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Screening for L-Arabinose Fermenting Yeasts†

BRUCE S. DIEN,*[,1] CLETUS P. KURTZMAN,[2] BADAL C. SAHA,[1] AND RODNEY J. BOTHAST[1]

[1]*Fermentation and Biochemistry Research and* [2]*Microbial Properties Research, National Center for Agricultural Utilization Research, Agricultural Research Service, US Department of Agriculture, 1815 North University Street, Peoria, IL 61604-3999*

ABSTRACT

Utilization of pentose sugars (D-xylose and L-arabinose) derived from hemicellulose is essential for the economic conversion of biomass to ethanol. Xylose-fermenting yeasts were discovered in the 1980s, but to date, no yeasts have been found that ferment L-arabinose to ethanol in significant quantities. We have screened 116 different yeasts for the ability to ferment L-arabinose and have found the following species able to ferment the sugar: *Candida auringiensis, Candida succiphila, Ambrosiozyma monospora,* and *Candida* sp. (YB-2248). Though these yeasts produced ethanol concentrations of 4.1 g/L or less, they are potential candidates for mutational enhancement of L-arabinose fermentation. These yeasts were also found to ferment D-xylose.

Index Entries: L-arabinose; pentose; ethanol; fermentation; yeasts.

INTRODUCTION

Ethanol has the potential to be an economic and environmentally sound alternative to petroleum for use in vehicular transportation. Corn starch is currently the primary feedstock for the US biofuel industry. However, a significant increase in ethanol production will depend on the fermentation of lignocellulosic biomass. Both the cellulosic and hemicellulosic components of the biomass will need to be fermented for the conversion to be economical *(1)*.

Fermentation of many hemicelluloses in agricultural biomass is problematic because it consists primarily of D-xylose and other pentoses that cannot be fermented by the yeast traditionally used to produce ethanol from starch. Much of the past research on hemicellulose fermentation has concentrated on D-xylose, because D-xylose is the most abundant pentose in almost all hemicelluloses *(2,3)*. However, L-

*Author to whom all correspondence and reprint requests should be addressed.

†Names are necessary to report factually on available data. However, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

arabinose is present in significant amounts in many hemicelluloses. One important example is the fiber that is separated during the wet milling of corn. Other examples include rice hulls, sugar beet residues *(4)*, sugar cane residues *(5)*, and the hemicelluloses of herbaceous species *(2)*.

Several bacteria *(4,6–11)*, three of which are recombinant, and a single filamentous fungus, *Paecilomyces* sp. NF1 *(12,13)*, have been reported to convert L-arabinose to ethanol efficiently. No yeast has been discovered to date that produces more than a trace amount of ethanol from L-arabinose *(2,14,15)*. Before the 1980s, yeasts were also thought to be unable to ferment all pentoses *(16–18)*. Many of the yeasts, which were subsequently discovered to ferment D-xylose, were screened by using microaerophilic culture conditions and directly testing for the presence of ethanol *(19–21)*.

Previous screenings for L-arabinose fermenters have been incomplete in that they have been limited to a few of the better known yeasts *(14)* or to some of the more efficient D-xylose-fermenting species *(2,14,15,22–27)*. We have now carried out an extensive screening of 116 yeast strains, from the ARS Culture Collection (National Center for Agricultural Utilization Research, Peoria, IL), some of which had previously been screened for arabitol production by this laboratory *(28)*. The yeasts were screened for L-arabinose fermentation under microaerophilic culture conditions, and ethanol was detected directly using gas chromatography (GC). The yeast strains screened were chosen on the basis of one or more of the following four sets of criteria:

1. Yeast species that aerobically catabolize D-xylose and L-arabinose and ferment glucose (101 strains).
2. Yeast strains that ferment glucose, aerobically catabolize L-arabinose, and do not catabolize D-xylose (six strains).
3. Yeast strains that are superior D-xylose fermenters (10 strains).
4. Yeast strains isolated from environments rich in L-arabinose (12 strains).

METHODS

Organisms

The yeast screened are listed in Table 1. All strains were obtained from the ARS Culture Collection (NRRL).

