Mol wt ($\times 10^3$)
<i>Kluyveromyces aestuarii</i>	61-29	4438	43
<i>K. africanus</i>	57-16	2517	42
<i>K. bulgaricus</i>	71-13	2762	41
<i>K. cicerisporus</i>	71-14	4857	41
<i>K. delphensis</i>	56-2	2170	33
<i>K. dobzhanskii</i>	50-45	2104	42
<i>K. drosophilum</i>	51-130	2105	39
<i>K. fragilis</i>	71-58	397	41
<i>K. lactis</i>	71-59	683	41
<i>K. lodderi</i>	70-3	2757	39
<i>K. marxianus</i>	55-82	712	41
<i>K. phaffii</i>	70-5	4417	42
<i>K. phaseolusporus</i>	50-80	2103	39
<i>K. polysporus</i>	57-17	2163	43
<i>K. vanudenii</i>	70-4	4372	35
<i>K. veronae</i>	55-41	2803	33
<i>K. wickerhamii</i>	54-210	2745	41
<i>K. wikenii</i>	71-15	5671	42

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TABLE 2. Molecular weights of exo- β -glucanases from species of various yeast genera and as determined by gel exclusion chromatography on Bio-Gel P-100 or P-150^a

Species	UCD-FS&T strain no.	CBS strain no.	Mol wt ($\times 10^3$)
<i>Debaryomyces cantarellii</i>	60-25 ^b	4349	43
<i>D. hansenii</i>	74-86 ^b	767	38
<i>D. phaffii</i>	60-24 ^b	4346	43
<i>Dekkera intermedia</i>	71-12		48
<i>Hanseniaspora valbyensis</i>	68-23 ^b	479	38
<i>Hansenula anomala</i>	C-366 ^b	5759	42
<i>Lipomyces starkeyi</i>	55-103		40
<i>Lodderomyces elongisporus</i>	68-17	2605	33
<i>Metschnikowia bicuspidata</i>	67-10 ^b	5575	43
<i>Nadsonia elongata</i>	61-42 ^b	2594	30
<i>N. fulvescens</i>	61-44 ^b	2996	32
<i>Pichia membranaefaciens</i>	57-22 ^b	107	38
<i>P. polymorpha</i>	57-25 ^b	186	40
Saccharomyces group I			
<i>S. cerevisiae</i>	74-83		51
<i>S. chevalieri</i>	61-22 ^b	400	53
<i>S. italicus</i>	C-105		54
<i>S. uvarum</i>	55-48 ^b	395	55
Saccharomyces group II			
<i>S. bisporus</i>	66-24 ^b	702	59
<i>S. rouxii</i>	51-49		49
Saccharomyces group III			
<i>S. rosei</i>	72-50 ^b	817	41
<i>S. saitoanus</i>	75-2 ^b	705	38
<i>Saccharomycodes ludwigii</i>	72-128		51
<i>Schizosaccharomyces japonicus</i> var. <i>japonicus</i>	71-26 ^b	354	28
<i>S. japonicus</i> var. <i>versatilis</i>	60-255		24
<i>S. malidevorans</i>	70-49 ^b	5557	63
<i>S. octosporus</i>	C-103		57
<i>S. pombe</i>	C-277		38
<i>Schwanniomyces alluvius</i>	54-83 ^b	4516	37
<i>S. castellii</i>	53-3	2863	36
<i>S. occidentalis</i>	73-2 ^b	1153	37
<i>S. personii</i>	61-9 ^b	2169	40
<i>Trigonopsis variabilis</i>	C-179		38
<i>Wickerhamia fluorescens</i>	60-5 ^b	4564	43
<i>Wingea robertsii</i>	60-22 ^b	2934	54

^a See footnotes of Table 1 for explanations of abbreviations.^b Type strain.

the β -(1 $\rightarrow$ 3) linkage is generally more pronounced than on β -(1 $\rightarrow$ 6)-linked glucans, but it is possible that the observed differences in velocity could be due to structural differences between laminaran and pustulan that are independent of their polymeric linkage. When the activation energies for these two substrates were calculated for the exo- β -glucanase from *K. aestuarii* (6), the two values were not far apart. However, the apparent specificity of that enzyme, as evaluated by comparing the V_{max}/K_m ratios obtained for laminaran and pustulan, is clearly in favor of the β -(1 $\rightarrow$ 3)-linked substrate. All yeast exo- β -glucanases that attack glucans from the nonreducing end also hydrolyze *p*-nitrophenyl- β -D-glucoside and a variety of other

synthetic β -glucosides. The significance of these physical-chemical properties in relation to evolution of the enzyme is not clear, especially since their actual function in yeast morphogenesis is not known.

