IMMUNOLOGY OF THE YEAST *HANSENULA WINGEI*

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ABSTRACT

BROOKBANK, JOHN W. (University of Florida, Gainesville) AND MARY R. HEISLER. Immunology of the yeast *Hansenula wingei*. J. Bacteriol. **85**:509-515. 1963.—Antisera produced in three groups of rabbits (18 animals in all) against mating types of *Hansenula wingei*, using three different modes of injection, failed to show specificity for mating type. However, the antisera were reactive with material obtained from the cells (presumably from the cell wall) by extraction at 100 C. A constituent of this fraction of boiled cells is in some way involved in the autoagglutination of unboiled cells upon the disruption of these cells in a Mickle disintegrator. The antisera are of broad specificity regarding their ability to agglutinate cells of other species of *Hansenula*, and have been shown to react with a minimum of one antigen found in supernatants of both *H. anomala* and *H. saturnus* boiled cells in double agar diffusion tests. *H. wingei* supernatants (boiled cells), in reaction with homologous antisera, show additional components not shown by *H. anomala* or *H. saturnus*.

Gametes of many animals and plants have been thought to combine, in a species-specific manner, through the interaction of macromolecules located at the cell surface; it has been suggested (see reviews by Tyler, 1948; Metz, 1957) that this reaction is analogous to the reaction between antibody and homologous antigen. Recently, Wickerham (1956) described a species of yeast of the genus *Hansenula* (*H. wingei*) in which the mating reaction was exceptionally marked, resulting in a hydrophobic mass of cells of opposite mating types (strains 5 and 21). This combination of cells ultimately results in the formation of a relatively stable diploid strain (D-10) which will not agglutinate with either haploid strain, or with itself. The nature of the reactants of the cell walls has been extensively explored by Brock (1958*a*, *b*; 1959), who characterized the mating substance of strain 21 as a protein, and that of strain 5 as a carbohydrate. Since combination of 5 and 21 cells is reversed by treatment with 8 M urea, Brock (1959) suggested that the cells are bound together through hydrogen bonds between specific protein and carbohydrate molecules, and, further, that the reaction is similiar to the reaction between antibody and homologous antigen. It has so far not proved possible to isolate the active fractions from the yeast cells.

The present study initially involved an attempt to produce, in rabbits, antibodies directed specifically against the mating substances of this yeast. This approach was utilized previously (Tyler, 1945) in an analysis of sperm attachment in echinoderm fertilization. In this instance, antisera against sea urchin sperm were produced in rabbits. The antisera were then photo-oxidized to render them "univalent" or nonagglutinating, and in this form they were able to block the fertilizing capacity of the sperm. Similiar results were obtained by Metz and Schuel (1961) and Shivers and Metz (1962), using antisera against sea urchin sperm, and against frog-egg jelly. The protease, papain, was employed to render these antisera nonagglutinating.

Investigations were carried out using antisera in conjunction with various cell fractions, the aim being to discover the portions(s) of the cells with which the antisera were reactive. Particular attention was paid to the supernatants of boiled cells, and to autoagglutinated cell walls prepared by disruption of the cells in a Mickle disintegrator. Cross reactions of the antisera with other species of the genus *Hansenula* have also been included.

MATERIALS AND METHODS

Preparation of antisera. Three methods of immunization were employed, using three groups of New Zealand albino rabbits weighing 2.5 to 4 kg.

Group 1. After a preinjection bleeding to provide normal sera, four rabbits were injected intravenously and subcutaneously with 0.5 ml per injection of fresh strain 5 or 21 cells at a concentration of 10% in 0.5% NaCl. Injections were made on alternate days for a total of ten injections. Intravenous injections were alternated with subcutaneous injections; 5 days after the final injection, the animals were bled by cardiac puncture, and the antisera recovered by conventional techniques. None of the sera in any group was heated to inactivate complement.

