Uptake of Phenol by *Trichosporon cutaneum*

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The soil yeast *Trichosporon cutaneum*, which is distinguished by having a strictly oxidative metabolism, can be induced to utilize phenol as a sole carbon source. The present paper shows that such phenol-induced cells contain a specific, energy-dependent uptake system for phenol. Phenol uptake is not directly linked to its o-hydroxylation inside the cell, the first step of phenol metabolism. The $K_m$ for uptake is $235 \pm 30 \mu M$, that for hydroxylation only $4.5 \pm 0.5 \mu M$. Further, the phenol analog 2,6-dimethylphenol, which cannot be hydroxylated, competes with phenol for the uptake system. The pH dependence of uptake indicates that phenolate is an essential form during the uptake process. The energy requirement for phenol uptake is indicated by effects of various inhibitors of energy generation, including proton-conducting uncouplers. Direct monitoring of proton movements in a pH-stat during phenol uptake indicates a phenol-proton symport. One proton is cotransported with every phenol molecule. Phenol competes with the uptake of sucrose and glyceral by cells grown on these substrates. Under such conditions the uptake of phenol seems to proceed through a different system, with lower affinity for phenol than in phenol-grown cells.

It is now well established that the selective uptake of nutrients into microbial cells is an active process characterized by stereospecificity, saturation kinetics, and energy requirement. Constitutive and inducible transport systems in yeast for sugars have been described previously (3, 9, 12, 17). An increasing body of evidence points to chemiosmotic phenomena playing a central role in coupling energy to transport processes. The participation of proton symport in the transfer of nutrients into microbial cells has been shown in both, bacteria (8) and some lower eucaryotes (4, 11, 23) including different yeasts (1, 4, 10, 19-21).

In previous work, we have isolated strains of the soil yeasts *Trichosporon cutaneum* and *Candida tropicalis*, which can be induced to metabolize phenol as a sole carbon source by a sequence of intracellular enzymes (5-7, 14, 16, 24). In the present work, we address the question of whether an active transport system for phenol exists in phenol-induced *T. cutaneum* cells, and if so, what is its energy source and its relation to the enzymes participating in phenol metabolism.

**MATERIALS AND METHODS**

**Chemicals and equipment.** All chemicals were commercial products of reagent grade, whenever available, and purchased as described (14). Triton X-100 was from Rohm & Haas (Philadelphia, Pa.). Uniformly $^{14}$C-labeled compounds were obtained from the Radiochemical Centre (Amersham, England). Biofluor scintillation counting solution was from New England Nuclear Corp. (Boston, Mass.). Carbonyl cyanide p-trifluoromethoxy-phenyl hydraczone (FCCP) and oligomycin were products of Boehringer GmbH (Mannheim, Germany). Membrane filters were from Sartorius GmbH (Göttingen, Germany). Proton uptake was monitored in a pH-stat, with Titirigraph (model SBR3; Radiometer, Copenhagen, Denmark).

**Organism and culture conditions.** *Trichosporon cutaneum* was grown at 28°C on a rotary shaker in the medium described by Neujahr and Varga (16), containing salts and 0.05% yeast extract. In most experiments, the cells were grown for 17 h with phenol as the main carbon source. The initial concentration was 0.03%, and it was readjusted once, 2 h before harvest, to ensure exponential growth.

In a series of experiments, alternative carbon sources were used instead of phenol, usually at the 1% level. These included glucose, sucrose, maltose, glycerol, and acetate. Also these cultures were grown overnight and harvested during the exponential phase of growth. The cells were collected by centrifugation at 4 to 8°C and washed twice with distilled water.

