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Chemical and Structural Alterations at the Cell Surface of *Candida tropicalis*, Induced by Hydrocarbon Substrate

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The surface-localized polysaccharide of alkane-grown cells of *Candida tropicalis* was identified as mannan containing approximately 4% covalently linked fatty acids. Glucose-grown cells lacked the mannan-fatty acid complex. The surface structure of alkane-grown cells showed a radial arrangement of the wall polymers, with protruding parts. The cell surface of glucose-grown cells was smooth, with a coherent outer limit. The mannan was localized by using concanavalin A. Masking of the mannan with concanavalin A reduced the binding affinity of the surface for alkane, indicating the involvement of the surface-localized mannan-fatty acid complex in the binding of alkanes.

The growth of microorganisms on *n*-alkanes requires the transport of hydrocarbon from the oil phase to the cells. The processes by which the alkanes are transported to the cells and subsequently assimilated by the cells are still not fully understood. Direct contact of cells with dispersed alkane appears to be the mechanism through which most of the substrate is transported from the medium to the cell surface (12). Submicron droplets of various sizes (from tiny droplets to substrate aggregates) were found adhering to the cell wall of the yeast *Candida tropicalis* when grown on alkanes (4). Similar observations were made by Ludvik et al. (10). The authors reported that the yeast cell wall was covered with a thin layer of hydrocarbons after growth on alkanes. We showed that the interaction between alkanes and the cell surface of *C. tropicalis* is a nonenzymatic adsorption (7). Glucose-grown cells exhibited a lower alkane binding affinity than did alkane-grown cells. Furthermore, glucose-grown cells showed a greater dependence of the amount of adsorbed alkanes on the quality of the emulsion than did alkane-grown cells. These cells required finely emulsified hydrocarbon to obtain substrate adsorption to the cell surface. It was concluded that the binding affinity of alkane-grown cells was due to the peripheral portion of the cell wall involving components to which the insoluble, hydrophobic substrate was readily adsorbed.

In an attempt to characterize these components, we isolated a polysaccharide-fatty acid complex from the surface of alkane-grown cells of *C. tropicalis* (8). Upon removal of this complex by Pronase digestion, the cells lost their binding affinity for alkanes. Therefore, the polysaccharide-fatty acid complex was related to the alkane binding at the cell surface.

Because alkane- and glucose-grown cells of *C. tropicalis* differed in their binding affinity for alkanes, this study was initiated to investigate the chemical and structural alterations at the cell surface induced by growth on alkanes.

MATERIALS AND METHODS

Chemicals. Concanavalin A (ConA) and peroxidase were purchased from Boehringer, Mannheim, German Federal Republic; Pronase was purchased from Serva, Heidelberg, German Federal Republic. All other chemicals were of analytical grade.

Organism and culture conditions. *C. tropicalis* ATCC 32113 was used for all experiments. The cells originated from a continuous culture with the conditions described previously (8).

Precipitation of the mannan-fatty acid complex from the Pronase digest. The proteolytic digestion of whole cells with Pronase was carried out as reported previously (8). After incubation, the cells were separated by centrifugation (3,500 × *g* for 10 min), and the supernatant was clarified by membrane filtration (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.). To the filtrate (approximately 20 ml), 2 ml of 1 M KOH was added, and the mannan was precipitated by the addition of an equal volume of Fehling's solution with continuous stirring (3). The mixture was left overnight, and the mannan-copper complex was removed by centrifugation and washed twice with 0.01 M NaOH.

Agglutination of ConA with mannan. The agglutination of Pronase-released mannan was assayed

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turbidimetrically at 420 nm (15) in 0.5 M acetate buffer, pH 6.0, containing 1 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 1 mM $MgCl_2$.

Agglutination with whole cells was carried out in the same buffer. The interaction was measured by determining the protein concentration versus the incubation time of the supernatant fluid after separating the cells from the incubation mixture by centrifugation. A decrease in the protein concentration indicated a binding of ConA to the mannan of the cell wall.

Electron microscopy. Because ConA binds specifically to mannan (5), the method of Bernhard and Avrameas (1) was used to localize the mannan by electron microscopy. The bivalent ConA, bound to the mannan component of the cell wall, was allowed to react with peroxidase, a glycoenzyme. The peroxidase reaction was initiated in the presence of 3,3'-diaminobenzidine (DAB). The cells were fixed in 2% glutaraldehyde in 0.1 M sodium-cacodylate buffer (pH 7.2) for 2 h at room temperature, dehydrated, embedded in Araldite-Epon, sectioned, and examined in a Philips EM 301 electron microscope.