Group 2. The cells were diluted to a concentration of 10^6 per ml in 0.5% NaCl; 1.5 ml of this suspension were emulsified with 1.5 ml of Arlacel A (Atlas Powder Co., Wilmington, Del.), 1.5 ml of hexadecane (Eastman), and 0.1 g of lyophilized *Mycobacterium*. Each rabbit received an initial injection of 2 ml of this emulsion subscapularly, after a preinjection bleeding. A total of six rabbits were injected, three with strain 5 and three with strain 21; 5 weeks after the injection of cells and adjuvant, the animals were given an intravenous challenge of 1 ml of a 10% suspension of cells in saline. After 1 week, the animals were bled by heart puncture for immune serum.

Group 3. A total of eight rabbits were injected with heated (100 C for 5 min) cells (10% suspension in 0.5% saline) over a period of 2 months. The cells were not washed after heating. A total of 12 injections were given, using the following routes of injection: marginal vein, footpad, peritoneal cavity, and skin of the back (subcutaneous). The final challenge was administered intravenously 10 days before the final bleeding. Preinjection control sera were obtained as in the other groups.

The two strains of *H. wingei* (5 and 21) were supplied by Thomas Brock of Indiana University, Department of Biology. Other *Hansenula* species were obtained from Warren Silver of the University of Florida, Department of Bacteriology.

The titer of the sera was estimated by serial dilution. The dilution to be tested was mixed with an equal volume of cells (optical density of 1.0 at 500 mμ) and centrifuged at 1,000 $\times$ *g* for 5 min. The cells were then resuspended by shaking, and the tubes examined against a dark background (a Steiger-Simpson slit-lamp, Clay Adams, N.Y., was used) for clumping of the cells. The highest dilution giving macroscopic agglutination was recorded. Absorptions of the sera were done by adding an equal volume of serum to packed cells (saline medium removed) in a calibrated centrifuge tube, and allowing the reaction to proceed for 10 min, with occasional stirring, before recovering the serum by centrifugation. Absorptions were assumed to be complete when additional absorbing cells failed to reduce the titer of the serum, or when the undiluted absorbed serum failed to agglutinate the cells employed for absorption.

Water-insoluble papain was kindly provided by John Cebra of the Department of Microbiology (Cebra et al., 1961). After precipitation of the globulin fraction (group 1 sera were used) with 40% saturated $(NH_4)_2SO_4$, the precipitates were redissolved in a volume of 0.5% NaCl equivalent to the original serum volume. These globulin solutions were dialyzed against 0.01 M ethylenediaminetetraacetic acid (EDTA, Eastman) at pH 8.6 until free from sulfate, as indicated by the absence of a precipitate in the presence of $BaCl_2$. The globulin solutions were then placed in reaction with insoluble papain (1:5 ratio of insoluble papain suspension to globulin) at room temperature for 1 hr. The globulin solutions were recovered by filtration through Whatman no. 1 paper and mixed with an equal volume of 0.1 M sodium thioglycolate in 0.01 M EDTA (pH 8.6). Incubation with thioglycolate was allowed to proceed for 2 hr at 37 C. The sera were then dialyzed to remove the thioglycolate. Tests were performed to ascertain the extent to which globulin preparations so treated were capable of agglutinating *Hansenula* cells (1 part cells to 4 parts globulin; v/v). In addition, untreated antisera were added to cells treated with papain-split globulin, to determine whether the treated preparations were able to inhibit the agglutination of homologous cells by unsplit antisera. The ability of cells specifically coated with globulin fragments to combine with globulin-treated and globulin-untreated cells of opposite mating type was also observed by mixing the different cells together in equal proportions, centrifuging, and checking for agglutination as in the serum-titer procedure.