Media

Media were sterilized by autoclaving for 15 min. Sugars were autoclaved separately. Yeast cultures were maintained on YM slants (0.3 % [w/v] yeast extract, 0.3% [w/v] malt extract, 0.5% [w/v] peptone, 1.0% [w/v] glucose, and 1.5% [w/v] agar) incubated at 28°C and stored at 4°C. Cultures were transferred at least once to slants before being screened for L-arabinose fermentation.

All precultures and fermentations used yeast peptone (YP) (1% [w/v] yeast extract, 2% [w/v] Bacto peptone) supplemented with either L-arabinose or D-xylose. Precultures were grown on the same sugar as used for the fermentation and, unless stated otherwise, the sugar concentration was 8% (w/v). Medium ingredients were purchased from by Difco (Detroit, MI), and sugars were purchased from Sigma (St. Louis, MO).

Culture Conditions for L-Arabinose Fermentation Screening

Yeasts were screened for L-arabinose fermentation under microaerophilic culture conditions. Yeast strains were transferred from slants to 12-mL sterile polypropylene test tubes (Fisher Scientific, Springfield, NJ) containing 3 mL of medium. The tilted culture tubes were incubated with shaking (220 rpm) at 30°C in a Innova 4000 New Brunswick (Edison, NJ) incubator/shaker. After approx 24 h, 0.5-mL aliquots were removed from the culture tubes and used to inoculate foam-capped (Fisher Scientific) 125-mL Erlenmeyer flasks containing 30 mL of medium. The culture flasks were incubated with shaking as described for 48–72 h before being used for inoculation.

L-Arabinose fermentations were done in 50-mL Erlenmeyer flasks containing 30 mL of medium and 12 mL of inoculum from the above precultures. The flasks were sealed with rubber septa (Sigma). Each rubber septum was pierced with a 20-gage needle, with cotton inserted into the syringe end, to vent CO_2. The cultures were incubated as described for 12 d.

Fermentations were sampled (1-mL vol) every approx 48 h. Samples were centrifuged (8000*g* for 6 min) and the supernatant fluids recovered. Samples were analyzed for ethanol immediately, and then stored at –20°C for later analysis of sugars and sugar alcohols.

After the initial screening was completed, all the available strains that corresponded to the yeast species that fermented L-arabinose were screened. These strains were tested for the ability to ferment D-xylose using the same culture conditions. Yeast species that fermented L-arabinose were also tested to make sure they did not ferment the basal medium. The fermentations were performed as described earlier, except the cells used for inoculating these fermentations were washed three times with YP, and the medium used for the fermentations was not supplemented with sugar. The inoculum was grown on YP supplemented with L-arabinose.

Variation of Culture Conditions for L-Arabinose and D-Xylose Fermentations

In an attempt to increase ethanol yield from L-arabinose and D-xylose, the fermentation temperature was lowered (from 30 to 25°C) and aeration increased. Lower temperature and moderate aeration have been reported to give larger ethanol yields for some xylose fermentations *(29–31)*. Yeast strains were grown in culture tubes as described previously at 25°C, and after approx 24 h, 0.5-mL aliquots were transferred to foam-capped 250-mL Erlenmeyer flasks containing 50 mL of medium. Cultures were incubated with shaking for approx 24 h and 1-mL aliquots were used to inoculate foam-capped 500-mL Erlenmeyer flasks, each containing 100 mL of medium. These cultures were grown with shaking for 24 h. The cells were harvested by centrifugation (6 min at 6000*g*) and resuspend in 4 mL of YP. Two-milliliter aliquots from each suspension were transferred to the final fermentation flasks. The 125-mL Erlenmeyer flasks used for the fermentation each contained 50 mL of YP medium supplemented with either 10% (w/v) L-arabinose or D-xylose and were capped with Silicone Sponge Plug Closures (Bellco Glass). Cultures were incubated at 25°C and shaken at 140 rpm for 12 d. Cultures were sampled once a day as described.

