MATING REACTION IN THE YEAST *HANSENULA WINGEI*

PRELIMINARY OBSERVATIONS AND QUANTITATION

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Wickerham (1956) has described a new species, *Hansenula wingei*, which exhibits a rather unusual mating reaction. When vegetative haploid cells of the two mating types are mixed, a strong agglutination reaction may occur. On agar, the cells set to a solid mass which resists further mixing and adheres tenaciously to the instrument used for mixing the cells. When the mass of cells is placed in water, they do not disperse but remain tightly clumped. After fusion of haploids has occurred, the agglutinability disappears and the diploids show no agglutination. This process markedly increases the frequency of zygote formation in this yeast, even though sporulation occurs only weakly in most diploids.

Since agglutination is usually considered to be a surface reaction (Mudd, 1933), and since cells with such a tremendous attraction for one another should exhibit some large differences in surface components, a detailed study of the mechanism of this reaction was undertaken. The present paper outlines a method for quantitating the reaction and indicates the effects of certain factors on agglutination. Future papers will attempt to define the differences in surface properties which are responsible for the attraction.

MATERIALS AND METHODS

Organisms. The yeasts used in this study were supplied by Dr. L. J. Wickerham. *H. wingei* NRRL Y-2340, strains 5 and 21, are agglutinative mating types, and most of the work was done with these. A diploid hybrid was isolated following procedures of Wickerham (1956) and this was labeled *H. wingei* strain D10. *Hansenula subpelliculosa* NRRL Y-1683 was also used.

Growth of cells. Stock cultures were maintained on a medium of the following composition: glucose, 30 g; yeast extract, 7 g; KH_2PO_4, 5 g; agar, 20 g; in 1000 ml distilled water. Cells for experimental purposes were grown on large trays or in screw cap bottles on this same medium, or in shallow layers of liquid medium without agar. The cells were washed from the agar surface with distilled water, filtered through cheesecloth to remove lumps of agar, and then washed two or three times in distilled water. Cells were harvested from liquid medium by centrifugation, and then washed. Once harvested, cells were stable in the refrigerator for several weeks with no diminution in the mating reaction, provided they were tested in the appropriate manner (as later described).

Qualitative assay for agglutinability. It became obvious fairly soon that agglutination occurred strongly only when the two strains were mixed at fairly high cell concentrations. Loopfuls of cells from agar agglutinated strongly when mixed, but when liquid cultures were used, in which the cell concentration was much lower, agglutination was weak or absent. It was possible to use cells concentrated from liquid media and good agglutination would occur upon mixing on a glass slide. For preliminary testing, this procedure was used. A drop of cells of approximately 10^{10} cells per ml of each strain was placed on a slide. Agglutination usually occurred within 5 sec after vigorous mixing. The effect of a number of substances on agglutination was studied qualitatively by adding a drop of solution of the test substance to the slide before adding the cells.

Quantitative assay for agglutinability. Since high concentrations of cells were necessary for agglutination, it seemed reasonable that it might be merely that the cells of one strain had to be in close proximity to the cells of the other strain, before agglutination could occur. To test this, dilute suspensions of cells of both strains were mixed in tubes. No agglutination occurred. The tubes were then centrifuged at 2000 rpm for 5 min, at which time all of the cells had been deposited on the bottom of the tube. When the sediment was broken up with a spatula, the cells were found to be tightly agglutinated and did not resuspend well in the liquid. They quickly settled out.