The interest of the results presented here lies essentially in their interpretation and their application to the knowledge of yeast phylogeny and to yeast taxonomy. The molecular weight distribution of exo- β -glucanases from ascomycetous yeasts is striking in its diversity (Table 2). However, it appears that yeasts which share certain basic characteristics have exo- β -glucanases that are not greatly different in size. For example, the strains of the species of *Debaryomyces*, *Hansenula*, *Kluyveromyces*, *Pichia*,

TABLE 3. Immunological comparison of *exo*- β -glucanases from the type strains of various species of yeasts, using *Kluyveromyces fragilis* UCD-FS&T 71-58 as the reference strain^a

Species	UCD-FS&T ^b strain no.	N ^c	Immunological distance ^d	SD $\times$ SR ^e
<i>Kluyveromyces wikenii</i>	71-15	4	-1	16
<i>K. cicerisporus</i>	71-14	4	-1	4
<i>K. bulgaricus</i>	71-13	3	2	4
<i>K. marxianus</i>	55-82	4	2	8
<i>K. aestuarii</i>	61-29	3	159	14
<i>K. polysporus</i>	57-17	4	163	59
<i>K. africanus</i>	57-16	4	168	38
<i>K. veronae</i>	55-41	2	186	54
<i>K. vanudenii</i>	70-4	2	199	26
<i>Debaryomyces phaffii</i>	60-24	2	202	333
<i>Kluyveromyces lactis</i>	71-59	4	213	72
<i>K. delphensis</i>	56-2	NR ^f		
<i>K. dobzhanskii</i>	50-45	NR		
<i>K. drosophilae</i>	51-130	NR		
<i>K. lodderi</i>	70-3	NR		
<i>K. phaffii</i>	70-5	NR		
<i>K. phaseolusporus</i>	50-80	NR		
<i>K. wickerhamii</i>	54-210	NR		
<i>Dekkera intermedia</i>	71-12	NR		
<i>Hansenula anomala</i>	C-366	NR		
<i>Hanseniaspora valbyensis</i>	68-23	NR		
<i>Lodderomyces elongisporus</i>	68-17	NR		
<i>Metschnikowia bicuspidata</i>	67-10	NR		
<i>Pichia membranaefaciens</i>	57-22	NR		
<i>Saccharomyces rosei</i>	72-50	NR		
<i>S. uvarum</i>	55-48	NR		
<i>Schwanniomyces castellii</i>	58-3	NR		

^a The homologous titration slope was 271.^b University of California, Davis, Department of Food Science and Technology.^c N, Number of points used in calculating the immunological distance.^d Immunological distance = 100 log immunological dissimilarity (cf. reference 3).^e SD $\times$ SR, Product of the standard deviation of immunological distances calculated from each pair of coordinates and the ratio of the slopes of each titration curve.^f NR indicates either that no detectable complement fixation was observed or that excessive anti-complementarity was encountered.TABLE 4. Immunological comparison of *exo*- β -glucanases from the type strains of various species of yeasts, using *Kluyveromyces phaseolusporus* UCD-FS&T 50-80 as the reference strain^a

Species	UCD-FS&T strain no.	N	Immunological distance	SD $\times$ SR
<i>Kluyveromyces delphensis</i>	56-2	4	10	4
<i>K. lactis</i>	71-59	3	15	2
<i>K. vanudenii</i>	70-4	3	20	1
<i>K. drosophilae</i>	51-130	3	27	2
<i>K. dobzhanskii</i>	50-45	3	134	58
<i>K. africanus</i>	57-16	2	186	1
<i>K. wickerhamii</i>	54-210	4	206	41
<i>K. aestuarii</i>	61-29	2	209	3
<i>K. fragilis</i>	71-58	NR		
<i>K. lodderi</i>	70-3	NR		
<i>K. polysporus</i>	57-17	NR		
<i>Debaryomyces cantarellii</i>	60-25	NR		
<i>Saccharomyces cerevisiae</i>	74-83	NR		
<i>S. rosei</i>	72-50	NR		

^a The homologous titration slope was 291. See footnotes *b* through *f* of Table 3 for explanations of abbreviations.

