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Component from the Cell Surface of the Hydrocarbon-Utilizing Yeast *Candida tropicalis* with Possible Relation to Hydrocarbon Transport

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A polysaccharide-fatty acid complex was isolated from the cell surface of *Candida tropicalis* growing on alkanes. This complex was solubilized by Pronase treatment of whole cells. A decrease in alkane-binding affinity was observed after Pronase treatment, resulting in 10 to 12% of the yeast dry cell weight being released as polysaccharide. The isolated polysaccharide contained 2.5% fatty acids. *C. tropicalis* and *Saccharomyces cerevisiae* grown with glucose contained only traces of fatty acids in the corresponding polysaccharide fraction. The fatty acids were not removed from the polysaccharide moiety by gel filtration. Extraction of the polysaccharide with chloroform-methanol showed that fatty acids were covalently bound to the polysaccharide. The amphipathic nature of the isolated polysaccharide and the hydrocarbon-induced formation suggest a possible role in alkane metabolism.

The mechanisms of hydrocarbon transport by microorganisms have been studied extensively during the past decade. Two possible mechanisms for the transport of a liquid hydrocarbon substrate into the microbial cell have been proposed: (i) direct contact of the cells with emulsified substrate droplets (8), and (ii) transport of solubilized hydrocarbons from hydrocarbon micelles (1, 6, 12, 14). In each case, the cell surface serves as a mediator between the growth medium and the cell.

A previous report (10) has demonstrated a nonenzymatic adsorptive interaction between the substrate and the cell surface. It was concluded that the affinity between the cell surface and the hydrocarbon was due to the peripheral portion of the cell wall involving components to which the insoluble, hydrophobic substrate was adsorbed.

The present report deals with the chemical nature of the cell surface as related to alkane binding. The effect of partial degradation of the cell wall on the binding affinity of hydrocarbons as well as on the isolation of a polysaccharide-fatty acid complex are detailed.

MATERIALS AND METHODS

Organism. *Candida tropicalis* ATCC 32113 was used for all experiments.

Cultivation conditions and harvesting. A fraction of *n*-alkanes ranging from tetradecane to octadecane (9) or glucose was used as the sole source of carbon and energy. The medium was the same as described by Hug et al. (7).

Unless otherwise stated, cells were obtained from a continuous culture in a 10-liter bioreactor with a working volume of 5 liters and a dilution rate of 0.05/h. The cultivation conditions were described in an earlier report (9). The continuous culture was started with a batch culture. The samples for the experiments described were taken before complete steady-state conditions were reached.

The cells were harvested directly from the bioreactor and washed twice with ice-cold distilled water.

Quantitative determinations. Dry cell weight was determined by drying samples at 105°C for 24 h. Total carbohydrate was estimated by the phenol-sulfuric acid method of Dubois et al. (4). The fatty acid content was quantified by using methylcaprate as the internal standard. Protein was estimated by the method of Lowry et al. (11), using bovine serum albumin as the standard.

Partial degradation of the cell wall. The cells were incubated with proteases (Table 1) with agitation in a thermostated water bath. Trypsin, α-chymotrypsin, and carboxypeptidase were purchased from Boehringer, Mannheim, West Germany; Pronase was purchased from Serva, Heidelberg, West Germany; and subtilisin was purchased from Sigma Chemical Co., St. Louis, Mo. The proteases were applied at an enzyme-to-cell ratio (dry weight) of 1:10. The cell concentration was usually 100 mg (dry weight) in 10 ml of buffer solution.

Hexadecane-binding assay. Samples of 0.5 ml were removed from the tube where the proteolytic digestion was carried out and mixed with 5 ml of ice-cold 50 mM citrate-phosphate buffer (pH 5.5). After

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TABLE 1. *Incubation conditions for the partial degradation of the cell wall with proteases*

