Candida aquaetextoris sp. nov., a New Species of Yeast Occurring in Sludge from a Textile Industry Wastewater Treatment Plant in Tuscany, Italy

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We describe *Candida aquaetextoris*, a new yeast species isolated from sludge that accumulates at the main wastewater treatment facility which processes discharges from textile factories located in the Prato metropolitan district, northern Tuscany, Italy. This yeast degrades 4-(1-nonyl)phenol, a toxic intermediate originating from the microbial attack of nonylphenol polyethoxylates, which are nonionic surfactants largely used in leather and textile industries. In the investigation we employed conventional and molecular taxonomy techniques to compare the new isolate to strains of physiologically similar species, such as *Candida maltosa* and *Candida tropicalis*, as well as strains of quite phenotypically different species, such as *Candida haemulonii*. The results demonstrate that the yeast which we identified represents a separate taxon. The type strain of *C. aquaetextoris* is strain Lmar1, which has been deposited in the Industrial Yeast Collection of the Division of Applied Microbiology, Department of Plant Biology, University of Perugia, Perugia, Italy, as strain DBVPG 6732.

In the course of an ecological study focusing on the biological degradation of nonionic surfactants in the whole treatment cycle of textile industry wastewater, a yeast strain which can use 4-(1-nonyl)phenol (pNP) as a sole energy and carbon source was isolated (1). This compound is a model of the isomeric nonylphenols that originate in wastewater depuration plants from microbial attack on the polyoxyethylene chains of the nonylphenol polyethoxylates, a family of nonionic detergents widely used by textile and leather manufacturers. The new isolate is also capable of growing on phenol and other phenol derivatives, as well as on several alkanes and alkylbenzenes.

Preliminary classification of the new strain, which was designated Lmar1^T (T = type strain), by using the rapid ID 32 C identification system (bioMérieux SA, Marcy l'Etoile, France) resulted in identification as *Candida maltosa*. The strain was then registered as strain DBVPG 6732^T. Since strain Lmar1^T was shown to utilize the unusual growth substrate pNP, in contrast to the various sources of energy and carbon reported for the type strain of *C. maltosa*, we decided to reexamine the new isolate and physiologically similar strains by using conventional and molecular taxonomy methods. Comparison of the phenotypic properties of the new isolate and its levels of DNA homology with *Candida* species in the present study revealed that strain DBVPG 6732^T represents a new species, for which the name *Candida aquaetextoris* is proposed.

MATERIALS AND METHODS

Samples of sludge collected from the secondary settlement tank at an activated sludge plant used for the treatment of textile industry wastewater were the biological matrix for yeast isolation. The new strain was obtained in axenic culture by using techniques described in detail elsewhere (5).

Comparative investigations of morphological and physiological characteristics, as well as molecular features, were carried out with other yeast strains, some of which were physiologically similar and some of which were not, obtained from

the Yeast Division of the Centraalbureau voor Schimmelcultures Collection, Delft, The Netherlands (Table 1).