**Uptake studies.** All cell preparations were harvested and used for uptake studies on the same day. Washed cells were suspended in buffers as indicated. Portions (1 ml) were placed in test tubes (16 by 64 mm) and shaken in a water bath at 28°C. Uptake was initiated by the addition of $[^{14}$C]phenol, and it was stopped by taking out samples of cells, which were either rapidly filtered or diluted with a volume (40- to 50-fold) of ice-cold distilled water and then filtered through Sartorius membrane filters (pore size, 0.45 μm). After being washed thoroughly with distilled water, the filters were transferred to vials for liquid scintillation counting without being dried beforehand. The water retained by the filters (35 to 40 μl per filter) did not produce any internal quenching, for the scintillation cocktail (20 ml per sample) was able to incorporate up to 13% water. Controls consisting of boiled cells, as well as of filters without cells, were included into each series of experiments. Also the alternative carbon sources were added as U$^{14}$C-labeled compounds, and their uptake was calculated from the radioactivity retained by the cells.

The kinetic parameters of uptake were determined by linear regression analysis of double-reciprocal plots of initial uptake velocity versus phenol concentration (Lineweaver-Burk plots). Occasionally, Eadie-Hofstee plots were used as an additional method.

**Determination of metabolic $^{14}$CO$_2$.** Washed-cell suspensions were incubated with $[^{14}$C]phenol, essentially as described above but with 3-ml suspensions in test tubes (14 by 120 mm) fitted with side arms. The side arm of the incubation tube was connected to a syringe containing 6 M HCl, and the upper end was plugged with cotton soaked in 20% KOH as an absorber for the $^{14}$CO$_2$. At the end of the incubation, HCl was added from the syringe to yield a pH of ca. 1 to
terminate the reaction and release dissolved $^{14}\text{CO}_2$. The mixture was vigorously and repeatedly stirred on a whirl-
mixer to ensure complete absorption of the released $^{14}\text{CO}_2$. The cotton plug was then transferred to a vial for liquid
scintillation counting. Calculations were based on the assumption that all of the radioactivity counted was in the form
of $^{14}\text{CO}_2$.

Permeabilization procedures and enzyme assays in situ. The cells were permeabilized essentially as described by Miozzi-
ari et al. (13). Cell pellets were resuspended to 10 to 20 mg (dry weight) per ml in 0.1 M HEPES (N-2-hydroxyethylpi-
erazine-N'-2-ethanesulfonic acid) (pH 7.5) containing 0.05% Triton X-100. The suspensions were kept frozen at
$-20^\circ$C for at least 40 h. After thawing in an ice bath, the suspensions were used without further treatment for enzyme
assays. Controls, in which the detergent was removed before the enzyme assays, gave identical results.

Enzyme activities were measured spectrophotometrically, phenol hydroxylase was measured as described by Neujahr and Gaal (14), and catechol 1,2-oxidase was measured as described by Varga and Neujahr (24). For the enzymes 1 U is
defined as the amount of enzyme which causes the conversion of 1 $\mu$mol of substrate or cosubstrate per min.

Determination of the ATP content of cells. Yeast cells corresponding to 1.7 mg (dry weight) were extracted with 3%
perchloric acid containing 2.5 mM EDTA at 0°C for 10 min. After centrifugation the supernatant was neutralized with
NaOH and used for ATP determination by the luciferin-
luciferase method (Adenosine-5'-triphosphate). Biolumines-
cence assay using luciferase (firefly), in Topics in biochemis-
try, 2nd ed., Boehringer Mannheim GmbH, Biochimica,

Proton movement studies. Cells were washed twice and suspended in distilled water. Proton movements were moni-
tored in a pH-stat by direct recording of the consumption of
2mM HCl required to reestablish the initial pH after addition
of phenol, energy inhibitors, and so on.

RESULTS

Kinetic characterization of the transport system and of the
enzymes initiating phenol metabolism. Studies of the time
course of $[^{14}\text{C}]$phenol uptake at pH 7.5 showed an increasing
incorporation for at least 20 min (Fig. 1A).

In order not to underestimate the rate of phenol uptake, it
was necessary to account for losses of $^{14}\text{C}$ as $^{14}\text{CO}_2$.
Measurements of $^{14}\text{CO}_2$ collected from cells incubated
with $[^{14}\text{C}]$ phenol for 10 min showed a loss of 3% of total uptake
(data not shown). For the estimation of initial uptake rates,
2-min incubations were selected. During that time the uptake
proceeded linearly and the loss of radioactivity as $^{14}\text{CO}_2$ was
guarded as negligible.

