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Factors Governing Adherence of *Candida* Species to Plastic Surfaces

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The ability of *Candida albicans* and *Candida* spp. to adhere to inert polymeric surfaces may allow these organisms direct ingress into the human host. Biophysical characterization of this adherence shows that the forces responsible for such adherence are attractive London-van der Waals forces (or hydrophobic forces) and electrostatic forces. The hydrophobic affinity of yeasts was determined by (i) a water-hydrocarbon two-phase assay and by (ii) measurement of the contact angle (θ) of a liquid droplet on a monolayer of yeast cells. The hydrophobicity of the yeasts correlated with the tendency of yeasts to adhere to polystyrene and was reduced in the presence of Tween 20. The adherence of yeasts to polymers of increasing hydrophobicity (determined by the contact angle method) was directly proportional to θ. Yeast surface charges were altered by selectively blocking amino and carboxyl groups. The more positively charged yeasts adhered in greater numbers. Increasing the molarity of NaCl increased yeast adherence. These forces probably contribute to the negative cooperativity (determined by Scatchard and Hill plot) that characterizes the adherence of yeasts to polymers.

Candida albicans and related *Candida* species are the most common fungal opportunistic pathogens encountered in clinical medicine (14). Access to the vascular space precedes the establishment of metastatic infections by this organism. Presumably the fungus achieves entry into the vascular system by one of two routes: (i) directly, through a breach in vascular patency, e.g., through an intravenous catheter (30); or (ii) by persorption through the gut (25). In an earlier report it was shown that once *Candida* spp. have gained access to the vascular system they are fully capable of adhering to endothelial tissue (16).

This report demonstrates the surface properties of *Candida* spp. which make them eminently suitable opportunistic pathogens which may invade the human host by attaching to plastic surfaces or similar materials that comprise the indwelling component of many intravenous devices, bladder catheters, and other prostheses. The fact that *Candida* spp. adhere to plastic intravenous catheters has been reported (28), but the mechanism has not been elucidated. The adherence of yeasts to inert surfaces is unlikely to be mediated by a receptor-adhesin interaction. *Candida* spp. possess a rigid, smooth cell wall devoid of pilus-like projections or flagella. Hence, the microorganism can be regarded for purposes of this study as a colloid in suspension. In the experiments reported here, it was possible to establish that surface energy (and, by extension, interfacial tension) is the principal general measurement relating adherence of *Candida* spp. to solid hydrophobic substrates (including samples of polymers used in intravenous catheters and prosthetic devices).

The adherence of *Candida* spp. to plastics was examined from two perspectives: (i) the role of yeast surface characteristics in adherence and (ii) the role of polymer surface characteristics in adherence.

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MATERIALS AND METHODS

Yeasts. All species and strains of *Candida* were clinical isolates with known ability to adhere to endothelial tissue (16). *Yarrowia* (*Candida*) *lipolytica*, a hydrophobic nonpathogenic yeast, was ATCC 8661. Yeasts were maintained on Sabouraud dextrose agar (Difco Laboratories, Detroit, Mich.) and transferred monthly. For experimental purposes, a loopful of yeasts was transferred to 50 ml of Sabouraud dextrose broth (Difco) and incubated at 26°C at 100 rpm for 21 h (these cells are in the early stationary phase of growth). Yeast cells were washed in distilled water, 0.85% NaCl, or Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) by centrifugation and suspended to the desired concentrations by use of a hemocytometer.

Adherence to solid surfaces. The following substrates were used: poly(tetrafluorethylene) (Teflon; E. I. du Pont de Nemours and Co., Wilmington, Del.); poly(ethyleneterephthalate) (Thermanox, Miles Laboratories, Inc., Naperville, Ill.); poly(methylmerthacrylate) (Plexiglass; Rohm & Haas, Co., Philadelphia, Pa.); and bacteriological grade polystyrene petri dishes (Miles). Pieces of substrate (2.5 cm^2) were washed vigorously with nonabrasive soap and rinsed thoroughly with distilled water. Pyrex glass petri dishes (Fisher Scientific Co., Dallas, Tex.) were acid cleaned, rinsed thoroughly with distilled water, and then dried in an oven at 100°C for 2 h.

