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Improved Assay For Surface Hydrophobic Avidity of *Candida albicans* Cells

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A simple method that distinguishes among hydrophobic avidity levels of highly hydrophobic isolates of the pathogenic fungus *Candida albicans* is described. This method involves mixing polystyrene microspheres at different concentrations with a constant concentration of yeast cells and plotting the data in accordance with the Langmuir isotherm equation. A 10-fold difference between the *C. albicans* isolates with the lowest and highest avidity (K_H) values was found. This method may also demonstrate that surface hydrophobic sites with different avidities are present within a yeast cell population.

Expression of cell surface hydrophobicity (CSH) plays an important role in pathogenesis of various fungi, especially *Candida albicans* and *Aspergillus* species (K. C. Hazen, unpublished data). Recent studies of *C. albicans* have shown that CSH is involved in the adherence of yeast cells to human epithelial cells and plastics, is associated with yeast-to-hypha transition, and may contribute to yeast cell avoidance of polymorphonuclear neutrophil killing mechanisms (2, 6–8). For a definitive analysis of how surface hydrophobicity participates in these pathogenic events, specific knowledge of the avidity and uniformity of cell wall hydrophobic sites within a yeast cell population would be beneficial.

Current methods to assess CSH in fungi (for a review, see reference 11) can distinguish among yeast strains that vary greatly in their hydrophobicities, that is, the percentages of cells that are hydrophobic. These methods, however, are incapable of detecting differences in the hydrophobic binding strengths of strains that appear highly hydrophobic (i.e., CSH levels greater than 75%). These differences could have interpretative significance when the role of CSH in fungal pathogenesis is investigated. One method, which measures the contact angle formed by a drop of sensing liquid on a layer of yeast cells (1), appears applicable but has several drawbacks. One problem is that the cell layer formed on a filter or glass slide must be uniform in depth to get a reliable contact angle, but uniformity may not be achievable with fungal cells. The cell layer must also be free of excess moisture before application of the sensing liquid, but drying may lead to alterations in surface characteristics. Development of a method that distinguishes among highly hydrophobic yeast strains in an aqueous environment is therefore needed.

We recently developed a CSH measurement assay that involves attachment of hydrophobic polystyrene microspheres to yeast cells (5, 9). The yeast cells are maintained in an aqueous environment throughout the procedure. This assay, which is designated the hydrophobic microsphere assay (HMA), is simple and gives easily quantifiable results. With appropriate modifications (described herein), the HMA may be used to assess differences in hydrophobic avidities among yeast cell populations that appear highly hydrophobic by standard CSH measurement techniques. The term avidity is used here in place of the term affinity, as the latter implies a property of individual molecules. Avidity implies that hydrophobic sites of yeast cell surfaces may result from an aggregate of molecules which act in concert to bind a polystyrene microsphere.

Our modifications of the HMA are based on enzyme-ligand binding theory, in which the Langmuir isotherm equation serves as a basis (12). The equation is as follows: $1/r = (K_a/[A] + 1)$, where r is the concentration of ligand molecules per standard concentration of macromolecules and $[A]$ is the concentration of bound ligands for a specific r. On the basis of these conditions, it is possible to determine the strength of binding or hydrophobic avidity (obtained through calculation of the association constant). If the hydrophobic avidity constant (herein designated K_H to distinguish it from the enzyme-ligand association constant K_a) is substituted for K_a in the Langmuir isotherm equation, then K_H can be obtained from the slope of a plot of $1/r$ versus $1/[A]$. To obtain yeast cell K_H, r is defined as the concentration of microspheres (ligand) and $[A]$ is defined as the percentage of cells (macromolecules) that have three or more attached microspheres. This assumes for convenience that a cellular hydrophobic site is made extant by the attachment of three or more microspheres to an entire cell. This assumption defines a hydrophobic site as the result of either the entire cell surface or specific areas of that surface. Therefore, hydrophobic sites of different avidities on a single cell cannot be unequivocally distinguished.