Protease	Buffer	Temp (°C)
Trypsin	0.05 M Tris-hydrochloride (pH 8.0)	25
α-Chymotrypsin	0.05 M phosphate (pH 7.0)	25
Subtilisin	0.01 M Tris-hydrochloride (pH 7.5)	37
Pronase	0.01 M Tris-hydrochloride (pH 7.8)	37
Carboxypeptidase	0.01 M Tris-hydrochloride (pH 7.8)	37

centrifugation (3,500 × *g* for 5 min), the cells were suspended in the same buffer containing 5 mM KCN, and the binding affinity was measured as described previously (10). The cells were then incubated with finely emulsified hexadecane. After incubation, the cells were centrifuged and washed. The hexadecane adsorbed at the cell surface was extracted from the washed cell pellet with diethyl ether and estimated by gas-liquid chromatography. The amount of adsorbed hexadecane was taken as a measure of the binding affinity of the cell wall to hydrocarbon.

Precipitation of the polysaccharides from the Pronase digest. After digestion, the cells were separated by centrifugation (3,500 × *g* for 10 min). The supernatant was clarified by membrane filtration (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.). The polysaccharides were precipitated with 3 volumes of absolute ethanol in the presence of 0.5 mM $Na_2B_4O_7$ (5) at 0 to 4°C and removed by centrifugation (3,500 × *g* for 10 min).

Estimation of fatty acids in the polysaccharide released. The fatty acids were transesterified from the precipitated polysaccharides with 5 ml of 20% methanolic H_2SO_4 at 60°C overnight. The fatty acid methyl esters were extracted with pentane, and samples were analyzed with a Beckman GC4 gas chromatograph fitted with DEGS-PS (10% on Supelcoport; 80/100 mesh) stainless-steel columns (0.32 by 250 cm). The temperature was programmed from 100 to 200°C at a rate of 11°C/min. The nitrogen flow was 20 ml/min.

Identification of fatty acids. For identification, a sample of fatty acid methyl esters was hydrogenated by the method of Appelquist (2). The saturated fatty acids were determined by test mixtures. Fatty acids were identified by comparing the hydrogenated and non-hydrogenated fatty acid composition.

Column chromatography. The polysaccharides were partially fractionated by gel filtration. The ethanol-precipitated polysaccharides were dissolved in 4 ml of 0.2 M KCl and applied to a Sepharose 6B column (2.6 by 32 cm; Pharmacia Fine Chemicals, Sweden). Elution was effected with 0.2 M KCl at a flow rate of 20 ml/h. Fractions (2 ml) were collected and analyzed for polysaccharides. The eluted polysaccharides were divided into three fractions (see Fig. 4). Each of these fractions was dialyzed against distilled water and evaporated to dryness in vacuo at 40°C. The residues were transesterified and analyzed for fatty acids.

Chloroform-methanol extraction of the polysaccharide-fatty acid complex. Ethanol-precipitated polysaccharide was dissolved in distilled water, and chloroform-methanol (1:2, vol/vol) was added to a final ratio of water-chloroform-methanol of 0.8:1:2 (vol/vol/vol). This monophasic system was left overnight at room temperature, back-extracted with 2 M KCl (20% of the volume), and separated into a chloroform-soluble phase, an interphase, and an aqueous phase. The interphase was separated from the aqueous phase by centrifugation (5 min, 3,500 × *g*) and dissolved in 0.01 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride buffer (pH 7.8). After two extractions with *n*-hexane, the polysaccharide was re-precipitated as described above. The polysaccharide was transmethylated and analyzed for fatty acids by gas chromatography. The aqueous phase was evaporated in vacuo at 40°C to a volume of about 10 ml, dialyzed against 0.01 M Tris-hydrochloride buffer (pH 7.8) overnight, and precipitated after two extractions with hexane. The polysaccharide from the aqueous phase was transmethylated and analyzed for fatty acids by gas chromatography.

The chloroform layer was evaporated and submitted to transesterification and subsequent gas chromatography. This fraction was also analyzed by thin-layer chromatography by using the solvent system petroleum ether-diethylether-glacial acetic acid (85:15:1, vol/vol/vol).

RESULTS

Effect of protease digestion of whole cells on the binding affinity of hexadecane. Earlier experiments (10) indicated that the affinity of the cell surface of *C. tropicalis* for hydrocarbon was due to the peripheral part of the cell wall. Therefore, the affinity of the cell surface for hexadecane was evaluated after partial degradation of the cell wall. The incubation of whole cells with the specified endopeptidases reduced the binding affinity of hexadecane to the cell surface (Fig. 1 and 2). However, no effect was observed with carboxypeptidase (Fig. 1).