Figure 1B illustrates the saturation of the uptake system as
a function of phenol concentration. Calculations from six
independent experiments gave a $K_m$ of $235 \pm 30$ nM and a
$V_{max}$ of $49 \pm 9$ nmol min$^{-1}$ mg$^{-1}$ of cells$^{-1}$. In the experiment shown, the $K_m$ (for phenol) was $205$
M and the $K_m$ (for phenol) in the presence of 1.5 mM 2,6-
dimethylophenol was 436 M, whereas the $V_{max}$ was 48 nmol
of phenol min$^{-1}$ mg$^{-1}$ of cells$^{-1}$ in both cases.

FIG. 1. Saturation kinetics of phenol uptake in T. cutaneum. (A)
Time course of phenol uptake. Incubation at 28°C in 0.1 M HEPES
(pH 7.5) containing 0.7 mg of cells (dry weight); $[^{14}\text{C}]$phenol
was added to a final concentration of 1.5 mM. Uptake was plotted
as nanomoles of $[^{14}\text{C}]$phenol mg of cells (dry weight)$^{-1}$. (B) Rate of
phenol uptake $(v)$ vs phenol concentration. Conditions were as in
Fig. 1A, with 2-min incubations and variable levels of phenol.
Uptake was plotted as nanomoles of phenol min$^{-1}$ mg of cells$^{-1}$
Inset: Double-reciprocal plot of $v$ versus the concentration of phenol
alone (○) and phenol in the presence of 1.5 mM 2,6-dimethylophenol
(●). Data obtained from six independent experiments gave a $K_m$
(for phenol) of $235 \pm 30$ nM and a $V_{max}$ of $49 \pm 9$ nmol min$^{-1}$ mg
of cells$^{-1}$. In the experiment shown, the $K_m$ (for phenol) was $205$
M and the $K_m$ (for phenol) in the presence of 1.5 mM 2,6-
dimethylophenol was 436 M, whereas the $V_{max}$ was 48 nmol
of phenol min$^{-1}$ mg$^{-1}$ of cells$^{-1}$ in both cases.

The pH dependence of phenol uptake rates in T. cutane-
num. Washed phenol-grown cells were suspended in 0.05 M buffers,
MES [2-(N-morpholinoethanesulfonic acid)]-NaOH (pH 5.5 to 6.5)
and HEPES-NaOH (pH 7.0 to 8.5). Inset: uptake rate at 1.5 mM
$[^{14}\text{C}]$phenol, in the pH range 5.5 to 8.5. The main diagram shows
uptake rate versus phenol concentration at pH 5.5 (○) and pH 7.5
(△). Replot of the main diagram in a Lineweaver-Burk fashion (not
shown) gave a $K_m$ of 0.2 mM at pH 7.5 and 99 mM at pH 5.5.
The corresponding $K_m$ values based on calculated phenolate concen-
trations, instead of total phenol, are 1 mM at pH 7.5 and 4 mM at pH
5.5.
catechol, interfered with the uptake of phenol in a competitive manner in most cases (data not shown). Resorcinol was an exception, as it did not affect the uptake of phenol when added under identical conditions. There was no effect on [14C]phenol uptake by m- or p-hydroxybenzoate or by tyrosine (data not shown).

The pH dependence of phenol uptake. Figure 2 shows the pH dependence of the rate of phenol uptake. At 1.5 mM [14C]phenol, a level which saturates the system at pH 7.5 (Fig. 1B), the uptake rate increased with increasing pH (inset). At pH 5.5, the system required much higher concentrations of phenol to become saturated than it did at pH 7.5 (main diagram). The $K_m$ values for phenol were estimated to 0.2 mM at pH 7.5 (cf., Fig. 1B) and 99 mM at pH 5.5. When recalculated on the basis of concentration of phenolate, rather than on total phenol, the difference between the $K_m$ values becomes much smaller, 4 $\mu$M at pH 5.5, compared with 1 $\mu$M at pH 7.5. These results indicate that phenolate may be an essential form during uptake.