Table 1
Yeasts Screened for L-Arabinose Fermentation

Species	NRRL no.	Species	NRRL no.
Ambrosiozyma monospora	Y-1081	*C. tenuis*	Y-12987
A. monospora	Y-1484	*C. tenuis*	Y-1498
A. monospora	Y-5955	*C. tenuis*	Y-17105
A. monospora	Y-5956	*C. tenuis*	Y-17106
A. monospora	Y-7403	*C. tropicalis*	Y-7661
Candida auringiensis	Y-11848	*C. verstilis*	Y-12819
C. auringiensis	Y-11849	*C. viswanathii*	Y-6726
C. auringiensis	Y-11850	*C. wickerhamii*	Y-2563
C. boidinii	Y-2332	*Citeromyces matritensis*	Y-11797
C. buinensis	Y-11706	*Debaryomyces (Wingea) robertsiae*	Y-6670
C. chilensis	Y-17141	*D. castellii*	Y-7423
C. conglobata	Y-1504	*D. hansenii*	Y-7426
C. dendronema	Y-7781	*D. hansenii*	YB-434
C. diddensiae	Y-2324	*D. nepalensis*	Y-7108
C. diddensiae	Y-7589	*D. nepalensis*	Y-7534
C. entomaea	Y-7785	*D. polymorphus*	Y-2022
C. entomophila	Y-7783	*D. pseudopolymorphus*	YB-4228
C. ernobii	Y-12940	*D. yamadae*	Y-11714
C. guilliermondii	Y-7572	*Geotrichum fermentans*	Y-1492
C. guilliermondii	YB-4760	*Kluyveromyces marxianus*	Y-1195
C. halophila	Y-2483	*K. marxianus*	Y-6373
C. hellenica	Y-17319	*K. marxianus*	Y-7571
C. insectorum	Y-7787	*Pachysolen tannophilus*	Y-2460
C. intermedia	Y-981	*Pichia tannicola*	Y-7499
C. kefyr	Y-1204	*P. acaciae*	Y-7773
C. membranamfaciens	Y-2089	*P. amylophila*	YB-1287
C. mogii	Y-17032	*P. angophorae*	Y-7842
C. naeodendra	Y-10942	*P. angusta*	Y-2214
C. nitratophila	YB-3654	*P. bimundalis*	Y-5343
C. parapsilosis	Y-7659	*P. bovis*	YB-4184
C. parapsilosis	YB-433	*P. burtonii*	Y-1933
C. peltata	Y-6888	*P. burtonii*	Y-2057
C. rhagii	Y-2596	*P. burtonii*	Y-7144
C. shehatae	Y-12856	*P. capsulata*	Y-1842
C. shehatae	Y-12858	*P. capsulata*	Y-2234
C. shehatae	Y-17029	*P. capsulata*	YB-2252
C. silvanorum	Y-7782	*P. ciferrii*	Y-1031
C. silvicola	YB-2251	*P. etchellsii*	Y-7121
C. silvicultrix	Y-7789	*P. etchellsii*	Y-7547
C. sp.	Y-10925	*P. guilliermondii*	Y-2075
C. sp.	YB-2090	*P. hangzkouana*	Y-17346
C. sp.	YB-2248	*P. haplophila*	Y-7860
C. sp.	YB-2249	*P. holstii*	Y-2155
C. sp.	YB-3082	*P. kodamae*	Y-17234
C. steatolytica	Y-7136	*P. methanolica*	Y-11993
C. succiphila	Y-11997	*P. methanolica*	Y-7685
C. succiphila	Y-11998	*P. methylovora*	Y-17250

Species	NRRL no.	Species	NRRL no.
P. mexicana	Y-11818	*P. silvicola*	Y-1679
P. mississippiensis	Y-11748	*P. silvicola*	Y-7005
P. mississippiensis	YB-1294	*P. stipitis*	Y-11545
P. naganishii	Y-7654	*P. stipitis*	Y-7124
P. nakazawae	Y-7903	*P. sydowiorum*	Y-10995
P. philogaea	Y-7813	*P. sydowiorum*	Y-10996
P. philogaea	Y-7814	*P. sydowiorum*	Y-7130
P. pinus	Y-11528	*P. trehalophila*	Y-6781
P. pinus	Y-7928	*Saccaromycopsis capsularis*	Y-17639
P. scolyti	Y-11540	*Saccaromycopsis capsularis*	Y-676
P. scolytii	Y-5512	*Zygoascus hellenicus*	Y-7923