Freeze fracturing. The use of cryoprotectants was avoided by using a rapid freezing technique (13). A 400-mesh gold grid was dipped into a pellet of suitable cell density and placed between two conventional gold disks. This sandwich was frozen and subsequently fractured at −100°C in a Blazer BAF 300 freeze-etching apparatus. Carbon-platinum replicas were examined in a Philips EM 301 electron microscope.

Analytical methods. Dry cell weight was determined by drying samples at 105°C for 24 h. Total sugar was estimated by the phenol-sulfuric acid method of Dubois et al. (2). The fatty acid content was quantified by gas chromatography, with methylcaprate as the internal standard (8). Protein was estimated by the method of Lowry et al. (9), with bovine serum albumin as the standard.

RESULTS

Mannan-fatty acid complex released by Pronase. The polysaccharide obtained by Pronase digestion of whole cells of *C. tropicalis* grown on alkanes contained covalently bound fatty acids (8). The polysaccharide-fatty acid complex was precipitated from the Pronase digest with ethanol. The mannan was isolated with Fehling's solution and represented approximately 30% of the total polysaccharide released by Pronase. The fatty acid content of the mannan was 43 μg/mg of polysaccharide. The fatty acid composition was identical to that reported for the ethanol-precipitated polysaccharide (8). No difference in the amount and composition of the mannan was found when it was precipitated directly from the Pronase digest.

Because the polysaccharide-fatty acid complex was formed only in alkane-grown cells (8), a substrate shift from glucose to alkane was carried out. The substrate was changed in the continuous culture without affecting the other parameters of the culture (6). Over the whole transient period, samples were taken, and the Pronase digest of the cells was analyzed for total polysaccharide, mannan, and fatty acid content (Table 1). In the polysaccharide fraction of glucose-grown cells, both mannan and fatty acids were present in much smaller amounts than in alkane-grown cells. The mannan and fatty acid content gradually increased during the transient period to a level characteristic for growth on alkanes. About 50% of the fatty acids were associated with the mannan.

The amphipathic nature of the mannan-fatty acid complex was evaluated by measuring its effect on a hexadecane emulsion (Fig. 1). The mannan-fatty acid complex markedly enhanced the clearing time of the emulsion, indicating amphipathic properties of the isolated component.

Localization of the mannan. The Fehling's solution-precipitated and washed mannan of alkane-grown cells was suspended in the specified buffer for the agglutination assay with ConA. The amount of agglutinated mannan increased as the amount of mannan in the reaction mixture increased up to 80 μg/ml, with a constant ConA concentration of 1 mg/ml (Fig. 2). Likewise, the

TABLE 1. *Changes in mannan and fatty acid content of the Pronase-released polysaccharide during a substrate shift from glucose to alkane*

Time after substrate change (h)[a]	Carbohydrate released by Pronase digestion (mg/g [dry wt])	Mannan (mg/g [dry wt])	Fatty acid content (mg/g [dry wt]) of:	
			Total ethanol-precipitated polysaccharide	Mannan
0	117	1.0	0.1	0.05
2	98	0.8	0.1	0.04
4	108	7.0	0.7	0.3
6	110	9.1	2.2	1.4
8	108	9.5	2.6	1.5
10	108	16.1	2.6	1.5
72	121	37.6	3.0	1.6

[a] In substrate shift from glucose to alkane cells.

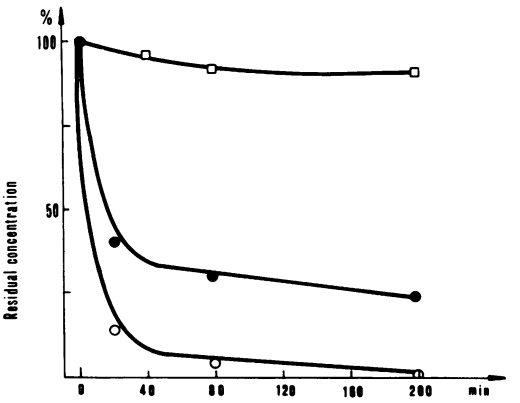

FIG. 1. *Effect of the mannan-fatty acid complex on the clearing time of a hexadecane emulsion (●). A 100-mg amount of hexadecane was added to 5 ml of an aqueous solution of the isolated mannan-fatty acid complex, and the clearing time of the emulsion was determined after emulsification by sonic treatment. The hexadecane concentration was recorded 1 cm above the bottom of the vessel. Symbols: □, clearing time in a solution of 0.05% Tween 80; ○, clearing time in a citrate-phosphate buffer, pH 5.5.*