After the above preparations, the surface substrates were submerged to an equal depth in a normal saline bath containing 5×10^6 yeasts per ml at 26°C. The bath was agitated at 50 rpm for 20 h. The yeasts were >99% viable as determined by exclusion of methylene blue dye (17) after the 20-h incubation. The substrates were removed with forceps, rinsed by dipping in distilled water four times, allowed to dry, and then stained with crystal violet. Adherent yeasts were counted from photomicrographs of stained preparations.

Kinetics. The adherence of yeasts to the wells of polystyrene microtiter trays (Dynatech Laboratories, Inc.,

TABLE 1. Comparison of *Candida* species and strains by means of three hydrophobic indices[a]

Species	Strain	% Δ Absorbance	Cosine θ	Adherence to polystyrene
C. albicans	1	15 ± 3.5	0.89 ± 0.03	$1.2 \times 10^6 \pm 0.3 \times 10^6$
C. albicans	1755	15 ± 0.6	0.89 ± 0	$1.0 \times 10^6 \pm 0.4 \times 10^6$
C. albicans	J	74 ± 1.5	0.92 ± 8.1	$3.6 \times 10^6 \pm 0.6 \times 10^6$
C. tropicalis	1	7 ± 5.8	0.84 ± 0.04	$3.4 \times 10^6 \pm 0.3 \times 10^6$
C. tropicalis	2	85 ± 7.5	0.90 ± 0.01	$4.9 \times 10^6 \pm 0.2 \times 10^6$
C. krusei	1	33 ± 3.0	0.91 ± 0.02	$3.6 \times 10^6 \pm 0.2 \times 10^6$*
C. krusei	2	1 ± 1.0	0.87 ± 0.03	$4.1 \times 10^6 \pm 0.2 \times 10^6$*
C. pseudotropicalis	1	81 ± 5.8	0.93 ± 0.02	$2.6 \times 10^6 \pm 0.8 \times 10^6$
C. pseudotropicalis	2	85 ± 3.5	0.96 ± 0.01	$4.3 \times 10^6 \pm 0.1 \times 10^6$
C. parapsilosis	1	23 ± 2.0	0.46 ± 0.06	$3.3 \times 10^6 \pm 0.8 \times 10^6$
C. parapsilosis	2	75 ± 4.6	0.69 ± 0.03	$3.9 \times 10^6 \pm 0.2 \times 10^6$
C. glabrata	2	69 ± 3.8	0.88 ± 0.02	$3.9 \times 10^6 \pm 0.6 \times 10^6$
C. lipolytica	8661	98 ± 1.0	0.90 ± 0.02	$2.2 \times 10^6 \pm 0.5 \times 10^6$

[a] For the three columns, the higher the value, the more hydrophobic the strain. The three methodologies are in agreement (as to magnitude), except for results marked with an asterisk (*). One standard deviation is shown for each determination.

Alexandria, Va.) was measured in the following fashion. A 0.1-ml sample of a suspension of the desired concentration of yeasts was placed in each well; the tray was placed on a rotating platform at 200 rpm at 26°C for 2 h; the wells were washed with 0.1 ml of distilled water four times with an octapette (Costar, Cambridge, Mass.). The wells were then filled with 0.1 ml of normal saline, and the optical density at 410 nm was obtained in a densitometer (Dynatech). This provided a reproducible and sensitive method of quantitating the adherence of yeasts to a representative hydrophobic surface. The same method was used to measure adherence of yeasts suspended in increasing molarities of NaCl ("salting-out" experiments).

To obtain a numerical estimate of the number of yeasts that adhered and the number of binding sites on the surface of a solid substrate, radiolabeled yeasts were allowed to adhere to 13-mm-diameter poly(ethyleneterephthalate) cover slips (Lux Scientific). *C. albicans* (strain J) was grown to the stationary phase (21 h) in 50 ml of Sabouraud dextrose broth to which 5 μCi of D-[6-^{14}C]glucose (57 mCi/mmol, New England Nuclear Corp., Boston Mass.) had been added. After incubation at 26°C at 100 rpm, the yeasts were washed in normal saline, enumerated by use of an hemocytometer, and suspended to desired concentrations with or without 1% Tween 20 (Sigma Chemical Co., St. Louis, Mo.). Cover slips were placed in the wells of a tissue culture tray (Costar). A 0.5-ml sample of yeasts at the desired concentrations was added to each well and incubated at 37°C for 90 min at 100 rpm. The cover slips were then washed four times with 1 ml of normal saline, removed, and placed in scintillation fluid, and the counts per minute of ^{14}C were determined in a Beckman LS 7000 scintillation counter (Beckman Instruments, Irvine, Calif.).