Analytical Methods

Ethanol concentrations were determined by gas–liquid chromatography on a Hewlett Packard 5890A gas chromatograph (Hewlett Packard, Wilmington, DL) with a 80/100 Porapak Q column (6 ft × 1.8 in., Supelco, Bellefonte, PA) using a flame ionization detector. The injection temperature was set at 200°C, the oven temperature at 150°C, and the detector temperature at 250°C. Integration was performed by a Hewlett Packard 3393A Integrator. Sugars (L-arabinose and D-xylose) and sugar alcohols (arabitol, xylitol, and ribitol) were determined by high-pressure liquid chromatography with an HPX-87C column (300 × 7.8 mm; Bio-Rad Laboratories, Hercules, CA) at 85°C. The column was eluted with distilled H_2O at a flow rate of 0.6 mL/min. The detector used was either a Waters R401 or 410 Differential Refractometer (Milford, MA). Detected peaks were separated baseline to baseline.

RESULTS

Of the 116 strains screened for L-arabinose fermentation (Table 1), seven strains produced detectable levels of ethanol (Table 2). Most of the 116 strains screened converted L-arabinose to arabitol, and for some strains, trace amounts of xylitol were also detected (data not shown). The greatest amount of ethanol produced was 3.4 g/L by *Candida* sp. (YB-2248). However, yeasts fermented the L-arabinose slowly, and maximum ethanol concentrations were measured at or near the end of the fermentations, 14 d after inoculation (data not shown). Most yeast strains consumed less than half of the L-arabinose supplied and not all of the *A. monospora* or *C. auringiensis* strains fermented L-arabinose. The yeasts that fermented L-arabinose did not produce any ethanol from YP medium not supplemented with L-arabinose (data not shown).

All 11 strains fermented D-xylose (Table 3). The ethanol concentration measured ranged from 2.1 to 20.4 g/L. As observed for L-arabinose fermentations, the maximum ethanol concentration for most strains was measured on the last day of the fermentation. These yeasts tended to leave less residual D-xylose than L-arabinose (Table 2) and most also produced xylitol as a coproduct (Table 3). In general, the better D-xylose fermenters were the yeasts that also fermented L-arabinose.

The fermentation temperature was lowered from 30 to 25°C, and aeration increased in an attempt to increase the yield of ethanol from L-arabinose. Only the

Table 2
Variation in Ethanol Production by L-Arabinose-Fermenting Yeasts[a]

Species	NRRL no.	Max. ethanol, g/L	Max. arabitol, g/L	% L-Arabinose used
A. monospora	Y-1081	0.8 ± 0.2	0 ± 0	14 ± 6
	Y-5955	1.8 ± 0.7	0 ± 0	19 ± 8
	Y-5956	nd	0 ± 0	18 ± 4
	Y-7403	nd	0 ± 0	13 ± 5
	Y-1484	2.3 ± 0.4	4 ± 0	36 ± 4
C. auringiensis	Y-11849	nd	37 ± 20	75 ± 26
	Y-11850	nd	11 ± 3	35 ± 3
	Y-11848	1.4 ± 0.6	17 ± 1	32 ± 1
C. succiphila	Y-11997	2.1 ± 0.3	8 ± 1	43 ± 2
	Y-11998	2.3 ± 0.4	8 ± 0	32 ± 6
Candida sp.	YB-2248	3.4 ± 0.1	4 ± 0	33 ± 5

[a]All strains were tested in duplicate. nd, not detected.

Table 3
Ethanol Production from D-Xylose Fermentation by L-Arabinose-Fermenting Yeast Species

Species	NRRL no.	Max. ethanol, g/L	Max. xylitol, g/L	% Xylose used
A. monospora	Y-1081	6.2	10	44
	Y-5955	2.1	5	20
	Y-5956	4.7	0	13
	Y-7403	7.6	7	44
	Y-1484	20.4 ± 1.6	31 ± 3	78 ± 8
C. auringiensis	Y-11849	6.6	0	33
	Y-11850	4.1	0	18
	Y-11848	3.6 ± 0.2	0 ± 0	20 ± 4
C. succiphila	Y-11997	5.5	16	71
	Y-11998	17.1 ± 0.8	18 ± 1	66 ± 7
Candida sp.	YB-2248	14.2 ± 1.1	25 ± 0	96 ± 5

[a]For each strain tested in duplicate, the average ± the half difference is shown.

strain that produced the highest concentration of ethanol at 30°C from each species was tested. The results are shown in Table 4. Both *A. monospora* and *C. succiphila* reached approximately twice the ethanol concentration under the new culture conditions compared to the previous fermentations (Table 2). However, *Candida* sp. (YB-2248) produced less ethanol, and *C. auringiensis* produce none. All four yeasts fermented L-arabinose slowly. Two of the strains (*C. auringiensis* and *C. succiphila*) consumed all of the L-arabinose, converting most of it into arabitol. In contrast, *A. monospora* did not produce arabitol as a coproduct.