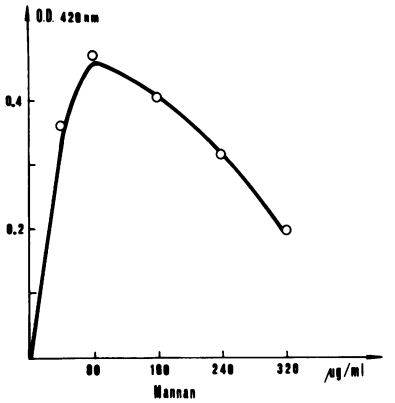

FIG. 2. *Interaction of ConA and mannan isolated from the cell surface of alkane-grown cells of C. tropicalis by Pronase digestion. ConA concentration, 1 mg/ml. O.D.$_{420}$ nm, Optical density at 420 nm.*

cells were tested for their capability to interact with ConA before and after Pronase digestion (Fig. 3). The Pronase-treated cells bound approximately 30% of the ConA as compared with those not digested with Pronase. Samples of ConA-treated cells were tested for alkane binding affinity (7). The alkane binding affinity decreased with the increasing saturation of the cell surface with ConA (Fig. 4). As reported previously, the alkane binding affinity also decreased when the surface-localized polysaccharide-fatty acid complex was removed by proteolytic digestion (8).

The mannan was ultrastructurally visualized by incubating the cells successively with ConA, peroxidase, and H_2O_2 in the presence of DAB (Fig. 5). The polymerized DAB formed a layer at the surface of the cell wall. This indicates that the mannan forms the outermost layer of the cell wall of cells grown on alkanes.

Structure of the cell surface. In addition to the substrate-dependent chemical differences at the cell surface of *C. tropicalis*, ultrastructural differences were observed. The cell wall of glucose-grown cells (Fig. 6) exhibited the same smooth surface as described for other freeze-fractured yeast species (11). In contrast, the cell walls of alkane-grown cells were characterized by an amorphous surface layer with radially oriented projections (Fig. 7A and B). The surface recovered a glucose-like appearance, becoming thinner after Pronase digestion (Fig. 8).

DISCUSSION

The mannan contained covalently bound fatty acids as demonstrated with the ethanol-precipi-

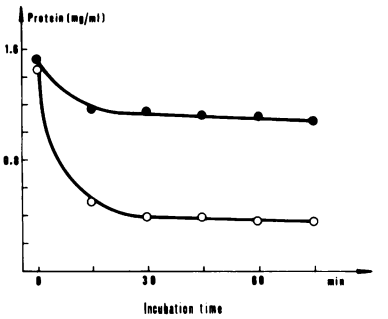

FIG. 3. *Binding of ConA by alkane-grown cells of C. tropicalis. Symbols: ○, control; ●, Pronase-treated cells.*

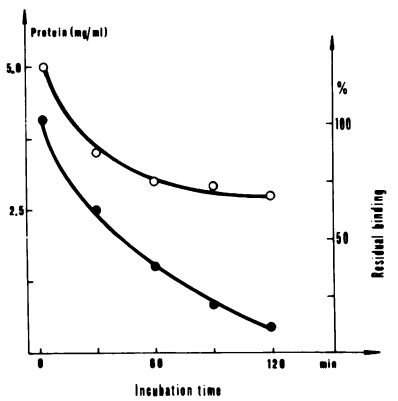

FIG. 4. *Effect of ConA on hexadecane binding to the cell surface of C. tropicalis. Symbols: ○, ConA binding; ●, hydrocarbon binding.*