Phenotypic characteristics of the new isolate were determined by methods commonly used in yeast taxonomy (6). Furthermore, a series of carbon compounds related in chemical structure to the nonylphenol polyethoxylates were also used in comparative assimilation tests. These compounds included (i) phenol derivatives, such as catechol, hydroquinone, 4-hydroxyacetylphenol, hydroxycinnamic acid, 3-(4-hydroxyphenyl)propanol, 3-(4-hydroxyphenyl)propionic acid, pNP, nonylphenol polyethoxylate (NP9EO), nonylphenoxyacetic acid, phenol, and resorcinol; (ii) benzoic acid derivatives, such as benzoate, diethylphthalate, dimethylphthalate, ethyl-4-hydroxybenzoate, methyl-4-hydroxybenzoate, phthalic acid, and protocatechuic acid; (iii) ethylene glycol and polymeric derivatives of ethylene glycol, such as diethylene glycol, polyethylene glycols (PEG) of different molecular weights, including PEG 300, PEG 600, PEG 1000, and PEG 2000, and triethylene glycol; and (iv) several alkanes and alkylbenzenes, such as *n*-hexadecane, *n*-hexane, *n*-nonane, *n*-octane, octylbenzene, phenoxyethanol, phenylglyssilic acid, 5-phenyl-1-pentanol, and 3-phenyl-1-propanol. These organic compounds were supplied to cultures of either isolate Lmar1^T or *C. maltosa* DBVPG 6143^T in Bacto Yeast Nitrogen Base without amino acids (Difco Laboratories, Detroit, Mich.) in order to determine whether they were utilized as carbon and energy sources. Chemicals were added to the yeast cultures as filter-sterilized (0.22- μ m-pore-size Nalgene filter) solutions. The final concentration of most compounds tested in the growth medium was 30 mg liter⁻¹; the exceptions were resorcinol, octylbenzene, and 5-phenyl-1-pentanol, which were added at a concentration of 50 mg liter⁻¹, and pNP, NP9EO, and phenol, which were added at a concentration of 100 mg liter⁻¹. Incubation was in flasks at 28°C on a rotatory shaker for 30 days in the dark. Yeast growth was evaluated at different times during cultivation (see Table 4) by measuring optical density at 660 nm. The slant culture method suggested by Middelhoven et al. (4) was also used to verify yeast growth at the expense of the potentially toxic compounds tested.

Resistance to 10% NaCl–5% glucose and resistance to 0.1 g of cycloheximide per liter were determined in liquid medium on a roller drum at 25°C by using the methods described by van der Walt and Yarrow (6). The maximum growth temperature was determined in tubes containing YEPD broth (1% yeast extract, 2% peptone, 2% glucose; Fluka Chemie AG, Buchs, Switzerland) equilibrated in a water bath.

Intact chromosomal DNA for pulsed-field gel electrophoresis (PFGE) with a contour-clamped homogeneous electric field system was prepared and analyzed as previously reported by Vaughan-Martini et al. (8). High-molecular-weight nuclear DNA for reassociation experiments was also obtained and studied by using techniques described in an earlier study (7). The incubation temperature for reassociation was 57°C.

RESULTS

Latin diagnosis of *Candida aquaetextoris* (Vallini, Frassinetti, et Scorzetti) sp. nov. In medio YM liquido (YM broth; Fluka) cum extracto levedinis, extracto malti, peptono et glucoso post dies 3 ad 25°C cellulae globosae, obovatae, elongatae

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TABLE 1. Yeast strains considered in the present study

Species	Original designation	Strain ^a		Isolation source
		DBVPG accession no.	CBS accession no.	
<i>Candida haemulonii</i> (van Uden et Kolipinski) Meyer et Yarrow (Yarrow et Meyer)	<i>Torulopsis haemulonii</i>	DBVPG 6958 ^T	CBS 5149 ^T	Gut of dolphin (<i>Haemulon sciurus</i>)
	<i>Candida haemulonii</i>	DBVPG 6959	CBS 8125	Swiss furniture polish
	<i>Candida haemulonii</i>	DBVPG 6960	CBS 6915	Unknown
<i>Candida maltosa</i> Komagata et al.	<i>Candida cloacae</i>	DBVPG 6136	CBS 5612	Mud
	<i>Candida maltosa</i>	DBVPG 6143 ^T	CBS 5611 ^T	Neutralizing tanks for monosodium glutamate
	<i>Candida maltosa</i>	DBVPG 6961	CBS 7327	Industrial wastewater
<i>Candida tropicalis</i> (Castellani) Berkhout	<i>Oidium tropicale</i>	DBVPG 3982 ^T	CBS 94 ^T	Bronchitic patient
Unknown	Lmar1 ^T	DBVPG 6732 ^T		Sludge from textile industry wastewater treatment plant

^a DBVPG, Industrial Yeast Collection, Division of Applied Microbiology, Department of Plant Biology, University of Perugia, Perugia, Italy; CBS, Centraalbureau voor Schimmelcultures, Delft, The Netherlands.

vel cylindricae ($2.5\text{--}5.5 \times 3.5\text{--}9.5 \mu\text{m}$), singulae, gemmantes, binae vel breviter catenatae sive in parvis racemis connectae. Post dies 5 sedimentum exiguum atque levis pellicula formantur.