TABLE 5. Immunological comparison of exo- β -glucanases from the type strains of various species of yeasts, using *Kluyveromyces aestuarii* UCD-FS&T 61-29 as the reference strain^a

Species	UCD-FS&T strain no.	N	Immunological distance	SD $\times$ SR
<i>Kluyveromyces drosophilorum</i>	51-130	3	161	2
<i>K. fragilis</i>	71-58	3	162	1
<i>K. wickerhamii</i>	54-210	2	163	2
<i>K. africanus</i>	57-16	2	170	11
<i>K. lactis</i>	71-59	2	173	1
<i>Dekkera intermedia</i>	71-12	NR		
<i>Hansenula anomala</i>	C-366	NR		
<i>Lodderomyces elongisporus</i>	68-17	NR		
<i>Pichia membranaefaciens</i>	57-22	NR		
<i>Saccharomyces rosei</i>	72-50	NR		
<i>S. uvarum</i>	55-48	NR		

^a The homologous titration slope was 263. See footnotes *b* through *f* of Table 3 for explanations of abbreviations.

TABLE 6. Immunological comparison of exo- β -glucanases from various species of yeasts, using *Saccharomyces cerevisiae* UCD-FS&T 74-83 as the reference strain^a

Species	UCD-FS&T strain no.	N	Immunological distance	SD $\times$ SR
<i>Saccharomyces italicus</i>	C-105	3	5	8
<i>S. uvarum</i>	55-48	4	11	7
<i>S. chevalieri</i>	61-22	3	16	10
<i>S. bisporus</i>	66-24	NR		
<i>S. rosei</i>	72-50	NR		
<i>S. rouxii</i>	51-49	NR		
<i>Debaryomyces cantarellii</i>	60-25	NR		
<i>Kluyveromyces fragilis</i>	71-58	NR		
<i>Lodderomyces elongisporus</i>	68-17	NR		

^a The homologous titration slope was 245. See footnotes *b* through *f* of Table 3 for explanations of abbreviations.

TABLE 7. Comparison of some physical-chemical properties of various yeast exo- β -glucanases

Species ^a	Laminaran		Pustulan		pNPG ^b		pH activity		
	V_{max}^c	K_m (mg/ml)	V_{max}	K_m (mg/ml)	V_{max}	K_m (mM)	pK _i	pH _{opt}	pK ₂
<i>Candida utilis</i> (12)		1.2		0.7		3.1		5.5	
<i>Cryptococcus albidus</i> var. <i>aerius</i> (11)		0.1		0.3		0.007	3.5	5.5	7.0
<i>Hansenula anomala</i> (1)	54	5	7.2	5.9	1.8	5.4		5.5	
<i>Kluyveromyces aestuarii</i> (6)	260	1.7	3.9	4.5	24	5.0	3.2	5.2	6.0
<i>K. fragilis</i> (1)	83	1.2	7.4	1.8	2.2	0.6	4.2	5.5	7.0
<i>K. phaseolusporus</i> (16)		2		35.7		6	5.2	6.0	7.1
<i>Saccharomyces cerevisiae</i> (1)	9.5	14	3.8	3.6	0.7	215		5.5	
<i>Schizosaccharomyces japonicus</i> var. <i>versatilis</i> (4)	350	6.2	52	166			3.6	5.2	6.8

^a The numbers after the species name are literature citations.

^b pNPG, *p*-Nitrophenyl- β -D-glucoside.

^c V_{max} , Micromoles of glucose released per minute at 30°C and pH 5.5.

Saccharomyces group III, and *Schwanniomyces* included in this survey all gave values clustering around 40×10^3 . Species of these genera not only share certain properties, particularly in their sexual life cycles, but also exhibit important differences; this has resulted in the present

state of confusion regarding their classification (7, 15, 20, 22). In contrast, the species of *Schizosaccharomyces* appeared highly diverse with respect to the size of their exo- β -glucanases. This possibly indicates that this genus is either polyphyletic or very ancient.