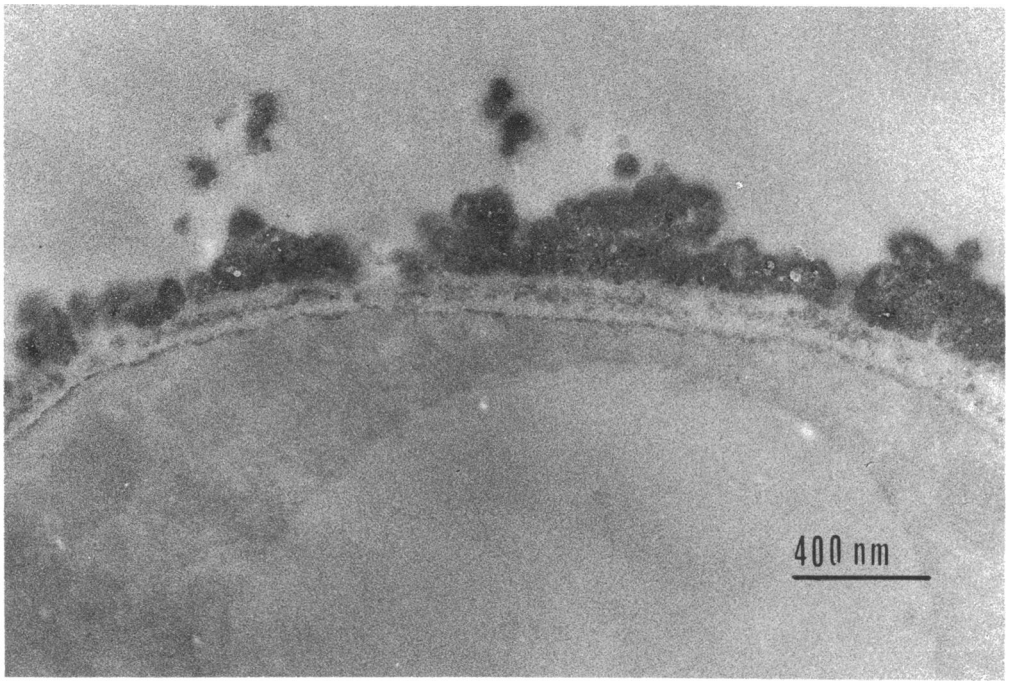

FIG. 5. *C. tropicalis incubated successively with ConA, peroxidase, and H_2O_2 in the presence of DAB. The black layer represents the polymerized DAB, indicating the localization of the mannan at the surface of the cell wall.*

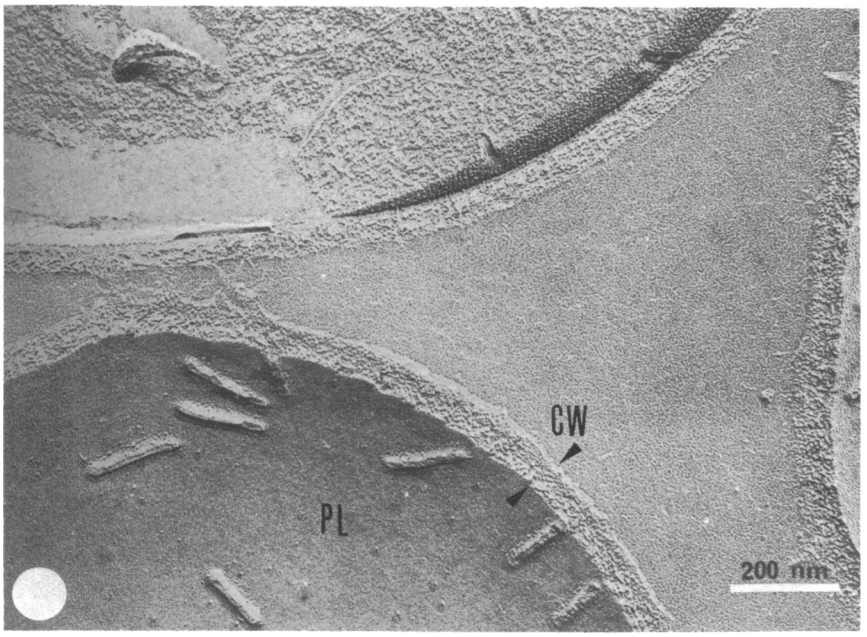

FIG. 6. *Freeze fracture of C. tropicalis grown on glucose. Notice the well-defined cell wall (CW) with a smooth surface. PL, Plasmalemma sculptured by invaginations.*

tated polysaccharide from alkane-grown cells of *C. tropicalis*. An enrichment of fatty acids (4.3%) in the mannan was observed as contrasted to 2.5% fatty acids in the total polysaccharide. This was consistent with the distribution of the polysaccharide after chloroform-methanol ex-