These yeast strains, along with *Pichia stipitis*, were also tested for fermentation of D-xylose (Table 5) under the same conditions. *P. stipitis*, which is one of the better D-xylose fermenters *(32,33)*, produced 35.0 g/L of ethanol. In comparison, the L-arabinose-fermenting yeast produced approximately a third of this amount

Table 4
Fermentation of L-Arabinose at 25°C with Moderate Aeration[a]

Species	NRRL no.	Max. ethanol, g/L[b]	Max. arabitol, g/L	% L-Arabinose used
A. monospora	Y-1484	4.1 ± 0.3 (7)	0 ± 0	27 ± 1
C. auringiensis	Y-11848	nd	73 ± 3	100 ± 0
C. succiphila	Y-11998	3.9 ± 0.6 (7)	81 ± 1	100 ± 0
Candida sp.	YB-2248	1.7 ± 0.7 (9)	6 ± 0	42 ± 1

[a]All strains tested in duplicate.
[b]Numbers in parentheses are the average number of days before maximum ethanol concentration was measured.

Table 5
Fermentation of D-Xylose at 25°C with Moderate Aeration[a]

Species	NRRL no.	Max. ethanol, g/L[b]	Max. xylitol, g/L	% D-Xylose used
A. monospora	Y-1484	18.2 ± 0.5 (9)	22 ± 3	100 ± 0
C. auringiensis	Y-11848	11.5 ± 1.3 (6.5)	4 ± 3	67 ± 5
C. succiphila	Y-11998	12.5 ± 1.0 (9)	12 ± 1	57 ±13
Candida sp.	Y-2248	12.3 ± 0.3 (9)	20 ± 1	96 ± 0
P. stipitis	Y-7124	35.0 ± 0.4 (3)	0 ± 0	100 ± 0

[a]Replicates were run for all strains.
[b]Numbers in parentheses are the average number of days into the fermetation when the maximum ethanol concentration was measured.

and fermented the sugar more slowly. *A. monospora* produced the most ethanol (18.2 g/L). Although *P. stipitis* produced ribitol as a coproduct (data not shown), the other yeast produced xylitol. Except for *C. auringiensis*, the yeasts produced similar concentrations of ethanol under both aeration conditions.

DISCUSSION

Rarity of L-Arabinose Fermenters

The majority of the 101 yeast species tested were able to convert L-arabinose to arabitol. The results of previous, more limited, screenings also have reported that yeasts and other fungi able to catabolize L-arabinose aerobically will commonly convert it to arabitol when oxygen limited *(2,14,28)*. Only four species, 4% of the yeast species screened, were able to ferment L-arabinose. The rarity of L-arabinose fermenters might arise from a putative redox imbalance in the L-arabinose pathway and/or the low incidence of L-arabinose-rich environments found in nature.

The L-arabinose and D-xylose pathways in yeasts and other fungi share the common intermediate xylitol, but whereas D-xylose is converted directly to xylitol *(18,34,35)*, the conversion of L-arabinose to xylitol requires two additional steps. L-Arabinose is converted to xylitol according to the following sequence: L-arabinose to arabitol to L-xylulose to xylitol *(18,36–38)*. Once xylitol is formed, whether from L-arabinose or D-xylose, it is oxidized to D-xylulose, phosphorylated to D-xylulose-5-phosphate, and enters the pentose phosphate pathway *(18,36,38)*. The

Table 6
Past Results for Fermentation of L-Arabinose by Fungi and Yeast

Species	Ethanol, g/L	References
Candida sp.	0.16	*15*
Candida sp. XF 217	0.68	*15*
C. tropicalis	Trace	*42*
Fusarsium oxysporum	1	*43*
Pachysolen tannophilus	Not reported	*26*
Paecilomyces sp. NF1	13.8	*12*
Schizosaccharomyces pombe	trace	*14*

formation of D-xylulose from L-arabinose compared to D-xylose requires an extra NADPH and NAD^+.