Cultura in medio YM cum agar post mensem 1 ad 20°C crenea-eburnea, butyrosa, glabra vel subrugosa nec lucida, moderate convexa, margine expresso. In agar farinae *Zea mays* (Bacto Corn Meal Agar; Difco) pseudomycelii extensio

FIG. 1. Morphological features of yeast strain Lmar1^T (= DBVPG 6732^T). (a) After cultivation for 3 days in YM broth at 25°C, cells occur singly, sometimes with lateral budding, in pairs, or in short chains and clusters. (b) Clump of cells starting pseudohypha formation. (c) Pseudomycelium development after 7 days on corn meal agar at 25°C, consisting of tufts of elongated cells with multilateral branching. (d) Detail of pseudohyphae with lateral budding.

TABLE 2. Growth characteristics of yeast strain Lmar1^T (*C. aquatextoris*)

Characteristic	Reaction of strain Lmar1 ^T
Fermentation of:	
Glucose	+ ^a
Maltose	+
Sucrose	+
Trehalose	D
Galactose	—
Lactose	—
Melibiose	—
Raffinose	—
Assimilation of:	
Glucose	+
Galactose	+
Sucrose	+
Maltose	+
Cellobiose	D
Trehalose	+
Melezitose	+
Xylose	+
Ethanol	+
Glycerol	+
Adonitol	+
D-Mannitol	+
D-Glucitol	D
α-Methyl-D-glucopyranoside	+
Lactic acid	+
Succinic acid	+
Citric acid	+
Malic acid	+
Hexadecane	+
D-Glucosamine	+
N-Acetyl-D-glucosamine	+
D-Gluconate	+
Sorbose	—
Lactose	—
Melibiose	—
Raffinose	—
D-Arabinose	—
L-Arabinose	—
D-Ribose	—
L-Rhamnose	—
i-Erythritol	—
Dulcitol	—
Salicin	—
i-Inositol	—
Methanol	—
Ethyl acetate	—
Ethylamine	+
L-Lysine	+
Cadaverine	+
KNO ₃	—
Phenol	+
pNP	+
Diazo Blue B color test	—
Growth at 40°C	+
Growth in the presence of cycloheximide (0.1 g liter ⁻¹)	+

^a +, positive; —, negative; D, delayed.

post dies 7 ad 25°C. Pseudohyphae crassae cum lateralibus ramis singulae vel saepe aggregatae, blastosporas proferentes singulas vel brevibus catenis parvisque acervis coniunctas.

Sexualis coniunctio non manifesta.

Glucosum, maltosum, sucrosum, et trehalosum fermentantur at non galactosum, lactosum, melibiosum, nec raffinolum.

Glucosum, galactosum, sucrosum, maltosum, cellobiosum, trehalosum, melezitosum, xylosum, ethanolum, glycerinum,

FIG. 2. Electrophoretic karyotypes of the yeast strains studied. Lanes STD, *Saccharomyces cerevisiae* standard obtained from Bio-Rad, Richmond, Calif.; lane 1, *C. haemulonii* DBVPG 6959; lane 2, *C. haemulonii* DBVPG 6960; lane 3, *C. haemulonii* DBVPG 6958^T; lane 4, strain Lmar1^T (= DBVPG 6732^T); lane 5, *C. tropicalis* DBVPG 3982^T; lane 6, *C. maltosa* DBVPG 6961; lane 7, *C. maltosa* DBVPG 6136; lane 8, *C. maltosa* DBVPG 6143^T. The strains in lanes 6 through 8 exhibited more than 90% DNA nucleotide sequence homology (3; this study). Electrophoresis of the 0.8% agarose gel was performed for 55 h at 4.5 V and an angle of 120°.

adonitolum, D-mannitolum, D-glucitolum, α-methyl-D-glucosidum, acidum lacticum, acidum succinicum, acidum citricum, acidum malicum, hexadecanum, D-glucosaminum, *n*-acetylglucosaminum, et D-gluconatum assimilantur neque autem sorbosum, lactosum, melibiosum, raffinolum, D-arabinosum, L-arabinosum, D-ribosum, L-rhamnosum, *i*-erythritolum, dulcitolum, salicinum, *i*-inositolum, methanolum, nec ethyl acetat.

