Phenotypic and genotypic characterization of two closely related subgroups of *Candida rugosa* in clinical specimens

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In this study, six clinical isolates (two from blood, two from urine and one each from a bronchoalveolar lavage and a vaginal swab) were identified as *Candida rugosa* based on carbohydrate assimilation profiles using API 20C AUX and ID32 C kits (bioMérieux). Sequence analysis of the D1/D2 domain of the yeasts differentiated the isolates into two subgroups, A and B (three isolates per subgroup), which were closely related (99.1–99.6 % nucleotide similarity) to *C. rugosa* strain ATCC 10571. Compared with the *C. rugosa* type strain, the intergenic transcribed spacer (ITS) nucleotide similarity for subgroup A was only 89.2 % (29 mismatches and one deletion) and for subgroup B was 93.7 % (20 mismatches). All isolates grew green colonies on Oxoid Chromogenic *Candida* Agar, with darker pigmentation observed for subgroup A. All isolates were able to grow at 25–42 °C but not at 45 °C. The isolates had identical enzymic profiles, as determined by API ZYM (bioMérieux) analysis, and produced proteinase. High amphotericin MICs ($\geq 1 \mu\text{g ml}^{-1}$) were noted for two isolates from each subgroup. Dose-dependent susceptibility to fluconazole (MIC 32 $\mu\text{g ml}^{-1}$) was noted in a blood isolate. The biofilms of the isolates demonstrated increased resistance to amphotericin and fluconazole. The greater ITS sequence variability of subgroup A isolates is in support of this yeast being recognized as a distinct species; however, further verification using more sophisticated molecular approaches is required. A sequence comparison study suggested the association of subgroup A with environmental sources and subgroup B with clinical sources. Accurate identification and antifungal susceptibility testing of *C. rugosa* are important in view of its decreased susceptibility to amphotericin and fluconazole. The ITS region has been shown to be a valuable region for differentiation of closely related subgroups of *C. rugosa*.

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INTRODUCTION

Candida rugosa has been recognized as an emerging fungal pathogen capable of causing invasive infection in immunocompromised patients. This yeast has been identified as a rare cause of bloodstream infections in North America and Europe (Minces *et al.*, 2009) and accounted for 0.4 % of *Candida* isolates in the global ARTEMIS DISK Antifungal Surveillance Program (Pfaller *et al.*, 2006). However, it has been noted with increased frequency of isolation in several geographical regions recently. *C. rugosa* has been identified as a frequent colonizer of high-risk patients and accounted for 44 % of 32 consecutive episodes of candidaemia in critically ill Brazilian patients (Colombo *et al.*, 2003; Rosas *et al.*, 2004). In a laboratory-based

surveillance study in India, *C. rugosa* was the predominant *Candida* species isolated from the blood cultures of >20 % of critically ill trauma patients (Behera *et al.*, 2010; Singh *et al.*, 2011).

The existence of genetic heterogeneity in *C. rugosa* isolates has been documented in a previous study where clinical isolates were clustered into nine fingerprinting groups using a repetitive sequence-based PCR technique (Redkar *et al.*, 1996). Recently, a novel yeast species closely related to *C. rugosa*, *Candida pseudorugosa*, exhibiting a similar API 20C AUX carbohydrate assimilation profile to *C. rugosa*, has been reported (Li *et al.*, 2006). Reports on the association of *C. rugosa* with catheters and total parenteral nutrition, nosocomial spread and decreased susceptibility to both polyenes and fluconazole (Dubé *et al.*, 1994; Minces *et al.*, 2009) have made identification and characterization of this emerging yeast pathogen important.

Molecular methods based on sequencing of the 26S rRNA gene D1/D2 domain or intergenic transcribed spacer (ITS)

Abbreviation: ITS, intergenic transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the *C. rugosa* sequences determined in this study are HQ412589 and HQ412590 (D1/D2 domain) and HM641831 and HM641832 (ITS sequence) for subgroups A and B, respectively.

region and use of carbohydrate assimilation profiling and chromogenic *Candida* agar are helpful for the correct identification of clinical isolates of yeast when routine phenotypic methods are insufficient (Ciardo *et al.*, 2006; Kurtzman & Robnett, 1997). To validate the identity of our isolates, phenotypic and molecular approaches including enzymic analysis were applied. The antifungal susceptibilities of the planktonic and sessile cells of these isolates are also presented in this study.