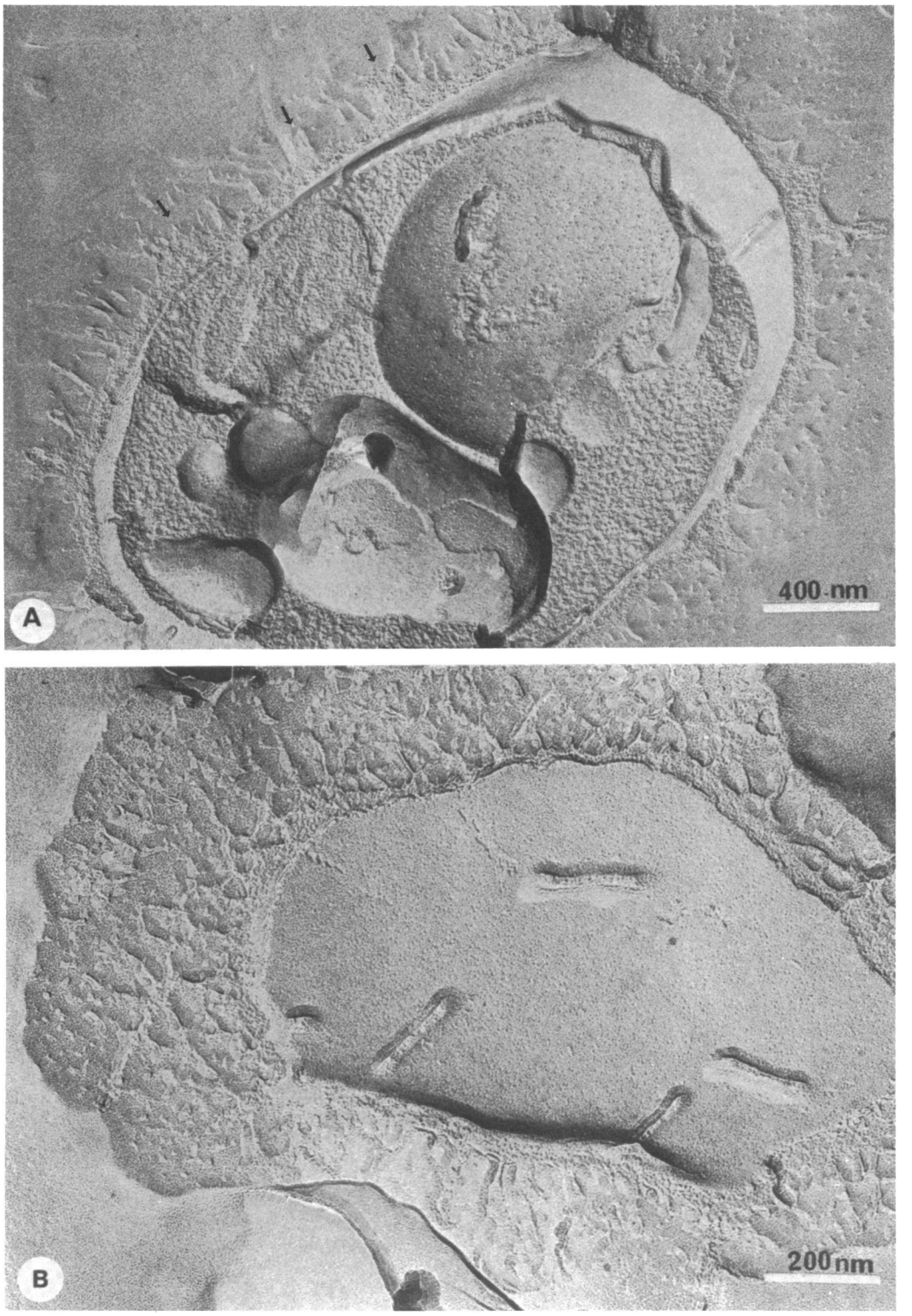

FIG. 7. *(A) Freeze fracture of C. tropicalis grown on alkanes. The cell surface shows numerous outgrowing projections (arrows), giving the surface a loose structure. (B) Fracture face through the tip of a hydrocarbon-grown cell of C. tropicalis, showing the radial orientation of the wall polymers.*