The greatest reduction in binding affinity was observed with Pronase (Fig. 2). After 60 min of incubation, hexadecane was no longer adsorbed to the cell surface. Therefore, Pronase was used in the following experiments for the investigation of the released cell surface components. The kinetics of polysaccharide release showed that 10 to 12% of the yeast dry weight was released as polysaccharides by Pronase treatment (Fig. 3). No further polysaccharide material was solubilized after 45 min of incubation. This was coincident with the decrease in hexadecane-binding affinity (Fig. 2). After this period of incubation, the binding affinity approached its minimum value, indicating the

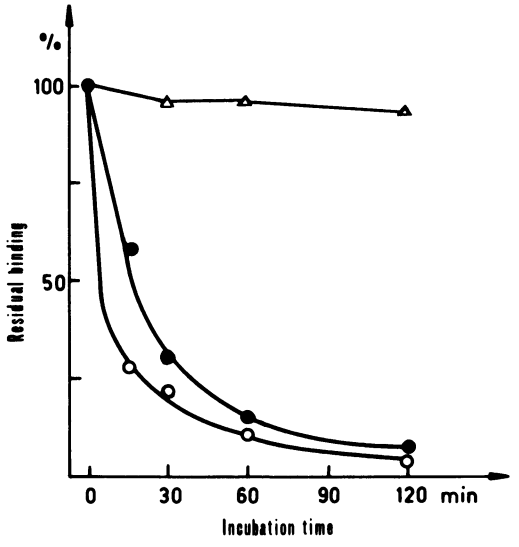

FIG. 1. *Influence of α-chymotrypsin (●), trypsin (○), and carboxypeptidase (△) on hexadecane binding to the cell surface of C. tropicalis.*

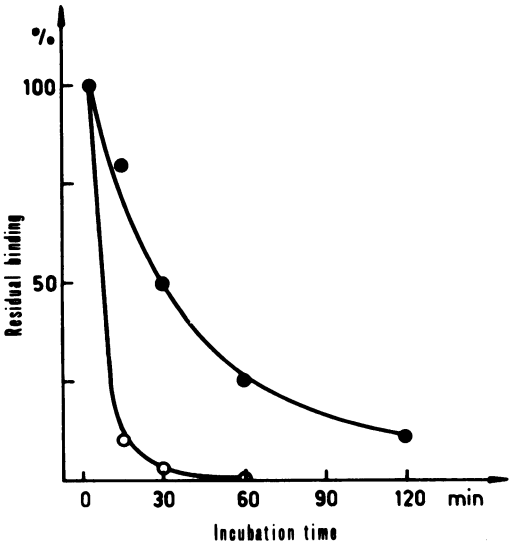

FIG. 2. *Influence of Pronase (○) and subtilisin (●) on hexadecane binding to the cell surface of C. tropicalis.*

involvement of the polysaccharide in the binding of hexadecane at the cell surface.

Polysaccharide-fatty acid complex released by Pronase. Polysaccharide represents the major constituent released from the yeast cell wall by proteases (3, 5). A polysaccharide fraction was precipitated by adding ethanol (3 volumes) to the clarified Pronase digest. The polysaccharides isolated constituted about 75% of the total polysaccharide released by Pronase. The composition of the polysaccharide was: protein/sugar, 0.3 mg/mg; and fatty acid/sugar, 25 μg/mg. Compared with the polysaccharides released from glucose-grown cells of *C. tropicalis* and *S. cerevisiae*, the polysaccharide fraction of alkane-grown cells of *C. tropicalis* contained a higher concentration of fatty acids (Tables 2 and 3). The data indicate that the incorporation of fatty acids into the peripheral polysaccharides was induced by growth on alkanes.