Adherence to water-hydrocarbon interface. The methods of Rosenberg and Rosenberg (27) were modified in the following manner. (i) The absorbance of 5×10^6 yeasts per ml in Hanks balanced salt solution was read at 530 nm in a spectrophotometer (Beckman DB-G). (ii) A 1.5-ml sample of this suspension was placed in a glass test tube (12 by 75 mm) and covered by 1 ml of a liquid hydrocarbon (either toluene, xylene, or hexadecane; Aldrich Chemical Co., Inc.; Milwaukee, Wis.). (iii) After equilibration to 37°C in a water bath, the biphasic samples were vortexed for 2 min and then allowed to settle for 10 min. (iv) A sample of the lower (i.e., water) phase was removed, and the absorbance for the sample was recorded. The percent change in absorbance (%Δ absorbance) was then calculated by dividing value (iv) by value (i). The greater the change in absorbance, the greater the shift in yeasts from the bulk medium to the interface, i.e., the more hydrophobic the yeast strain.

Contact angle measurements. The method of van Oss et al. (32) was used to measure contact angles (θ) on lawns of yeasts. Yeasts were grown as described above for 21 h and washed in normal saline by centrifugation, and the supernatant was decanted, leaving a yeast paste. This paste was layered onto previously prepared microscope slides which had been covered by a thin film of 2% agar made with 10% (vol/vol) glycerol in 90% normal saline. These slides were allowed to dry in air for 3 h. With a micropipette, 1 μl of a sensing liquid was then placed upon the yeast lawn, and the contact angle was measured. Some strains of the yeasts were so hydrophilic that no contact angle could be measured with normal saline as the sensing liquid. To circumvent this, α-bromonaphthalene (Aldrich) was used as the sensing liquid (4). Angle θ was obtained by measuring it directly from the image of the bubble of sensing liquid projected onto a screen by a horizontally mounted drawing microscope (23). Distilled water was the sensing liquid used for the measurement of θ on solid substrates.

Electrostatic charges. The role of electrostatic charges in the adherence of yeasts to hydrophobic surfaces was investigated by altering the surface charges of the yeasts. Carboxyl groups were neutralized by treatment with carbodiimide (29), and amino groups were neutralized by treatment with 1.5% Formalin (15). Confirmation of these surface charge alterations was obtained by staining treated yeasts with a 0.025% solution of cationized ferritin for 1 min (8) and a 1% solution of Alcian blue (Polysciences, Inc., Warrington, Pa.) (5). The stained specimens were then compared with control specimens by transmission electron microscopy. Specimens for transmission electron microscopy were fixed in cold buffered glutaraldehyde, rinsed in buffer, postfixed in osmium tetroxide, and dehydrated with acetone and water. Specimens were embedded in Epon, sectioned with a microtome, and examined in a Philips 301 electron microscope (Philips Electronic, Inc., Houston, Tex.).

RESULTS

The relative hydrophobic affinity of the *Candida* species and strains was obtained by the water-hydrocarbon biphasic assay (Table 1). In this assay, emulsions of hydrocarbon

were formed during vortexing, in effect increasing the hydrocarbon interfacial area available for adherence. Emulsion droplets were visible macroscopically as a creamy layer at the interface and were stable for days, possibly maintaining integrity by repulsive electrostatic charges (20). This assay, at best qualitative, does permit a reproducible ranking of the different strains of yeasts by measuring yeast adherence to a liquid hydrocarbon. Increasing the volume of hydrocarbon or using log-phase yeasts enhanced extraction or separation of the yeasts (i.e., the %Δ absorbance increased) to the oil phase. Adherence was greatest with toluene (> xylene > hexadecane). Systematic studies involving changes in the hydrocarbon, the volume, or the temperature did not change the relative ranking of the yeasts.