The characteristic formation of xylitol from D-xylose under oxygen-limited conditions and the inability of most yeasts to ferment D-xylose are theorized to be consequences of yeasts' inability to regenerate the NAD^+ used in the oxidation of xylitol to D-xylulose *(34,39,40)*. Yeasts that do ferment D-xylose well have D-xylose reductase activity that is cospecific for NADH and NADPH *(39)*, which allows for the regeneration of NAD^+. Conversion of L-arabinose to D-xylulose requires two oxidation steps, which are thought to use NAD^+ *(18,36,38)*. The inability of yeasts to regenerate NAD^+ might, therefore, hinder the ability of yeast to ferment L-arabinose to ethanol and instead favor arabitol production *(2)*.

Another possible reason for the rarity of L-arabinose fermenters might be the low incidence of L-arabinose-rich environments found in nature. Although L-arabinose is present in many hemicelluloses, the predominant pentose is almost always D-xylose *(2,3)*. Therefore, an environment relatively rich in L-arabinose is likely to be much richer in D-xylose. An exception is the water-soluble hemicellulose fraction of larch wood *(41)*. In this regard, it is significant that one of the L-arabinose fermenters (*Candida* sp. YB-2248) was isolated from larch wood. In fact, all the better D-xylose fermenters were isolated from D-xylose rich environments *(20,23)*.

Efficiency of L-Arabinose Fermentations

Of the yeast screened, *A. monospora* produced the highest concentration of ethanol (4.1 g/L) from L-arabinose. Those yeasts that fermented L-arabinose did so slowly. Previous results reported for yeasts and other fungi fermenting L-arabinose are listed in Table 6. The ethanol concentrations reported here are greater than those previously reported, except for the fungus *Paecilomyces* sp. NF 1.

Aeration was increased and temperature lowered in an attempt to increase final ethanol concentrations. Changing the culture conditions doubled the maximum ethanol concentrations measured for *A. monospora* and *C. succiphila*. However *Candida* sp. (YB-2248) and *C. auringiensis* produced more ethanol at the lower aeration and higher temperature.

L-Arabinose Fermenters Also Ferment D-Xylose

The yeast strains we found that fermented L-arabinose also fermented D-xylose. Only *C. succiphila* has previously been identified as a D-xylose fermenter

(20). The direct correlation between the ability to ferment L-arabinose and D-xylose is probably a consequence of the similarity of the biochemical pathways for fermentation of both of these sugars. A near-perfect correlation has been observed between aerobic catabolism of L-arabinose and D-xylose by yeasts *(18,44)*. Of the 214 yeast species identified by Barnett et al. (1983) *(45)* that aerobically catabolize L-arabinose, only 13 do not catabolize D-xylose *(5)*. As an aside, the six yeasts present in the ARS Culture Collection that utilize L-arabinose and not D-xylose were included in the screen, and none fermented L-arabinose.

The L-arabinose-fermenting yeasts fermented D-xylose more efficiently, producing on average five times more ethanol from D-xylose than L-arabinose. Like the L-arabinose fermentations, the D-xylose fermentations were also run under different aeration conditions and at 25°C. *P. stipitis* was included in the experiment for comparison. The maximum ethanol concentrations produced by the L-arabinose fermenters were approximately half of that formed by *P. stipitis* and the rates of ethanol formation much slower. The L-arabinose fermenters also produced the coproduct xylitol. *P. stipitis* did not produce xylitol as a coproduct during this fermentation, but has been reported under some culture conditions *(29)*.

The seven strains that have been discovered to ferment L-arabinose do not yet produce enough ethanol to be of commercial interest. However, these strains might be improved by mutagenesis. Previous to the discovery of *Pachysolen tannophilis* and other more efficient D-xylose fermenters, mutagenesis was used to increase the ethanol yield of an unknown *Candida* sp.-fermenting D-xylose fivefold *(15)*. If, instead natural enrichment is used to find a better L-arabinose fermenting yeast, the yeasts should probably be screened first for D-xylose fermentation and second for L-arabinose fermentation. All the yeasts found here to ferment L-arabinose fermented D-xylose better.

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