Upon this observation was based a quantitative assay to determine the effect of various substances and treatments on agglutination. Test tubes were selected that could be placed in a colorimeter. Solutions of test substances or water were placed in tubes and then suspensions of strains 5 and 21 were added. The suspensions were mixed well and centrifuged at 2000 rpm for 5 min. With a small spatula the sediment was scraped off the bottom of the tube and dispersed in the liquid. The tubes were allowed to stand at room temperature for 5 min. If the cells had agglutinated during centrifuging, practically all of the cells would settle out during the period of standing and the supernatant would be clear. With partial or no agglutination, considerable turbidity would remain. The turbidities were then read in a Klett-Summerson colorimeter. In control tubes containing only one cell strain, no reduction in turbidity would occur in times much longer than the 5 min allowed. Results can then be expressed in per cent reduction in turbidity compared to the control. This method has certain errors that are apparently inherent in an assay based on rate of fall of particles (Jansen and Mendlik, 1951), but seems to be reasonably acceptable. It has the advantages of simplicity and speed and does not require large concentrations of cells. In many cases, it was not necessary to read the turbidity, since the differences were quite large and simple visual estimates could be made. This was done in a number of cases using small tubes (10 by 75 mm) with 0.5 ml of each cell type to conserve cells. In this test, the concentration of cells was not critical, so long as equal numbers of each strain were used.

RESULTS

Effect of proportion of cells. When varying amounts of broth suspensions of cells were mixed, an optimum degree of agglutination occurred when the cells of the two strains were present in equal proportions (figure 1). This, of course, would be expected if the cells of the one type combine only with cells of the opposite type. By staining one strain with Congo red and then mixing with unstained cells of the other strain, the clumps of agglutinated cells could be examined microscopically. In all cases, approximately equal numbers of cells of each type were present in the clump, and each stained cell seemed to be adjacent to unstained cells only.

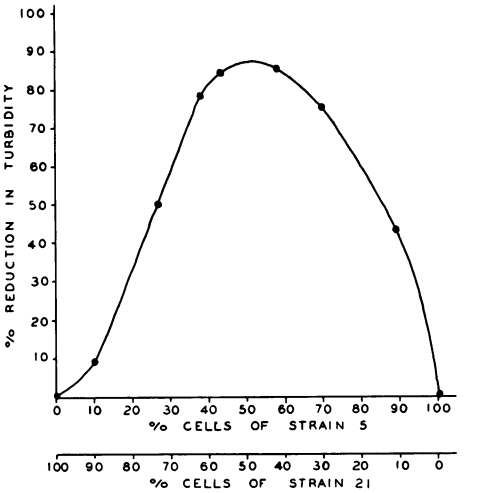

Figure 1. Quantitative assay of agglutination of broth-grown cells, when mixed in various proportions. Higher percentage reduction in turbidity means greater degree of agglutination.

Specificity of the reaction. Cells of either type do not agglutinate with the diploid hybrid D10. Nor do they agglutinate with another species, *H. subpelliculosa.* Thus the reaction seems to be highly specific. Wickerham (1956) has studied this specificity in some detail using qualitative techniques.

Microscopic observation. When the cells agglutinate, there is obviously a strong attraction between them, since the cell wall actually becomes deformed. In figure 2 a wet mount of an agglutinated mass is shown. The cells come together in compact groups which show the complete absence of intercellular space, and probably become 14-sided, since this is the shape usually assumed by spheres when compressed so that no intercellular space remains (Matzke, 1939). This deformation of the cell wall indicates how strong the attraction between the two types is. When the cells fuse, they resume the oval shape typical of this species.

Cofactors necessary for agglutination. It became obvious immediately that twice washed cells of the two strains do not agglutinate when mixed, even when present in high concentrations. When washed cells were tested with the centrifuge technique, the cells resuspended completely and showed no signs of agglutinability. However, this agglutinability could readily be restored by adding small amounts of cations or proteins like gelatin and peptone. At concentrations of these factors which had no effect on the suspension