Another independent measure of hydrophobic affinity of the yeasts was obtained by the contact angle method (Table 1). Values are reported as cosine θ. Therefore, as an angle approaches 0, the cosine approaches 1. Neufeld et al. (23) have reported values as high as 140° for *Acinetobacter* spp. The hydrophilic property of some *Candida* spp. cultures required using a less polar standard such as α-bromonaphthalene. The hydrophobicity ranking with this method is similar (within species) to the ranking obtained by the water-hydrocarbon biphasic method.

The results of the two methods described above correlated with the adherence of yeasts to bacteriological grade polystyrene petri dishes (Table 1). The only discrepancy among the three methods was polystyrene adherence by *Candida*

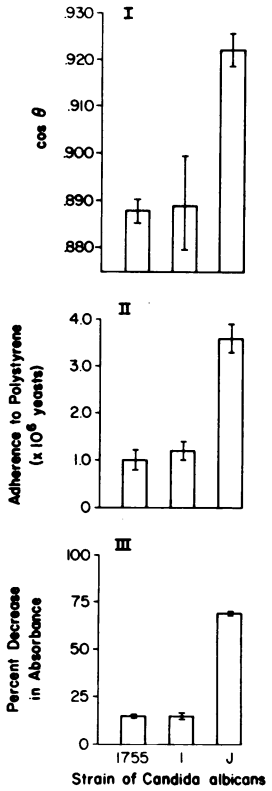

FIG. 1. Comparison of hydrophobic assays. The hydrophobicity of three strains of *C. albicans* (1, 1755, and J) are shown to correlate in rank order and magnitude. The methods included (I) contact angle measurement of layers of yeast cells, (II) adherence to polystyrene, and (III) adherence to the xylene-Hanks balanced salt solution interface. Bars show 1 standard deviation.

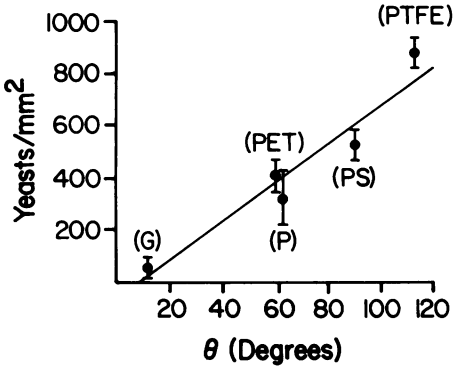

FIG. 2. Adherence of *C. albicans* to different hydrophobic surfaces; θ, determined with distilled water. Abbreviations: G, Pyrex glass; PET, poly(ethyleneterephthalate); P, poly(methylmethacrylate) (Plexiglas); PS, polystyrene; PTFE, poly(tetrafluorethylene) (Teflon). Bars show 1 standard deviation.

krusei, which may indicate that this strain possesses an additional mechanism functional in adherence, such as protruding slime, etc. Nevertheless, the three different methodological approaches to the measurement of hydrophobic affinity yielded congruent results of similar magnitude and rank order. This is shown graphically for *C. albicans* in Fig. 1.

The importance of the surface properties of the solid substrates was examined in a separate experiment. Substrates with a wide range of hydrophobicity (determined by the contact angle method) were immersed in a saline bath containing *C. albicans* (Fig. 2). It is clearly evident that there is a nearly linear relationship between the cell number adhering per unit area and the contact angle of the substrate, i.e., the more hydrophobic the surface, the greater the cell adherence per unit area.

The kinetics of the adherence of yeasts to a representative polymer were explored by measuring the adherence of ^{14}C-labeled *C. albicans* cells to poly(ethyleneterephthalate) cover slips. A representative binding or adsorption isotherm at 26°C is shown in Fig. 3. Experiments in which 2.5×10^7 yeasts (i.e., a number which would saturate the cover slip surface) and to which 1% Tween 20 was added reduced adherence to 5% of control values. Without Tween 20, 1.9×10^6 yeasts adhered; with Tween 20, 1×10^5 yeasts adhered. Figure 4 shows the same data prepared as a Scatchard plot.