Effect of inhibitors of energy metabolism on phenol transport, ATP content, and phenol-degrading enzymes. Preincubation of cells for 2 or 15 min with NaN3, FCCP, and 2,4-dinitrophenol (2,4-DNP) resulted in decreased phenol uptake after a subsequent 2-min incubation with [14C]phenol. Figure 3 shows the results obtained after a 2-min preincubation.

Oligomycin, when added 2 min before phenol, did not affect the uptake (Fig. 3). However, the 15 min preincubation with oligomycin decreased phenol uptake to 35% of control. The ATP level of such cells decreased to 50% (data not shown). The ATP level of cells preincubated for 15 min with NaN3 or FCCP was unchanged.

Cells preincubated for 15 min with NaN3, FCCP, 2,4-DNP, and oligomycin showed decreased uptake values also after 30-min incubations with [14C]phenol. Such cells were permeabilized and assayed for the enzymes initiating phenol metabolism, viz phenol hydroxylase and catechol-1,2-oxidase (EC 1.13.1.1). No inhibition of the enzymes could be detected.

These results indicate that phenol uptake is an energy-dependent process. The effects of the proton-conducting uncouplers FCCP and 2,4-DNP point to the involvement of a proton gradient.

Proton movements associated with phenol transport. Suspensions of washed cells in distilled water gave stable base lines in pH-stat at pH 5.4 to 5.9. Addition of phenol to such suspensions triggered the consumption of protons (Fig. 4). Table 1 summarizes the results obtained by simultaneous recording of proton movements and [14C]phenol uptake. These results indicate phenol-proton symport with a ratio during uptake of 1:1.

Phenol uptake was not significantly affected by the presence of NH$_4^+$, Na$^+$, or K$^+$ in concentrations up to 0.5 M. Simultaneous recording of proton movement and [14C]phenol uptake added together with FCCP or NaN3 showed that both inhibitors enhanced the uptake of phenol as well as the uptake of protons (cf. Fig. 4). However a stoichiometric relation between protons and phenol could no

![Figure 3](http://jb.asm.org/)

**FIG. 3.** Effect of energy inhibitors on phenol uptake. The cells were incubated with the indicated inhibitor (FCCP [ ]; NaN$_3$ [ ]; 2,4-DNP [ ]; oligomycin [ ]) for 2 min before the addition of 1.5 nM [14C]phenol. Samples were taken after 2-min incubation with phenol. Other conditions were as described in the legend to Fig. 1A. Higher concentrations of oligomycin caused aggregation of the cells. After 15 min of preincubation with 0.01 mM oligomycin, phenol uptake was reduced to 35% of control with a concomitant 50% reduction of ATP level. The ATP level of cells similarly incubated with FCCP or NaN$_3$ remained unaffected.

![Figure 4](http://jb.asm.org/)

**FIG. 4.** Effect of phenol, FCCP, and 2,4-DNP on the uptake of protons by T. cutaneum. Washed cells, 1.4 mg (dry weight), were suspended in 1 ml of distilled water at 28°C. Stable base lines were obtained at pH 5.4 to 5.9. Addition of phenol was to a final concentration of 83 mM; addition of FCCP was to 0.02 mM; addition of NaN$_3$ was to 5 mM.

### TABLE 1. Protons cotransported with phenol

<table>
<thead>
<tr>
<th>Phenol (mM)</th>
<th>Phenol uptake (nmol mg of cells [dry wt]$^{-1}$)</th>
<th>Proton uptake (nmol mg of cells [dry weight]$^{-1}$)</th>
<th>Phenol-to-H$^+$ ratio</th>
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<tr>
<td>45</td>
<td>30</td>
<td>25</td>
<td>1.2</td>
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<td>53</td>
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longer be found (data not shown). Possibly, the uncoupler-triggered collapse of the proton gradient caused phenol to enter the cell in an uncontrolled process.