Double-diffusion plates were prepared by first siliconing the bottom of a disposable petri

TABLE 1. *Results of homologous and heterologous absorption on the titer of two group 3 antisera*

Serum	Versus strain	Titer	Absorbed with	Test on	Absorbed with	Test on	Absorbed with	Test on
I immune	5	1:128	5	5−	21	5−	D-10	5−
				21−		21−		21−
I normal		0	5	5−	21	5−	D-10	5−
				21−		21−		21−
M immune	21	1:256	5	5−	21	5−	D-10	5−
				21−		21−		21−
M normal		0	5	5−	21	5−	D-10	5−
				21−		21−		21−

TABLE 2. *Effect of treatment with "split" group 1 globulin on mating agglutination**

Cell combination	Mating agglutination	Sera tested†
5p + 21c	+	Anti-5
5c + 21p	+	Anti-21
5p + 21p	+	Anti-5 and anti-21
5c + 21c	+	—

* Two normal untreated sera and two normal papain-treated sera were without effect on mating agglutination. Explanation of symbols used: p = papain-treated globulin added to cells; c = cells in saline without globulin treatment; + = strong mating reaction, virtually all cells involved.

† Two samples of each serum were used in the mating agglutination experiments.

dish to prevent seepage of reactants under the agar. A 0.5% solution of Ionagar no. 2 (Consolidated Laboratories, Chicago Heights, Ill.) was poured over the silicone to a depth of 0.5 cm. A die was used to cut six wells 1 cm in diameter and 1 cm apart, as well as a 1-cm center well equidistant from all others. The wells were filled once with the serum or solution to be tested, and were not refilled. Plates were read daily and accurately sketched or photographed. Ring precipitin tests were performed in the usual way, i.e., layering of antigen over antiserum in 2-mm tubing and observing the interface against a dark background with oblique light.

Fractionation of the cells for use in precipitin and double-diffusion experiments employed the following procedures. Cells were washed free from culture medium (agar, glucose, yeast extract, phosphate) with saline. They were then placed in Mickle disintegrator vessels (Aloe Scientific Division, St. Louis, Mo.) with Ballotini beads and shaken at maximal amplitude and speed for 30 min. After centrifugation, the supernatant was arbitrarily designated "soluble internal material" (SIM). The autoagglutinated cell-wall precipitate was washed four times and heated to 100 C for 5 min. The supernatant was then referred to as the "cell-wall extract" (CWE). The remaining cell walls (W) were then washed four times and used as absorbing antigens. In addition, whole cells (*H. wingei*, *H. anomala*, and *H. saturnus*) were boiled (usually at 100 C for 5 min) prior to Mickle disintegration. The supernatant of such boiled cells is referred to as "boiled-cell supernatant" (BCS).

RESULTS

Titer. The titer of the antisera in groups 1 and 2 ranged between 1:8 and 1:64. In group 3, the titers ranged somewhat higher (1:64 to 1:256). It can be stated, from the following lines of evidence, that none of the antisera contained antibody directed against the mating substance of either strain. (i) Absorptions (Table 1): strain D-10 (diploid) cells were capable of absorbing all agglutinins present in antisera directed against strain 5 or 21. Since D-10 cells possess no surface mating substances, it appears that antibody against these materials is not detectable in antisera against strains 5 and 21 by this procedure. (ii) Papain experiments (Table 2): globulin fractions of antisera of group 1 were treated with insoluble papain and thioglycolate to the point where the globulin fractions were

TABLE 3. *Reaction of group 2 antisera with heterologous species of Hansenula**

Serum	Versus strain	Absorbed with	Test on
a Immune	21	21	21−
		21	5−
		Ha9†	21−
		Ha9	Ha319†−
		Ha9	Ha9−
		Ha319†	21±
		Ha319	Ha9−
		Ha319	Ha319−
a Normal		21	Neg‡
		Ha9	Neg
		Ha319	Neg
c Immune	5	5	5−
		5	21−
		Ha9	5−
		Ha9	Ha319−
		Ha9	Ha9−
		Ha319	5−
		Ha319	Ha9−
		Ha319	Ha319−
c Normal		5	Neg
		Ha9	Neg
		Ha319	Neg

* All unabsorbed sera were strongly positive at 0 dilution.

† Explanation of symbols used: Ha9 = *Hansenula anomala* strain 9 cells; Ha319 = *Hansenula anomala* strain 319 cells.

‡ All negative.

rendered nonagglutinating. Cells (5 or 21) coated with these nonagglutinating preparations, and thereby unable to agglutinate in the presence of whole, undigested antisera, were capable of combining with globulin-treated or -untreated cells of opposite mating type, indicating that mating substances were not involved in combination with antibody fragments.