Application of the Langmuir isotherm requires that equilibrium be established after the microspheres are mixed with yeast cells (K_H is a form of an equilibrium association constant). Previous studies (9) have demonstrated that the number of microspheres which attach to yeast cells when mixed by the standard HMA procedure does not change with further mixing or static incubation. This indicates that free yeast cells and microspheres are in equilibrium with the yeast cell-microsphere complexes.

The modified microsphere assay, herein designated the hydrophobic microsphere avidity assay (HMAA), was conducted under the same conditions as the standard HMA except that yeast cells were exposed to various microsphere concentrations. The standard HMA is described in detail elsewhere (4, 5, 7, 9). Yeast cells were grown at 23°C to

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TABLE 1. [A] and K_H of six isolates of *C. albicans* exposed to various concentrations of microspheres

Isolate	[A][a] at microsphere concn (per ml)				K_H[b]
	4.5×10^8	2.25×10^8	9.0×10^7	4.5×10^7	
LGH1095	91.2	80.1	50.7	24.4	6×10^{-7}
CK4	92.4	87.0	45.9	11.5	1×10^{-7}
LGH870	98.7	93.4	62.8	31.5	8×10^{-7}
9938	78.1	44.7	9.2	0	9.2×10^{-8}
UMC9385	81.7	50.4	15.6	0	1×10^{-7}
LGH86	94.6	68.8	35.4	0	5×10^{-7}

[a] Percentage of yeast cells (200 cells per sample) with three or more attached microspheres. The yeast cell concentration was kept constant (10^6/ml) for all isolates and microsphere concentrations tested. Results for each *C. albicans* isolate represent the means from at least three experiments, each having triplicate samples. Standard deviations for all cases were less than 6%. At 2.25×10^7 microspheres per ml, [A] was 0 for all isolates.

[b] Avidity constant, calculated from the slope of the straight line resulting from the plot of 1/[A] versus 1/r for each isolate (see Fig. 1) and expressed as microspheres per site. The unit label ml is dropped from the denominator; it is constant throughout. A second K_H, 6×10^{-7}, was calculated for isolate CK4.

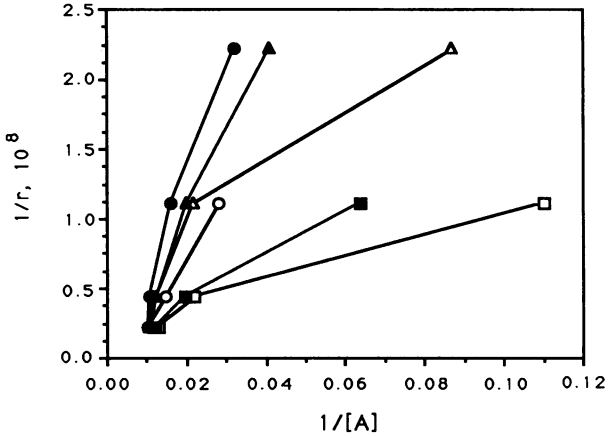

FIG. 1. Langmuir isotherm representation of data from Table 1. No datum points were drawn when [A] equalled zero. The slope of each line provides the K_H for the isolate. Regression analysis of the data for each isolate, except CK4, indicated excellent correlation ($r^2 > 0.97$). The r^2 value for CK4 was 0.91, suggesting that this isolate has two K_H values. Symbols: ●, LGH870; ▲, LGH1095; ■, UMC9385; ○, LGH86; △, CK4; □, 9938.