METHODS

Yeast isolates. Table 1 shows the origin and details of six yeast isolates (strains STC1–6), which were initially identified as *C. rugosa* by API 20C AUX tests (code 6442104; bioMérieux) in this study. The yeasts were obtained from blood ($n=2$), urine ($n=2$), a vaginal swab ($n=1$) and a bronchoalveolar lavage ($n=1$) from six independent inpatients at the University of Malaya Medical Center, Kuala Lumpur, Malaysia, from 2005 to 2007. The yeasts were inoculated onto potato dextrose agar and incubated at 25, 30, 35, 42 and 45 °C for up to 7 days. Chromogenic testing of the isolates was performed on Oxoid Chromogenic *Candida* Agar (OCCA). An ID32 C kit (bioMérieux) was used for further identification of the yeast strains, following the manufacturer's instructions.

Enzymic profiling of *C. rugosa*. API ZYM (bioMérieux) analysis was used to define the enzymic profiles of the yeasts, as recommended by the manufacturer. A proteinase agar clearance assay was performed according to the method of Ruma-Haynes *et al.* (2000).

Sequence determination and analysis. Yeast genomic DNA was extracted as described by Makimura *et al.* (1994). The ITS region and the 26S rRNA gene D1/D2 domain were amplified with primer pair ITS1 (5'-GTCGTAACAAGGTTTCCGTAGGTG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990) and primer pair NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3'), respectively (Kurtzman & Robnett, 1997). The amplicon was purified using a PCR SV kit (GeneAll) and the subsequent sequencing reaction was performed with a Big Dye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems) on an ABI PRISM 377 Genetic Analyzer (Applied Biosystems), using forward and reverse primers. The sequences were used for a BLAST search in GenBank. The neighbour-joining method in MEGA version 4.0 was used to determine the phylogenetic status of the isolates (Kumar *et al.*, 2004). The reliability of different phylogenetic groupings was evaluated using bootstrap tests (1000 bootstrap replications). Sequences of *C. rugosa* isolates (Table 2) and reference strains of *Candida* species were retrieved from GenBank.

Determination of planktonic MICs. The planktonic MICs of amphotericin, fluconazole, ketoconazole and flucytosine for the yeasts were determined using a microbroth dilution method as described by the Clinical and Laboratory Standards Institute (CLSI, 2008). MICs were recorded after incubation at 35 °C for 48 h. The MIC of amphotericin was defined as the lowest concentration at which there was an absence of growth. The MICs of the other drugs were defined as the lowest drug concentrations that achieved 80% growth inhibition compared with growth of the drug-free control.

Determination of biofilm formation and MICs. The biofilm-forming abilities of the isolates were determined using a standardized method as described by Ramage *et al.* (2001). Briefly, an overnight yeast culture was suspended in RPMI 1640 broth supplemented with L-glutamine (Sigma) to a final concentration of 10^7 cells ml⁻¹

Table 1. Origin and details of the isolates determined in this study

Strain	Age, gender, clinical specimens	Colony on potato dextrose agar	Colony on OCCA	MIC (µg ml ⁻¹)			
				Planktonic cells		Biofilms	
				Fluconazole	Flucytosine	Ketoconazole	Amphotericin
Subgroup A							
STC1	63, female, blood	Smooth, flat	Dark-green	32	8	0.015	≥ 8
STC2	64, male, urine	Smooth, flat	Dark-green	4	2	0.015	≥ 8
STC3	14, female, vaginal swab	Smooth, flat	Dark-green	1	0.25	0.007	≥ 8
Subgroup B							
STC4	65, female, blood	Dry, wrinkled	Light-green	4	<0.125	0.007	≥ 8
STC5	80, male, bronchoalveolar lavage	Smooth, flat	Light-green	1	0.25	0.015	≥ 8
STC6	76, female, urine	Smooth, flat	Light-green	1	0.5	0.007	≥ 8
							Fluconazole
							≥ 128
							≥ 128
							≥ 128
							≥ 128
							32
							≥ 128

Table 2. Origin and details of isolates of the *C. rugosa* complex and its closely related species used for phylogenetic analysis in this study