Properties of the polysaccharide-fatty acid complex. The polysaccharide-fatty acid complex was submitted to gel filtration to determine whether the fatty acids were bound to the polysaccharide (Fig. 4). The column eluate was

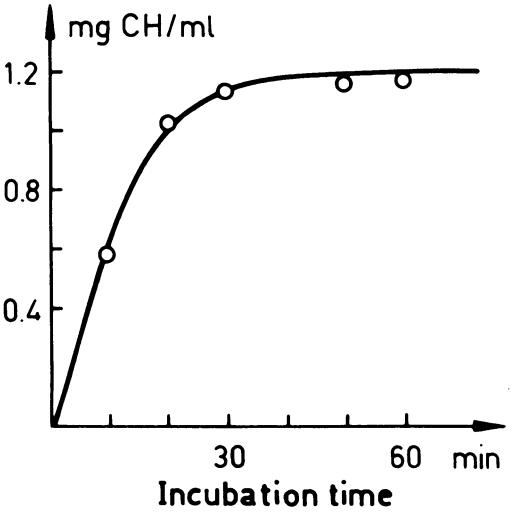

FIG. 3. *Release of polysaccharide by Pronase. 10 mg of dry cells per ml was incubated with 1 mg of Pronase per ml. CH, Carbohydrate.*

TABLE 2. *Fatty acid content of polysaccharides released by Pronase from C. tropicalis grown on glucose and n-alkane, respectively, and from S. cerevisiae grown on glucose*

Yeast	Carbohydrate released (mg/g [dry cell wt])	Fatty acid content (% of carbohydrate)[a]
C. tropicalis		
Glucose	115	0.1
Hydrocarbon	120	2.5
S. cerevisiae[b]	45	Trace

[a] Fatty acids were estimated after precipitation of the polysaccharides with ethanol.

[b] Cultivated in a 1-liter Erlenmeyer flask in a volume of 250 ml.

TABLE 3. *Fatty acid composition after transesterification of the total polysaccharide released by Pronase treatment and of the polysaccharide fractions formed after gel filtration*

Fatty acid	Total	Fraction 1	Fraction 2	Fraction 3
12:0	0.6[a]	0.8	1.6	0.4
14:0	4.7	6.1	6.5	3.6
15:0	1.1	1.3	1.5	1.5
16:0	33.6	36.0	40.1	43.6
16:1	19.9	16.5	10.4	11.2
17:1	0.2			4.7
18:0	4.5	7.4	14.2	11.0
18:1	22.2	26.8	20.8	17.4
18:2	12.0	4.0	4.7	6.5

[a] Numbers are percentages.

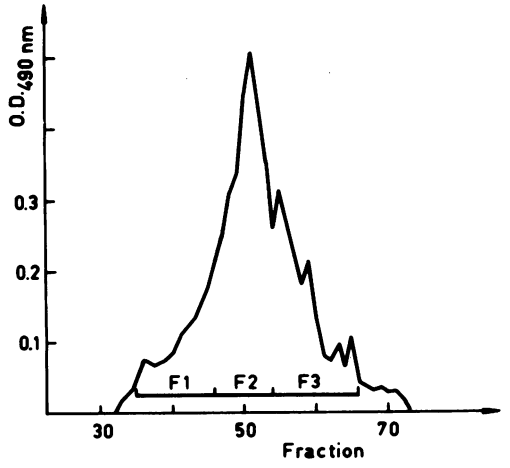

FIG. 4. *Gel filtration on Sepharose 6B of the ethanol-precipitated polysaccharide-fatty acid complex. Column, 2.6 by 32 cm; flow rate, 20 ml/h; sample volume, 2 ml. Samples (0.25 ml) were analyzed for polysaccharide. The bars indicate the fractions (F1, F2, F3) that were pooled.*

arbitrarily divided into three parts, and the amount of carbohydrate and fatty acids in each fraction was determined (Table 4). Fatty acids were present in each fraction, with no specific enrichment of particular fatty acids (Table 4). Compared with the fatty acid composition of the total polysaccharide released by Pronase, the fatty acid pattern of all three fractions showed an increase in $C_{16:0}$ and $C_{18:0}$ fatty acids and a decrease in the amounts of the corresponding unsaturated fatty acids (Table 3).