A few discrepancies were noted between molecular weights reported here and those previously reported in the literature. For example, Abd-El-A1 and Phaff (1) calculated values of 22×10^3 , 30×10^3 , and 40×10^3 for the *exo*- β -glucanases from strains of *K. fragilis*, *H. anomala*, and *S. cerevisiae*, respectively. These authors used column chromatography, as we did, but their columns were packed with cross-linked dextran (Sephadex) gels (for which these enzymes show significant affinity) eluted with buffers of lower ionic strength (0.2 M). This results in lower apparent molecular weights. We obtained values of 41×10^3 , 42×10^3 , and 51×10^3 , respectively, for the same yeast strains. During the purification of *exo*- β -glucanases from strains of *K. aestuarii* and *K. phaseolusporus*, it was observed (Lachance, Ph.D. dissertation) that, when these enzymes were chromatographed on different grades of Sephadex, their elution volumes indicated apparent molecular weights of approximately 22×10^3 . The enzyme from a strain of *K. phaseolusporus* similarly eluted from Sephacryl S-200 with an apparent molecular weight of 19×10^3 (21). The molecular weights reported here and determined on polyacrylamide columns are thus of a relative, although more realistic, nature. The influence of carbohydrate content of the different *exo*- β -glucanases on their chromatographic behavior is not known.

The standard deviation of molecular weights of *exo*- β -glucanases shown to be very similar by microcomplement fixation, or those from yeasts with high degrees of DNA complementarity, was 1.7×10^3 .

Immunological comparisons of *exo*- β -glucanases. Even though *exo*- β -glucanases in the genus *Kluyveromyces* appeared rather homogeneous with respect to their molecular weights, an appreciable degree of diversity was detected by the microcomplement fixation technique. A range of immunological distances from 0 to 27 was found for eight of our comparisons, but all other values were 130 or higher, indicating that the primary structure of *exo*- β -glucanase is not highly conserved in evolutionary time. If the relationship of four units of immunological distance for every 1% amino acid replacement, as found for bird lysozyme (3), holds equally for yeast *exo*- β -glucanases, many of these enzymes in *Kluyveromyces* differ from each other by at least 40% of their sequences. It follows that *exo*- β -glucanases may not be proteins of choice for deriving general yeast phylogenies.

The accuracy of high immunological distance values (i.e., >130) can be questioned, but the one reciprocal test (*K. fragilis* $\times$ *K. aestuarii*) to result from this study gave concordant values of

159 (SR $\times$ SD = 14; Table 3) and 162 (SR $\times$ SD = 1; Table 5). Higher values should nevertheless be considered with caution (V. M. Sarich, personal communication).

Various degrees of immunological similarity between *exo*- β -glucanases from different yeasts may take on different significances, depending on whether or not gene exchange is possible among the species compared. Our survey provided no clue as to the importance of intraspecific gene polymorphism in yeast, since it is not known whether or not in nature the "species" compared are capable of effective genetic recombination among each other. Wickerham and Burton (25, 26) induced, under laboratory conditions, exchange of genetic material, even in species that have low degrees of DNA complementarity (8). However, they gave no evidence on fertility or segregational patterns of the F_1 generation of these crosses.

Conversely, natural genetic exchange between "species" of *Kluyveromyces* may be a minor factor, so that, in practice, the divergence observed between the informational macromolecules (DNA, ribonucleic acid, and proteins) of these yeasts is almost exclusively the result of accumulated mutations through evolutionary time.

When the results of our immunological survey were compared with the relationships proposed by van der Walt (19) and by Poncet (14), an appreciable degree of agreement was apparent, but major discrepancies were also noted. For example, high degrees of immunological similarity were found between *exo*- β -glucanases from species with different spore shapes or from species which showed relatively little similarity by factor analysis of their expressed phenotypes. In other cases, species deemed identical by factor analysis showed low degrees of immunological cross-reactivity. There was, however, (with one exception [see below]) an excellent correlation between DNA/DNA complementarity data (2, 8, 9) and the immunological comparison. This is in support of treating the species of *Kluyveromyces* as distinct genetic lineages rather than of considering their structurally different glucanases as alleles of the same polymorphic gene.