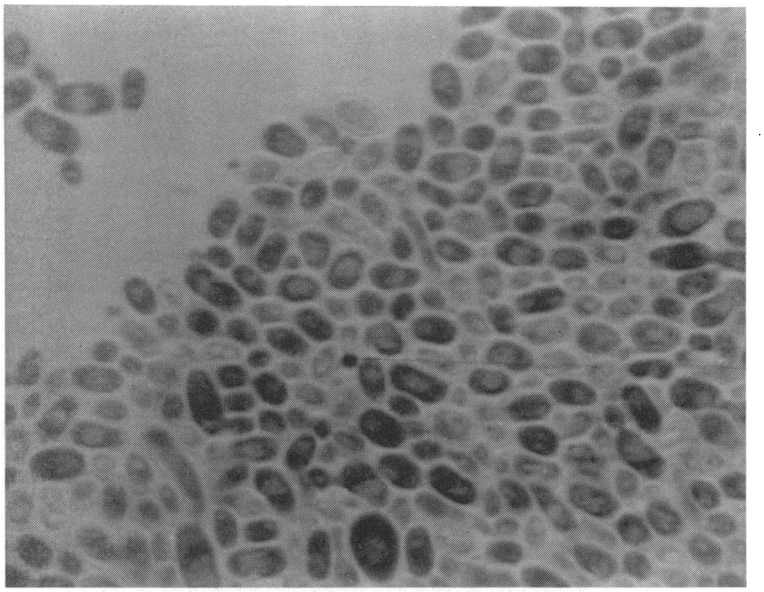

Figure 2. Light photomicrograph of wet mount of heated methylene blue stained cells from strongly agglutinating mass, 1500 ×.

stability of the individual strains, the mating reaction was strongly activated. Table 1 shows the effects of some of these factors on agglutination. The relative effects of the various cations do not follow the Hofmeister series.

The chelating agent disodium ethylenediaminetetraacetic acid at concentrations as high as 0.07 M had no effect on magnesium or calcium induced agglutination. At this concentration of disodium ethylenediaminetetraacetic acid, all cation would be expected to be bound (Puck and Tolmach, 1954). Of course, disodium ethylenediaminetetraacetic acid would not be expected to chelate gelatin or protamine.

TABLE 1

Effect of added cofactors on the agglutination of mixed cells of strains 5 and 21. Value is minimum concentration of substance necessary to give agglutination equivalent to unwashed cells

Substance	Molarity
NaCl	8×10^{-3}
$CaCl_2$	3×10^{-4}
$FeCl_3$	3.6×10^{-5}
$MgSO_4$	5×10^{-6}
Protamine sulfate*	2.3×10^{-5}
Gelatin†	6×10^{-5}

* Assuming a molecular weight of 7,000.

† Assuming a molecular weight of 40,000.

Mating reaction in heated cells. When washed cells of the two strains were heated at 100 C for 5 min, they agglutinated strongly when mixed, even in the absence of added cations. This observation indicates the heat stability of the structures necessary for agglutination. When these heated cells were washed again, they lost the power to agglutinate. When these washed/heated/washed cells were tested in the presence of added cations, they again agglutinated strongly. This seems to indicate that the initial heating destroyed the permeability of the cell membrane and allowed ions and proteins to leak out, and these substances could then function like added cofactors.

More surprising, however, when these washed/heated/washed cells were again heated for 5 min at 100 C, they again agglutinated without added cations, and the reaction was even more intense than any previously observed reaction. Further, no amount of washing would prevent them from agglutinating strongly. Apparently the second heating caused some irreversible changes, possibly by destroying some surface component that was partly masking the mating reaction component. If washed cells were initially heated for longer periods of time (20 min), they agglutinated strongly even after extensive washing.

Effect of dielectric constant on the reaction. If the attraction between the two mating types

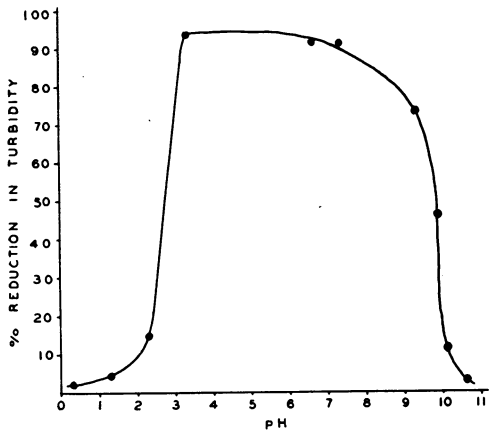

Figure 3. Effect of pH on agglutination of heated cells in the absence of added cofactors. Higher percentage reduction in turbidity means greater degree of agglutination.