Since a total of 18 rabbits were utilized, each contributing normal and immune sera, a table containing all absorptions, titers, etc., would grow cumbersome. Table 1 therefore includes pertinent data from two rabbits of group 3. All other antisera gave essentially the same pattern, save for the variations in titer mentioned previously. Antisera against strains 5 and 21 could not be distinguished from one another by any means so far employed. Control sera were

TABLE 4. *Ring-precipitation reactions of two group 3 antisera tested against various cell fractions**

Fraction	Anti-serum	Versus strain	Reaction
SIM (5)	L	21	− (± undiluted)
SIM (21)	L	21	− (± undiluted)
SIM (5)	I	5	− (− undiluted)
SIM (21)	I	5	− (− undiluted)
CWE (5)	L	21	− (± undiluted)
CWE (21)	L	21	− (± undiluted)
CWE (5)	I	5	− (± undiluted)
CWE (21)	I	5	− (± undiluted)
BCS (5)†	L	21	++
BCS (21)	L	21	++
BCS (5)	I	5	++
BCS (21)	I	5	++

* Corresponding normal sera were negative. All fractions were tested at a dilution of 1:32 and a titer of 1:256. Abbreviations: SIM, soluble internal material; CWE, cell-wall extract; W, cell walls; BCS, boiled-cell supernatant.

† Absorption (three times) of above antisera with W fraction of strains 5 or 21 failed to remove agglutinins for homologous whole cells.

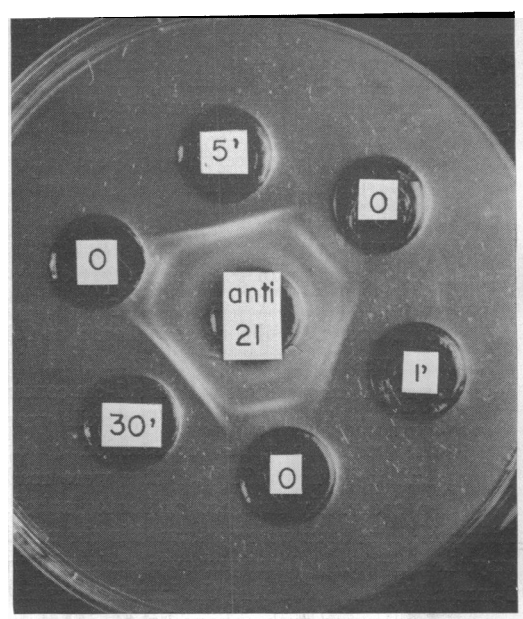

FIG. 1. *Antiserum vs. strain 21 (M) against unboiled cell supernatant, and BCS of strain 21, boiled for 1, 5, and 30 min. Incubated for 1 week at 25 C.*

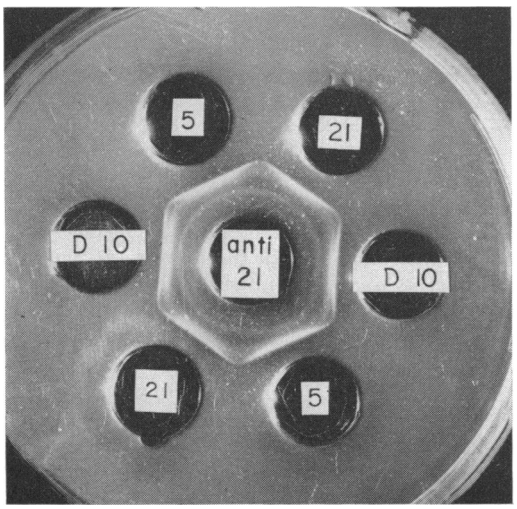

FIG. 2. *Antiserum vs. strain 21 (M) against BCS of 5, 21, and D-10 (in duplicate) to show identity of the bands derived from each mating type and the diploid. Incubation for 1 week at 25 C.*

without effect on cells of any of the strains of *H. wingei*, and were also negative when tested on other species of *Hansenula*.