The few comparisons performed with *Saccharomyces* strains with reference to *Saccharomyces cerevisiae* (Table 6) were also in agreement with the DNA reassociation patterns obtained with these strains (2).

Assuming that all presently defined "species" of *Kluyveromyces* are derived from a common ancestor and have not undergone reticulate evolution, the immunological distances found between the *exo*- β -glucanases of each of these species can be taken as an index of their time of

divergence. From this, the following conclusions can be drawn.

(i) The choice of ascospore shape as a basis for establishing two lines of development in the genus *Kluyveromyces* appears unjustified, because in two instances, "species" with reniform spores were shown to be immunologically closely related to "species" producing spherical ascospores (i.e., *K. fragilis* and *K. marxianus*, with *K. bulgaricus*, *K. cicerisporus*, and *K. wikenii*, respectively; *K. delphensis*, *K. drosophilarum*, and *K. phaseolosporus*, with *K. lactis* and *K. vanudenii*, respectively); conversely, many "species" with similar spore morphologies showed little resemblance in their exo- β -glucanases.

(ii) Combining our results with those obtained by DNA/DNA base sequence comparisons (2, 8, 9), the following should be retained as separate species: *K. aestuarii*, *K. africanus*, *K. dobzhanskii*, *K. lodderi*, *K. phaffii*, *K. polysporus*, *K. veronae*, and *K. wickerhamii*.

(iii) The close phenotypic similarities (14, 19) observed between (a) *K. aestuarii* and *K. lactis*, (b) *K. delphensis* and *K. phaffii* or *K. africanus*, (c) *K. lodderi* and *K. polysporus*, and (d) *K. phaseolosporus* and *K. wickerhamii* are not supported by the immunological data or by DNA/DNA reassociation experiments (7); they appear to be the results of either evolutionary parallelism or, more likely, convergence.

(iv) The intermediate immunological distances found between the exo- β -glucanases from *K. phaseolosporus*, on the one hand, and those from *K. delphensis*, *K. lactis*, *K. vanudenii*, and *K. drosophilarum*, on the other hand, are indications that these yeasts share a relatively recent common ancestry.

K. lactis and *K. vanudenii*, which were shown to share 97% (relative values) of their DNA base sequences (8), do not significantly differ in their respective immunological distances from *K. phaseolosporus* (Table 4). These two organisms could therefore be considered as representatives of the same species. *K. drosophilarum*, the most distant from *K. phaseolosporus* among the strains of intermediate status (Table 4), showed 70% relative DNA reassociation with the latter (8).

The relatedness determined between *K. delphensis* and *K. phaseolosporus* was unexpected, in view of their relatively low degree of phenotypic similarity (14, 19) and low inferred DNA complementarity (H. Presley and H. J. Phaff, unpublished data).

(v) Four nomenspecies (*K. bulgaricus*, *K. cicerisporus*, *K. wikenii*, and *K. marxianus*) were found to have exo- β -glucanases identical in primary structure to that of *K. fragilis*. Martini (8) demonstrated a very high degree of DNA com-

plementarity among the first three species, and Bicknell and Douglas (2) demonstrated the same between *K. fragilis* and *K. marxianus*. This suggests that these five yeasts are representatives of the same species.

A biological definition of yeast species will be possible in the future, when enough knowledge is acquired about genetic recombination among natural yeast populations. This will necessitate a thorough study of gene distribution for naturally occurring genetic polymorphisms over geographic gradients. Some progress in this area of research has been made for natural populations of *Pichia heedii* (13) and *P. amethionina* (16). In each of these yeasts genetic polymorphism was demonstrated over geographic gradients, and the physiological strains were capable of interbreeding.

Awaiting further knowledge, yeast taxonomists are still faced with the necessity of defining groups of yeast strains as "species," biological or not. Macromolecular comparisons provide taxonomists with an excellent tool for temporarily accomplishing this task. The classification of yeasts that have highly similar macromolecular sequences and that have undergone rapid divergence in some phenotypic properties (allowing occupation of new niches) remains a difficult problem, to be solved only by the judicious application of subjective judgments by experienced taxonomists.

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REPRINT REQUESTS

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