Ethylaminum, L-lysinum, et cadaverinum assimilantur at non kalium nitricum.

Ad crescentiam vitaminarum additae necessariae sunt.

Maxima crescentiae temperatura in medio liquido YEPD (Fluka) cum extracto levedinis, peptono, et glucoso: 40°C.

Crescere potest in 0.1 g cicloheximido per litrum.

Peculiaris crescentia in pNP.

Typus cum nomine Lmar1 ex statione purgationis aquae tetrini in Italia isolatus est Pisis anno 1991 apud CNR-Centro di Studio per la Microbiologia del Suolo. In italica collectione zymotica Sectionis Applicatae Microbiologiae, Dipartimenti Biologiae Vegetalis, Studiorum Universitatis Perusiae, depositus est sub numero DBVPG 6732^T.

Description of the type strain of *Candida aquatextoris* sp. nov. *Candida aquatextoris* (a.quae.tex.to'ris. L. gen. n. *aquae*, of water, referring to the process discharge; L. gen. n. *textoris*, of a weaver, referring to the activity of fabric manufacturers; L. gen. n. *aquatextoris*, of textile plant processing water, referring to the occurrence of the microorganism in wastewater from the textile industry). In YM broth (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% glucose; Fluka) after 3 days at 25°C the cells are globose, ovoid, and elongate to cylindrical (2.5 to 5.5 by 3.5 to 9.5 μm) and occur singly, budding, in pairs, or in short chains and clusters. A sediment starts to accumulate after about 5 days together with a light pellicle or ring.

After growth on YM agar plates at 20°C for 1 month colonies are white-cream, butyrous, smooth to slightly rough, unglistering, and moderately convex with entire margins.

Cultures on corn meal agar (Difco) form abundant pseudo-

TABLE 3. Discriminating characteristics of yeast strain Lmar1^T (*C. aquaetextoris*) and other species compared in the present study

Species	Strain	Assimilation of ^a :					Fermen- tation of ^b :		Maximum growth temp in YEPD broth (°C)	Pseudohyphae	Electrophoretic bands	
		Sorbose	Raffinose	Salicin	Phenol	pNP	Galactose	Maltose			No.	Size range (kb)
<i>Candida haemulonii</i>	DBVPG 6958 ^T	— ^c	+	—	ND	—	—	—	33	—	6	2,200–1,200
	DBVPG 6959	—	D	—	—	—	—	—	37	—	5	2,100–1,500
	DBVPG 6960	+	+	+	—	—	—	—	35	—	4	2,000–950
<i>Candida aquaetextoris</i>	Lmar1 ^T (= DBVPG 6732 ^T)	—	—	—	+	+	—	+	40	+	6	2,200–1,200
<i>Candida maltosa</i>	DBVPG 6143 ^T	+	—	+	+	—	—	+	37	+	5	2,200–1,450
<i>Candida tropicalis</i>	DBVPG 3982 ^T	+	—	D	+	—	+	+	40	+	4	2,200–1,250

^a The tests to determine assimilation of sorbose, raffinose, and salicin were performed as described by van der Walt and Yarrow (6). The tests to determine assimilation of phenol and pNP were performed as described by Corti et al. (1).

^b The fermentation tests were performed as described by van der Walt and Yarrow (6).

^c —, negative; +, positive; D, delayed; ND, not determined.

mycelium after 7 days at 25°C. The extensive pseudomycelium consists of thick, elongated cells with multilateral branching that crowd together to form tufts. Pseudohyphae bear blastospores singly, in short chains, and in clusters (Fig. 1).

A sexual phase has been not observed.