Analysis of the fractions formed after chloroform-methanol extraction showed that fatty acids remained bound to the polysaccharide. By thin-layer chromatography of the chloroform-soluble residue, only hexadecane and orcinol-positive lipid were detected. The orcinol-positive component did not move in the solvent system used. The carbohydrate content of this fraction was confirmed with the phenol-sulfuric acid method (4). Transesterification of the chloroform-soluble residue yielded 62% of the total fatty acids present in the original complex. The aqueous phase contained 26% of the originally applied polysaccharide and 11% of the fatty acids. At the interphase, 56% of the polysaccharide accumulated, with a fatty acid portion of 27%. The recovery of polysaccharide in these two fractions was 80 to 85% of the total.

TABLE 4. *Polysaccharide and fatty acid composition of the three fractions formed after gel filtration of the polysaccharide released from the cell surface of C. tropicalis by incubation with Pronase*

Fraction	Polysaccharide content (μg)	Fatty acid content (μg)	Fatty acid content of polysaccharide fraction (%)
1	970	30	3.6
2	1,960	55	2.8
3	1,270	27	2.1
Total	4,200	112	2.7

An important property of the polysaccharide-fatty acid fraction was its emulsifying characteristics. Hexadecane was added to an aqueous solution of the isolated polysaccharide-fatty acid complex, and the clearing time of the emulsion after sonic treatment was measured. The hexadecane concentration was determined at a point 1 cm above the bottom of the vessel. The isolated component markedly enhanced the clearing time of the emulsion, indicating emulsifying activity.

DISCUSSION

We have demonstrated that the cell surface of *C. tropicalis* grown on a hydrocarbon substrate exhibits a binding affinity for alkanes (10). The isolated polysaccharide-fatty acid complex from the cell surface of *C. tropicalis* represents a part of the alkane-binding system. Experimental data supporting this role are: (i) the binding affinity of hexadecane was reduced upon removal of the polysaccharide-fatty acid complex from the cell surface; (ii) the formation of the complex was induced by hydrocarbon substrate; and (iii) the complex exhibited emulsifying properties for hexadecane.

The polysaccharide-fatty acid complex was solubilized from the cell surface by proteolytic digestion. These polysaccharides are complexed

with proteins, and endopeptidase digestion products contain peptide residues (3, 5). The polysaccharide-fatty acid complex contained approximately 30% protein. Carboxypeptidase digestion did not release a significant amount of polysaccharide and did not affect the binding affinity.

The presence of covalently bound fatty acids in the surface-localized polysaccharide of the cell wall was specific for *C. tropicalis* grown on alkanes. It was demonstrated that the fatty acids cannot be removed by gel filtration. The specified distribution of the fatty acids after chloroform-methanol extraction indicated that the polysaccharide-fatty acid complex is present in forms of various hydrophile-lipophile balances. Differences in the proportion of hydrophilic to lipophilic portions were expressed in different solubility properties.

The composition of the cell wall proteins of *C. tropicalis* during growth on glucose and *n*-alkanes was analyzed by Rylkin et al. (13). The growth substrate did not significantly influence the amino acid composition of the wall protein. Cell wall proteins of glucose-grown cells contained more lysine, histidine, arginine, and methionine, but less serine, threonine, and glutamic acid, than those grown on *n*-alkanes. Generally, no increase in the lipophilic nature of the protein resulted from a change in the amino acid composition. A contribution of the protein component to the hexadecane-binding affinity cannot be assumed to be a result of a change in amino acid composition. However, it is possible that fatty acids are bound to protein and exhibit a mode of action similar to that of the polysaccharide-fatty acid complex.

The involvement of the polysaccharide-fatty acid complex in alkane binding relates to the water-insoluble characteristics of long-chain *n*-alkanes. In a bioreactor, the hydrocarbon exists in a biphasic suspension due to hydrophile-hydrophobe repulsion. If the cell surface contains a biopolymer that exhibits amphipathic properties, the hydrocarbon will adsorb preferentially to this component, becoming monodisperse in a hydrophobic environment. The loss of the binding affinity after removal of the polysaccharide-fatty acid complex suggests such a role for the isolated complex.

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