**Uptake of phenol in cells grown on alternative carbon sources.** Phenol competed with the uptake of glycerol and sucrose by cells grown on these respective carbon sources (Fig. 5). No such effect was observed with cells grown on maltose, glucose, or acetate.

Small amounts of \(^{14}C\)phenol were taken up by glycerol- or sucrose-grown cells. The uptake systems showed saturation kinetics, with much lower affinity for phenol than phenol-grown cells. The \(K_m\) for phenol at pH 7.5 was estimated to be 5 mM for sucrose-grown cells and 8 mM for glycerol-grown cells. The corresponding \(V_{max}\) values were 47 and 49 nmol min\(^{-1}\) mg of cells (dry weight)\(^{-1}\), respectively.

DISCUSSION

Phenol degradation in *T. cutaneum* begins with \(\alpha\)-hydroxylation by the flavoprotein enzyme phenol hydroxylase (14). Comparison of \(K_m\) and \(V_{max}\) values for phenol uptake and hydroxylation suggests that the uptake does not proceed against a concentration gradient. However, the transport system does not seem to be directly linked to phenol metabolism. Thus, 2,6-dimethylphenol, which can not be hydroxylated by phenol hydroxylase and further metabolized by the cells, gives competitive inhibition of \(^{14}C\)phenol uptake, whereas resorcinol, a good substrate of phenol hydroxylase (15), does not interfere with phenol uptake.

The proton-conducting uncouplers FCCP and 2,4-DNP inhibit phenol uptake (Fig. 3) without affecting the ATP level of the cells or phenol metabolism. A stoichiometrically related proton uptake accompanying phenol is demonstrated (Table 1). These results indicate that phenol transport is driven by a gradient of protons across the plasma membrane.

A decrease in the cellular ATP level caused by prolonged incubation of cells with oligomycin occurs concomitantly with inhibition of phenol uptake (see legend to Fig. 3), whereas short-time incubations with this compound do not significantly affect uptake (Fig. 3). We interpret these results as consistent with the idea that ATP is not directly involved in phenol transport but is needed for the generation of the proton gradient. Such interpretation is supported by the observation of other authors that the generation of a proton gradient for nutrient uptake in yeast is accomplished by an ATP-driven proton pump, the yeast plasma membrane ATPase (2, 22).

Our results show that Na\(_2\)S affects the proton-pumping activity in a way similar to that of FCCP (Fig. 4) but has no effect on the ATP content of the cells (legend to Fig. 3). The effect of Na\(_2\)S on phenol uptake could thus be explained by assuming that this agent acts as a proton-conducting uncoupler, in addition to its previously known effects on specific enzymes. Information in the literature is not complete in this respect, although some support for this idea is given by several authors (18, 19, 21).

Phenol is cotransported with protons with a stoichiometry of 1:1 (Table 1). The involvement of phenolate in phenol transport, as indicated by the pH dependence of uptake (Fig. 2), could explain how electroneutrality is maintained in the system since there was no indication of export of a positive ion (K\(^+\), Na\(^+\), or NH\(_4^+\)).

The data presented here indicate that phenol-grown *T. cutaneum* cells possess a transport system for phenol, which is characterized by saturation kinetics, considerable specificity with respect to phenol analogs, and by an energy requirement. The remaining criterion of active transport, that of the system being able to work against a concentration gradient, has not been demonstrated. Under normal physiological conditions, it would be suicidal for the cells to concentrate phenol intracellularly. The ability of the transport system to do so could possibly be demonstrated using the nonmetabolizable analog 2,6-dimethylphenol. However,
the prohibitive cost to obtain the latter as labeled compound ($5,000 [U.S.] for a small quantity) has prevented us from testing this possibility.

The question of how the transport system for phenol in T. cutaneum cells is induced and regulated is now the subject of further studies. The results obtained with cells grown on alternative carbon sources indicate the occurrence of additional transport system(s) for phenol, with much lower affinity for phenol than the uptake system in phenol-grown cells.

LITERATURE CITED