were due to an attraction of positive and negative charges, one might expect an increase in degree of reaction in solutions having a lower dielectric constant than water (Laidler and Ethier, 1953). Tests were run in solutions containing various concentrations of acetone, ethanol, and dioxane. In all cases a decrease or complete inhibition of agglutination occurred at concentrations of solvent between 30 and 40 per cent. No irreversible effects of the solvent occurred, since the cells could be recovered and were fully agglutinable in aqueous solutions. In fact, heated cells extracted with excess solvent were strongly agglutinable when resuspended in water. Klotz (1957) has considered the case of attraction between two molecular species of like charge (protein and organic anion) and has shown that in this situation, if the dielectric constant is decreased, there should be an increase in the repulsive factor and a decrease in attraction. However, as he points out, in the use of solvents like dioxane to decrease the dielectric constant, one must also consider the fact that these substances also compete with the negative ions for attraction to each other, so that two factors occur simultaneously to decrease the attraction. In the present case, it can only be stated that the solvents decrease the extent of agglutination, while the reason for this must remain obscure. However, it would seem possible that the attraction is not due to the bulk charge of one strain being opposite to that of the other.

Effect of pH. Study of the effect of pH using the quantitative method yielded the results shown in figure 3. A broad pH optimum extending from pH 3 to 9 was obtained. The pH was controlled using HCl-KCl or NaOH-KCl mixtures in which the ionic strength was kept constant at 0.2 μ. The fact that low and high pH values do inhibit the reaction seems to indicate that the attraction is at least partly electrostatic. At pH 10.5 partial irreversible destruction of the agglutination components occurred.

DISCUSSION

These preliminary observations serve mainly to define the most fruitful areas for further work. The attraction between the mating types is obviously quite strong, since the cell walls are actually deformed in the process. However, the forces do not act over very large distances. A system has been developed in which the reaction can be studied quantitatively in the absence of any complex medium constituents.

The fact that the reaction is not diminished, and may be intensified by heating at temperatures which kill the cells, indicates that the structures responsible are quite stable. A study of the charge of the cell surfaces will be necessary to decide whether electrostatic attraction is involved. Modification or extraction of essential surface components may help to delineate the nature of the substance involved. It is possible that the degree of hydration of the cell surface is a factor in agglutination, since the cells in suspension exhibit the stability of highly hydrated particles, while the clump formed by agglutination is quite hydrophobic.

It is interesting to compare this system with that of phage adsorption in *Escherichia coli* (Puck *et al.*, 1951; Tolmach and Puck, 1952; Puck and Tolmach, 1954). Phage adsorption also requires divalent cations, is unaffected by disodium ethylenediaminetetraacetic acid, is an almost instantaneous reaction, and has a broad pH optimum. In this system an initial electrostatic reaction has been postulated involving amino and carboxyl groups, even though the bulk charge of both virus and cell are predominantly negative.

The centrifuge technique presented here may be useful wherever it is desirable to greatly increase the probability of contact of cells, either in a mating system or otherwise. Such a technique

might also be useful to increase the amount of gene recombination in bacterial systems.

SUMMARY

Haploid mating types of a new yeast, *Hansenula wingei*, exhibit an interesting and unique mating reaction. When mixed under appropriate conditions, a strong agglutination occurs, which results in a tight mass of cells in which the cell walls are actually deformed. Although the attraction is quite strong, it does not act over large distances. A centrifuge technique is described to establish a quantitative reaction, which may have more general use to increase the probability of contact between microbial cells. Washed cells do not react when mixed unless cations or proteins are present. Heated cells react when mixed without added factors, and there are apparently two separate heat induced reactions. In the first, permeability is destroyed and cations and proteins leak out and are available for inducing the reaction. In the second heat reaction, some irreversible change occurs and mating occurs without any added factors. Disodium ethylenediaminetetraacetate at concentrations which would chelate all magnesium or calcium present does not inhibit the reaction. A broad pH optimum occurs, from pH 3 to 9. Further work will be necessary to determine if a purely electrostatic attraction is involved.

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