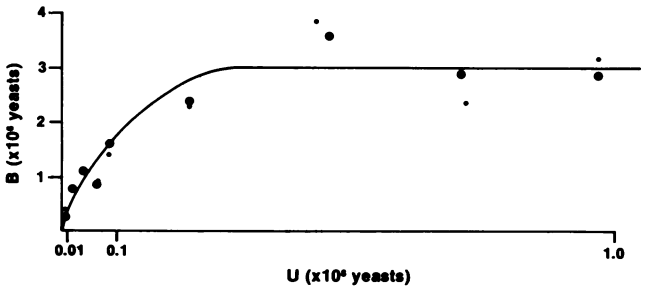

FIG. 3. Binding isotherm at 26°C of *C. albicans* to poly(ethyleneterephthalate) cover slips. Abbreviations: B, bound yeasts × 10^6; U, unbound yeasts × 10^8. Solid balls and bulls-eyes indicate data from two different experiments. Each point represents the mean of four values.

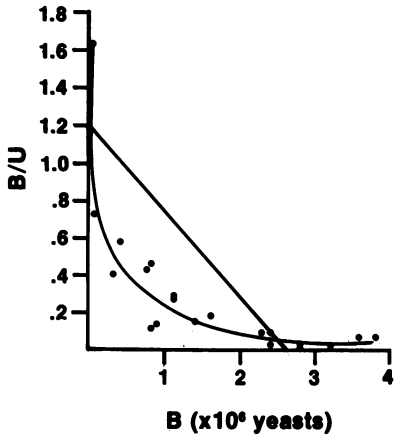

FIG. 4. Kinetics of the binding of yeasts to a plastic surface (same data points as in Fig. 3, treated by Scatchard plot). Abbreviations: B/U, bound/unbound yeast ratio; B, bound yeasts $\times 10^6$. The straight line is the best-fit straight line. The curved line is the real line drawn through actual data points. The deviation from the straight line indicates negative cooperativity.

The bowing of the line in the Scatchard plot is typical for receptor-ligand interactions displaying negative cooperativity (22, 26). A Hill plot of data from a similar experiment gives an h value of 0.67, a value which is also indicative of negative cooperativity. The negative cooperativity implies that a repulsive force previously not measured was present in addition to the attractive van der Waals or hydrophobic forces. This repulsive force is most likely electrostatic (1, 13).

To test the supposition that electrostatic interactions contributed to adherence, yeasts whose surfaces had been electrically altered were allowed to adhere to polystyrene. The net surface charge of yeasts clearly influenced adherence (Table 2). The control or untreated yeasts possess a net negative surface charge in common with all living cells. However, the more positively charged yeasts, i.e., those treated with carbodimide, are considerably more adherent than more negatively charged Formalin-treated yeasts. However, the addition of increasing molarities of NaCl to suspensions of yeasts actually enhanced the adherence of yeasts to polystyrene (Fig. 5). This phenomenon has been referred to as the salting-out of hydrophobic moieties (18, 21). It is surmised that electrostatic interactions, although present and capable of altering the kinetics of adherence (e.g., contributing to the negative cooperativity demonstrated by this and the Scatchard plot), were overshadowed by the simultaneous attractive hydrophobic forces.

TABLE 2. Effect of electrostatic alteration on adherence of yeasts to polystyrene[a]

Treatment	Optical density (± SD)	n
Control	6.6 ± 1.0	8
Carbodiimide	11.4 ± 1.3	8
Formaldehyde	3.4 ± 2.0	8

[a] Yeast suspensions were placed in microtiter wells for 2 h at 200 rpm and 26°C and then washed vigorously, and the optical density of wells at 410 nm was recorded. Carbodiimide-treated yeasts are more positively charged (net) than controls; formaldehyde-treated yeasts are more negatively charged (net) than controls. The greater the optical density, the greater the number of adherent yeasts.