stationary phase in Sabouraud dextrose broth and washed three times with ice-cold deionized water or 0.05 M sodium phosphate buffer (pH 7.2). In the HMA, the cells were adjusted to 2×10^6/ml in sodium phosphate buffer. A stock suspension of blue, unmodified polystyrene microspheres (diameter, 0.801 μm; Serva Fine Biochemicals, Westbury, N.Y.) was prepared by mixing 6 μl of a 10% solids suspension in 2 ml of sodium phosphate buffer, resulting in a concentration of 9×10^8 microspheres per ml. Equal volumes (100 μl) of the yeast cell and microsphere suspensions were placed in acid-washed glass test tubes (12 by 75 mm) and equilibrated at room temperature (final concentrations, 10^6 cells per ml and 4.5×10^8 microspheres per ml). The mixture was vigorously vortexed for 30 s on a flat-top mixer (Maxi-Mix 1; Thermolyne, Dubuque, Iowa), and the percentage of yeast cells with three or more attached microspheres was determined by bright-field microscopy (200 cells per sample). In the HMAA, the final microsphere concentration varied from 2.25×10^7 to 4.5×10^8/ml.

To examine the usefulness of the HMAA, six isolates of *C. albicans* were tested. These isolates were originally obtained from clinical samples and have been maintained in our stock collection for 2 to 3 years (9, 11). When grown to stationary phase at room temperature, the isolates are highly hydrophobic by the standard microsphere assay, i.e., they each have a CSH level of greater than 75% (Table 1). Similar CSH levels (±5%) were also obtained by the microorganism aqueous-to-hydrocarbon assay (Table 1), which has been described elsewhere (9). When isolates were tested with the modified assay and the data were plotted as 1/r versus 1/[A], it was apparent from the slopes that these isolates exhibit different surface hydrophobic avidities (Fig. 1). When the slopes were calculated, the results indicated that the most strongly hydrophobic sites belong to *C. albicans* isolate LGH870 (Table 1). The plot also indicates that one isolate, CK4, possesses at least two hydrophobic sites of different avidities. This interpretation is based on the dramatic change in slope at the lower microsphere concentration (higher 1/r value; for a review of these arguments, see reference 4). The avidity of one group of sites on isolate CK4 appears similar to the avidity of the sites expressed by isolate LGH1095. Whether these sites are located on different subpopulations or coexpressed on individual cells cannot be determined.

These results illustrate one significant advantage of the kinetic microsphere assay over other hydrophobicity measurement assays. By comparing K_H values, it is possible to obtain quantitative data about the hydrophobic sites of each strain without bias from the cells not expressing surface hydrophobicity under standard assay conditions. The plot (Fig. 1) of the data of Table 1 in accordance with the Langmuir isotherm equation shows that all isolates, except LGH86, give a slight change in slope as the value of 1/[A] approaches zero. However, with the exception of isolate CK4, the change in slope appears negligible, as the correlation coefficient (r^2) for each line was greater than 0.97. This suggests that all the isolates possess hydrophobic sites with different avidities, with the more avid sites representing a small percentage of the available sites. Further studies are needed to confirm this point.

Our results indicate that the HMAA is effective at differentiating among strains of *C. albicans*, and presumably other yeasts, that appear similarly hydrophobic by standard hydrophobicity assessment methods. The assay should have the additional benefit of detecting isolates or mutants that produce cells differing in their hydrophobic sites. The HMAA utilizes the Langmuir isotherm equation in a manner different from conventional applications of the equation, in which only the number of bound versus unbound cells on a surface is determined (3). Such conventional application was not possible in this case, as it would require that the precise number of attached microspheres for each microsphere concentration be determined (see above). Precise quantitation is not possible because highly hydrophobic cells are typically so covered with microspheres that they cannot be counted. Also, precise quantitation of attached microspheres is too time consuming, resulting in surface changes during the enumeration process that could lead to spurious results (8). For these reasons, the HMAA does not allow the number of hydrophobic sites on a cell to be determined. However, the eventual availability of radioactive microspheres may circumvent these problems.

We anticipate that the HMAA will help investigators to determine which yeast isolates are most suitable for physiological and pathogenetic studies on yeast cell hydrophobicity. For example, it may be possible to determine the effects

of high- and low-hydrophobic avidity sites on yeast-host cell interactions and on the interactions of amphipathic antifungal compounds with yeast cells. The assay may also provide quantitative information useful for biochemical analysis of the hydrophobic sites in a yeast cell population.

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