Heterologous reactions. The reaction of two antisera of group 2 with *H. anomala* (9 and 319) is shown in Table 3.

The reactions of two of the highest titer group 3 antisera with various fractions of cells of strains 5 and 21 are indicated in Table 4.

Double-diffusion tests were performed on all sera of groups 1 and 3. Typical results are shown in Fig. 1 and 2. One of the lower titer group 1 antisera failed to produce any precipitation pattern. This could conceivably have been due to dissolution of any precipitate by antigen excess. Generally speaking, a single, heavy band appeared after 24 to 48 hr (band 1), followed after another 24 hr by the appearance of a lighter band inside the first band (band 2). After a week, a circular band(s) sometimes appeared around the center well. Though its appearance was not consistent, it could conceivably be regarded as another antigen(s) shared by all yeasts tested, though the lack of a hexagonal pattern speaks more for a precipitation artifact. Thorough washing of cells before boiling would tend to rule out contamination by cross-reacting antigens of the yeast extract in the culture medium.

DISCUSSION

From the data above, it seems clear that none of the antisera is strain-specific. The presence of antibodies directed against the mating substances (but not the active sites of these molecules) cannot be completely excluded. Failure to obtain specific antibodies for mating substances is not without precedent. Metz (1954) described a similar attempt to produce antisera specific for mating types of *Paramecium aurelia*, with negative results. The mating substances of this ciliate are also insoluble in all reagents so far employed to remove them from the cilia. Insolubility, or indigestibility, of mating substances may be of considerable importance when considering the apparent inability of these substances to induce antibody formation in rabbits (Campbell and Cushing, 1957).

With regard to the extensive cross specificity observed between the different species of the genus *Hansenula*, the results of Benham (1931) seem pertinent. In this study, it was shown that *H. anomala* shared certain combining groups with certain species of *Candida* and *Saccharomyces*. It has also been shown (Wickerham and Burton, 1954) that *C. pelliculosa* is in fact the imperfect stage of *H. anomala*. In short, it seems that most of the yeasts (*Hansenula*, *Candida*, *Saccharomyces*) which have been serologically compared show differences by which they may be distinguished, as well as a sharing of some cellular thermostable antigenic characteristics (see Tsuchiya et al., 1957*a*, *b*; Beutmann, 1958, for additional information and references). Regarding the double-diffusion plates, it can be stated that band 2, which can be shown to appear with or without boiling the (*H. wingei*) cells, is shared by *H. anomala*, *H. saturnus*, and all strains of *H. wingei* considered here (Fig. 1). The heavier band (band 1) which appears in BCS of *H. wingei* does not appear to correspond to any substances released from *H. anomala* or *H. saturnus* upon boiling. It may be that the corresponding material from these last-mentioned yeasts is quantitatively less soluble, or perhaps unstable, at 100 C, if it is not absent altogether. That a similiar material may be present on the unheated cells of heterologous species is indicated by the ability of these cells to absorb completely antisera directed against *H. wingei*. In this connection, it should be noted that had Tsuchiya et al. (1957*b*) tested

the supernatants of their boiled-cell antigens, further (intra- and interspecific) distinctions between *Candida* and *Hansenula genera* might have been encountered. In addition, it was found that absorption of the antisera with boiled or unboiled *H. wingei* cells abolished all precipitin lines in double-diffusion tests, indicating that boiling had not released all antigenic materials from the cell surface, though, apparently, sufficient material was removed to prevent autoagglutination on disruption of the cells (see below). The identity of the two bands formed by BCS of strains 5, 21, and D-10 is shown in Fig. 2.