At 28°C in Durham tubes glucose, maltose, sucrose, and trehalose are readily fermented within 48 h, while galactose, lactose, melibiose, and raffinose are not fermented at all even after 3 weeks of incubation.

Glucose, galactose, sucrose, maltose, cellobiose, trehalose, melezitose, xylose, ethanol, glycerol, adonitol, D-mannitol, D-glucitol, α-methyl-D-glucopyranoside, lactic acid, succinic acid, citric acid, malic acid, hexadecane, D-glucosamine, N-acetylglucosamine, and D-gluconate are assimilated, while sorbose, lactose, melibiose, raffinose, D-arabinose, L-arabinose, D-ribose, L-rhamnose, D-erythritol, dulcitol, salicin, D-inositol, methanol, and ethyl acetate are not assimilated.

Assimilation of ethylamine, L-lysine, and cadaverine does occur.

KNO₃ is not assimilated.

Vitamins are required for growth.

The maximum temperature for growth is 40°C in YEPD broth (Fluka).

Growth also occurs in the presence of 0.1 g of cycloheximide per liter.

Distinctively, pNP can be used as a growth substrate.

The growth characteristics of the new yeast are presented synoptically in Table 2.

The habitat of *C. aquaetextoris* is typically sludge derived from the treatment of textile industry wastewaters which contain nonionic nonylphenol polyethoxylate surfactants and their intermediate degradation metabolites, such as nonylphenol. The type strain is Lmar1, which was isolated in the laboratories of the Soil Microbiology Center (National Research Council, Pisa, Italy) from activated sludge samples collected at the main treatment plant for textile industry wastewater depuration located in the Prato metropolitan area, Tuscany, Italy. The type strain has been deposited in the Industrial Yeast Collection at the Division of Applied Microbiology, Department of Plant Biology, University of Perugia, Perugia, Italy, as strain DBVPG 6732.

Although some physiological similarities were found when *C. aquaetextoris* was compared with strains of *C. maltosa* and *Candida tropicalis*, there are enough differences to construct a

TABLE 4. Comparison of hydrocarbon assimilation by yeast strain Lmar1^T (*C. aquaetextoris*) and the *C. maltosa* type strain^a

Compound	Assimilation by:	
	Strain Lmar1 ^T (= DBVPG 6732 ^T)	<i>C. maltosa</i> DBVPG 6143 ^T
Phenols		
Cathecol	++ ^b	+
Hydroquinone	+	+
4-Hydroxyacetophenol	+	—
Hydroxycinnamic acid	+	ND
3-(4-Hydroxyphenyl)propanol	+	±
3-(4-Hydroxyphenyl)propionic acid	+	—
p-NP	+	—
NP9EO (commercial formulation) ^c	+	±
Nonylphenoxyacetic acid	—	—
Phenol	++	++
Resorcinol	++	+
Benzoic acid derivatives		
Benzoate	—	—
Diethylphthalate	—	—
Dimethylphthalate	—	—
Ethyl-4-hydroxybenzoate	—	—
Methyl-4-hydroxybenzoate	—	—
Phthalic acid	—	—
Protocatechuate	—	—
Ethylene glycol and polymeric derivatives		
Diethylene glycol	—	—
Ethylene glycol	—	—
PEG 300	—	—
PEG 600	—	—
PEG 1000	—	—
PEG 2000	—	—
Triethylene glycol	—	—
n-Alkanes and alkylbenzenes		
n-Hexadecane	++	++
n-Hexane	—	—
n-Nonane	±	—
n-Octane	—	—
Octylbenzene	+	—
Phenoxyethanol	—	—
Phenylglyssilic acid	—	—
5-Phenyl-1-pentanol	+	—
3-Phenyl-1-propanol	—	—

^a The assimilation tests were performed in Yeast Nitrogen Base broth without amino acids.

^b ++, good growth within 3 days; +, good growth within 7 days; ±, weak growth after 7 days; —, no growth; ND, not determined.

^c The average number of ethoxy units was approximately nine.