DISCUSSION

It has been shown by this series of experiments that the adherence of *Candida* spp. yeasts to plastic surfaces is predominately controlled by what have been collectively called hydrophobic forces, but are more properly designated as attractive London van der Waals forces (31). The kinetics of the interaction between polymers and yeasts demonstrates negative cooperativity (i.e., as yeasts are bound to plastic there is present a simultaneous repulsive force). The work herein suggests that this force is electrostatic. Plastic surfaces possess various degrees of negative net surface charge (18); similarly, all living cells (including yeasts) possess a net negative surface charge. Hence, the negative-negative charge interactions, e.g., the interactions that would occur between plastic and yeast and between yeast and yeast would be expected to create a condition of negative cooperative binding. The electrical forces are minor to the hydrophobic forces, since adherence to a considerable extent occurs even in the presence of this repulsive force. The minor nature of this force is evident from the salting-out of yeasts rather than inhibition of yeast adherence in the presence of increasing concentrations of ionized salts. These findings are in accordance with the DLVO theory of colloid-surface interactions (7).

It is obvious, however, that other factors must be important influences upon the adherence of yeasts to plastic. For example, the size or shape of the microorganism is different for the different species of *Candida*, which may explain why the nonpathogenic yeast *C. lipolytica*, which has a contact angle (cosine θ) and adherence to polystyrene close to that of *C. albicans* 1, has a markedly greater adherence to hexadecane emulsion droplets. On the other hand, the comparison of strains 1 and 1755 of *C. albicans* shows that, within species where size and shape are probably close to identical, the values of %Δ absorbance, cosine θ, and adherence to polystyrene are also nearly identical. Our data are in agreement with those presented by Minagi et al., who recently reported on the adherence of *C. albicans* and *Candida tropicalis* to denture materials (19).

The interaction of yeasts with a polymer or any other inert surface is unlikely to be mediated by such specific microorganism-substrate interactions as those proposed for adhesin-receptor mechanisms such as the mannose-sensitive fimbriae

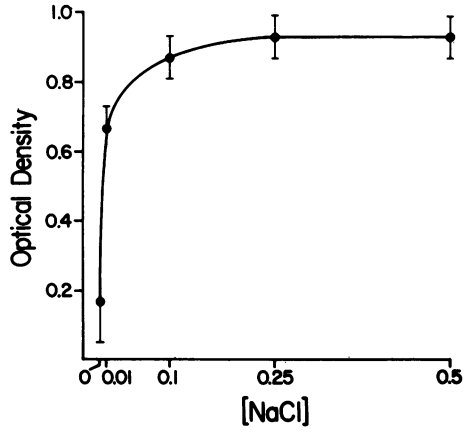

FIG. 5. Salting-out of yeasts to polystyrene. The optical density at 410 nm of an inoculum of 5×10^6 yeasts per ml is shown at different molarities of NaCl.

of certain *Escherichia coli* strains. The work described herein has demonstrated the molecular forces involved in the adherence of yeasts to polymeric surfaces. The adherence conforms to the concept of the minimal interfacial free energy (or interfacial tension [1, 2, 24]). As shown by this work, adherence occurs whenever the sum total of interfacial tensions (γ_T) i.e., yeast-liquid (γ_{ye}), yeast-solid (γ_{ys}), and solid-liquid (γ_{se}) is reduced. In thermodynamic terms the change in total free energy is negative. The applicable equations are developed lucidly by Boyce et al. as they apply to this situation (6). The principle of reduction of interfacial tension is best demonstrated in this work by yeasts adhering in direct proportion to the contact angle of the surface. Other work has shown that *Pseudomonas* spp. (11) and *Staphylococcus* spp. (12) conform to the same thermodynamic model. The accumulation of yeasts at an interface is further assisted by their exclusion from bulk water, a tendency that is increased in the presence of ionized salts. This principle is best shown by the experiments showing the yeasts collecting at the hydrocarbon-water interface and the salting-out experiments.

The investigation of the surface properties of the polymers (plastics) and their microdomain and the interaction with microorganisms may provide information that could be used to affect or alter the adherence of pathogens to intravascular cannulas and other vascular prostheses. Of equal importance is the need to recognize the nature of yeast adherence in experimental laboratory containers, which, if not controlled for, can produce spurious results. How much of the in vitro phenomenon will be applicable to in vivo conditions remains to be demonstrated. On the basis of some previous reports it may be anticipated that the ingredients of the liquid milieu are critical factors affecting adherence (3, 9, 10).

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