It appears that the antigen responsible for band 1 in the double-diffusion tests may be the antigen responsible for autoagglutination of cell walls after disruption of the cells. Heated cells, after extensive washing, do not autoagglutinate on disruption, and the BCS show both bands 1 and 2 (Fig. 1). Unheated-cell supernatants fail to give band 1 in agar diffusion experiments (Fig. 1), and these cells autoagglutinate on disruption. Further, it was shown that autoagglutinated cell walls were not able to absorb antisera (group 3) against whole heated cells, indicating that the antigens responsible for cell agglutination in the presence of antibody were most probably involved in the autoagglutination of the cell walls, and thus were not available as absorbing antigens. It can therefore be tentatively stated, both from serological evidence, and from the fact that heating actually enhances the mating reaction (Brock, 1958), that heating removes the major portion of the autoagglutinins, and that the material so released is not related to the mating reaction.

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LITERATURE CITED

BENHAM, R. W. 1931. Certain Monilias parasitic on man. Their identification by morphology and by agglutination. J. Infect. Diseases **49**:183–215.

BEUTMANN, W. 1958. Immunbiologische und Chemische Untersuchungen an Hefen. Arch. Mikrobiol. **29**:227–256.

BROCK, T. D. 1958*a*. Mating reaction in the yeast *Hansenula wingei*. Preliminary observations and quantitation. J. Bacteriol. **75**:697–701.

BROCK, T. D. 1958*b*. Protein as a specific cell surface component in the mating reaction of *Hansenula wingei*. J. Bacteriol. **76**:334–335.

BROCK, T. D. 1959. Mating reaction in *Hansenula wingei*. Relation of cell surface properties to agglutination. J. Bacteriol. **78**:59–68.

CEBRA, J. J., D. GIVOL, H. I. SILMAN, AND E. KATCHALSKI. 1961. A two stage cleavage of rabbit γ-globulin by a water-insoluble papain preparation followed by cysteine. J. Biol. Chem. **236**:1720–1725.

CUSHING, J. E., AND D. H. CAMPBELL. 1957. Principles of immunology. McGraw-Hill Book Co., Inc., New York.

METZ, C. W. 1954. Mating substances and the physiology of fertilization in ciliates, p. 284–334. *In* D. H. Wenrich [ed.], Sex in microorganisms. American Association for the Advancement of Science, Washington, D.C.

METZ, C. W. 1957. Specific egg and sperm substances and activation of the egg, p. 23–69. *In* A. Tyler, C. W. Metz, and R. C. von Borstel [ed.], The beginnings of embryonic development. American Association for the Advancement of Science, Washington, D.C.

METZ, C. W., AND H. SCHUEL. 1961. Inhibition of agglutinability and fertilizing capacity of sea urchin sperm by papain-digested antibody. Am. Zool. **1**:463.

SHIVERS, C. A., AND C. B. METZ. 1962. Inhibition of fertilization in frog eggs by univalent fragments of rabbit antibody. Proc. Soc. Exptl. Biol. Med. **110**:385–387.

TSUCHIYA, T., Y. FUKAZAWA, S. HAYASHI, J. HAYASHI, AND M. DOI. 1957*a*. Serological relationships among ascosporogenous and asporogenous yeasts. I. *Saccharomyces cerevisiae* and *Candida robusta*. Japan. J. Microbiol. **1**:125–131.

TSUCHIYA, T., Y. FUKAZAWA, J. HAYASHI, Y. NISHIKAWA, AND M. DOI. 1957*b*. Serological classification of the genus Hansenula. Japan. J. Microbiol. **1**:339–346.

TYLER, A. 1945. Conversion of agglutinins and precipitins into "univalent" (non-agglutinating or non-precipitating) antibodies by

photodynamic irradiation of rabbit-antisera vs. pneumococci, sheep-red-cells and sea-urchin sperm. J. Immunol. **51**:157–172.

TYLER, A. 1948. Fertilization and immunity. Physiol. Rev. **28**:180–219.

WICKERHAM, L. J. 1956. Influence of agglutination on zygote formation in *Hansenula wingei*, a new species of yeast. Compt. Rend. Trav. Lab. Carlsberg Ser. Physiol. **26**:423–443.

WICKERHAM, L. J., AND K. A. BURTON. 1954. A simple technique for obtaining mating types in heterothallic diploid yeasts, with special reference to their uses in the genus Hansenula. J. Bacteriol. **67**:303–308.