TABLE 5. DNA base sequence homology between yeast strain Lmar1^T (*C. aquatextoris*) and other species compared in the present study

Strain	% Homology with:			
	<i>C. haemulonii</i> DBVPG 6958 ^T	<i>C. maltosa</i> DBVPG 6143 ^T	Strain Lmar1 ^T (= DBVPG 6732 ^T)	<i>C. tropicalis</i> DBVPG 3982 ^T
<i>C. maltosa</i> DBVPG 6961	21	93	32	ND ^a
Lmar1 ^T (= DBVPG 6732 ^T)	18	12		23

^a ND, not determined.

conventional taxonomic key (Table 3). Strain Lmar1^T (= DBVPG 6732^T), which is unable to assimilate sorbose and salicin as sole carbon and energy sources but is capable of utilizing and degrading *p*NP, is distinct from *C. maltosa* DBVPG 6143^T. The same differences, as well as a lack of galactose fermentation, distinguish the new isolate from the type strain of *C. tropicalis*, strain DBVPG 3982. On the other hand, strain Lmar1^T is clearly separated from *Candida haemulonii* on the basis of several phenotypic characteristics. The ability of strain Lmar1^T to assimilate a variety of phenols, benzoic acid derivatives, ethylene glycol derivatives, *n*-alkanes, and alkylbenzenes is compared with the growth responses of the *C. maltosa* type strain on the same substrates in Table 4. Besides utilizing compounds such as catechol and resorcinol more readily than the *C. maltosa* type strain, strain Lmar1^T assimilates 4-hydroxy-acetylphenol, 3-(4-hydroxyphenyl)propionic acid, *n*-nonane, octylbenzene, and 5-phenyl-1-pentanol, while *C. maltosa* DBVPG 6143^T does not.

The results of the comparative PFGE analysis demonstrated the presence of highly related strains, as well as distinct strains (Fig. 2). Three cultures classified as *C. maltosa* (namely, strains DBVPG 6136, DBVPG 6143^T, and DBVPG 6961) had nearly identical electrophoretic karyotypes. Conversely, *C. haemulonii* DBVPG 6958^T, DBVPG 6959, and DBVPG 6960 exhibited distinct chromosomal profiles. Similarly, the remaining strains examined, *C. tropicalis* DBVPG 3982^T and strain Lmar1^T (= DBVPG 6732^T), each had a unique chromosomal profile.

When some strains of each species were examined by optical DNA-DNA reassociation by using the procedure of Kurtzman and coworkers (2), the relationships inferred from the evidence obtained in the PFGE analysis were for the most part confirmed (Table 5). Two strains of *C. maltosa*, DBVPG 6143^T and DBVPG 6961, which had identical chromosomal patterns, exhibited a level of DNA base sequence homology of more than 90%. The third *C. maltosa* strain, DBVPG 6136, has previously been shown to be homologous to the type strain of that species, DBVPG 6143 (= ATCC 28241) (3). All of the other strains compared did not show significant renaturation rates, as anticipated previously on the basis of their clearly different electrophoretic karyotypes.

DISCUSSION

Yeast strain Lmar1^T, which was previously assigned to the species *C. maltosa* (1) on the basis of morphological and physiological characteristics, is described as a member of a new species on the basis of its distinctive molecular features. The PFGE chromosomal profile and the low levels of base sequence homology in DNA-DNA reassociation tests indicate

that there is a wide divergence between strain Lmar1^T and the phenotypically similar species of the genus *Candida*.

C. aquatextoris is the name proposed for the new isolate which assimilates *p*NP, a very toxic metabolite from primary biodegradation of nonionic nonylphenol polyethoxylate surfactants occurring in textile industry wastewater. *C. aquatextoris* also utilizes some hydrocarbon compounds, such as 4-hydroxy-acetylphenol, 3-(4-hydroxyphenyl)propionic acid, *n*-nonane, octylbenzene, and 5-phenyl-1-pentanol, which do not support growth of the type strain of *C. maltosa*.

In addition, the lack of assimilation of sorbose, raffinose, and salicin is another characteristic which discriminates *C. aquatextoris* from the type strains of *C. maltosa* and *C. tropicalis*.

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