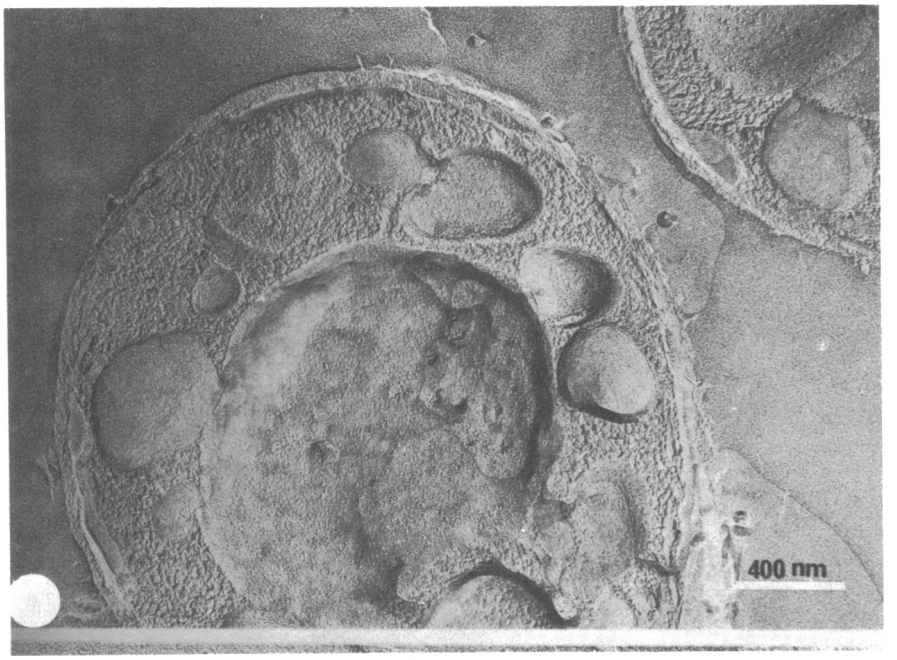

FIG. 8. *Freeze fracture of C. tropicalis grown on alkanes after Pronase digestion. The cell wall has recovered a distinct outer limit but shows clearly the effect of Pronase treatment.*

traction (8), which indicated that the polysaccharide-fatty acid complex was present as a heterogeneous mixture. The Pronase-released polysaccharide of glucose-grown cells contained about 40 times less mannan than that of alkane-grown cells. The fatty acid content of both cell types reflected the same order of magnitude (Table 1).

The interaction of ConA with mannan was used for the localization of the released mannan-fatty acid complex (Fig. 5). The specificity of the reaction could be demonstrated with both the agglutination of Pronase-released mannan with ConA (Fig. 2) and the binding of ConA to the cell surface before and after Pronase treatment (Fig. 3). The saturation of the cell surface with ConA reduced the binding affinity of the cell surface to alkanes (Fig. 4). This provides further evidence for the involvement of the peripheral polysaccharide-fatty acid complex in the adsorption of alkanes at the cell surface. The binding was lost upon the masking of the amphipathic polysaccharide-fatty acid complex with a hydrophilic protein.

The localization of the mannan in the cell wall was limited by its low resolution (5). The structural differences between glucose- and alkane-grown cells of *C. tropicalis*, as well as the effect of Pronase, were examined by using the freeze-fracture technique. The cell wall of yeast contains discrete layers of wall polymers, giving a characteristic stratification (11). In the cell wall of alkane-grown *C. tropicalis*, the wall polymers appear oriented radially with fibrous projections. Similar structures were described in alkane-grown yeasts by Osumi et al. (14).

The following experimental data suggest a possible role of the surface-localized mannan-fatty acid complex in alkane binding: (i) the alkane binding affinity of the cell surface was reduced upon the removal of the mannan-fatty acid complex and upon the masking of the cell surface with the hydrophile ConA, (ii) the formation of the mannan-fatty acid complex was induced by alkane substrate, and (iii) the mannan-fatty acid complex exhibited amphipathic properties.

The involvement of the mannan-fatty acid complex in alkane binding at the cell surface relates to the water insolubility of long-chain alkanes. Due to the repulsion of hydrocarbons by water, the alkane exists in a biphasic suspension. The dispersed hydrocarbon will adsorb preferentially to the cell surface containing a biopolymer exhibiting hydrophile-lipophile interactions, becoming monodisperse in a hydrophobic environment. The particular structure of the cell surface of alkane-grown cells favors the binding of the hydrocarbon by adsorption. The differences in the alkane binding affinity of glu-

cose- and alkane-grown cells appear to be due to the surface-localized components and the structure of the cell wall. Glucose-grown cells lack the mannan-fatty acid complex and exhibit a smooth surface. The cell surface structure becomes unfavorable for the adsorption of hydrocarbon, which is expressed as a lower alkane binding affinity and an enhanced dependence on finely emulsified hydrocarbon.

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