Strains	Source	GenBank no. (gene)	Reference
<i>Candida rugosa</i> strain ATCC 10571	ATCC strain	GU144663 (D1/D2 domain/ITS)	A. J. Mota and others (unpublished)
<i>Candida pseudorugosa</i> XH1164	Sputum, China	DQ234791 (D1/D2 domain), DQ234792 (ITS)	Li <i>et al.</i> (2006)
<i>Candida</i> sp. L2683B	Blood, Brazil	FJ768918 (D1/D2 domain), FJ768913 (ITS)	G. M. Chaves and others (unpublished)
<i>Candida</i> sp. L69D	Blood, Brazil	FJ768917 (D1/D2 domain), FJ768909 (ITS)	G. M. Chaves and others (unpublished)
<i>Candida rugosa</i> UOA/HCPF 2	Blood, Australia	GQ376074 (ITS)	W. Meyer and others (unpublished)
<i>Candida rugosa</i> LYSM3	Soil from forest, Thailand	AB498988 (D1/D2 domain)	R. Kaewwichian and others (unpublished)
<i>Candida rugosa</i> EB2	Water from mangrove forest, Thailand	AB436404 (D1/D2 domain)	C. Boonmak and others (unpublished)
<i>Candida rugosa</i> 8	Marine yeast, China	EF375701 (D1/D2 domain)	L. Wang & Z. Chi (unpublished)
<i>Candida rugosa</i> EF1	Water from mangrove forest, Thailand	AB436406 (D1/D2 domain)	C. Boonmak and others (unpublished)
<i>Candida rugosa</i> Zhuan8	Marine yeast, China	EF197805 (ITS)	N. Guo and Z. Chi (unpublished)
Subgroup A (STC1)	Clinical specimen, Malaysia	HQ412589 (D1/D2 domain), HM641831 (ITS)	This study
Subgroup B (STC4)	Clinical specimen, Malaysia	HQ412590 (D1/D2 domain), HM641832 (ITS)	This study

(OD₅₃₀ of 0.38). An aliquot of 100 µl of the yeast suspension was then transferred to each well of a flat-bottomed, 96-well polystyrene microtitre plate (Nunclon). After incubation with shaking at 75 r.p.m. at 37 °C for 1.5 h, unattached cells were removed by washing twice with 150 µl PBS. Fresh RPMI 1640 (100 µl) was then added to each well to allow proliferation of the biofilm for 24 h. The metabolic activity of the biofilm was measured using a 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) reduction assay as described by Jin *et al.* (2003). The biofilm in each well was washed twice with 200 µl PBS and a solution containing XTT with menadione was added. The A₄₉₀ of the resultant solution (100 µl) after incubation for 1 h in the dark was measured with a spectrophotometer. The biofilm MIC was determined as described by Melo *et al.* (2007). Biofilms were washed after 24 h, and amphotericin and fluconazole were added at concentrations of 0.015–8 and 0.25–128 µg ml⁻¹, respectively. Two hundred microlitres of each drug solution was added to the respective wells of the microtitre plate and incubated at 37 °C for 48 h. Biofilm MICs were determined on the basis of a 50% reduction in metabolic activity compared with the activity of the biofilm in the microtitre well without the drug. Experiments were performed in duplicate for each isolate.

RESULTS

Phenotypic characterization

Five isolates produced morphologically similar flat, butyrous and creamy colonies on potato dextrose agar, whilst one isolate (strain STC4, subgroup B) grew dry and wrinkled colonies. Identification using an ID32 C test kit revealed similar carbohydrate assimilation profiles (code 1300310015) for the six isolates and identified the isolates

as *C. rugosa*. The isolates were differentiated into two subgroups based on their pigmentation on OCCA. Strains STC1–3 (designated subgroup A) developed darker green colonies compared with the rest of the isolates (designated subgroup B). All isolates were able to grow at 25, 30, 35 and 42 °C but not at 45 °C.

Enzymic characterization

Identical enzymic profiles were obtained by API ZYM analysis for a representative isolate from each subgroup in this study, with the detection of alkaline phosphatase, esterase, leucine arylamidase, acid phosphatase, esterase lipase and lipase (C14) from the isolates. Proteinase production was detected from all isolates on proteinase clearance agar after 7 days of incubation.

Sequence analysis of the D1/D2 domain

A total of 466 and 467 nt were determined for the D1/D2 domain of subgroup A isolates (accession no. HQ412589) and subgroup B isolates (accession no. HQ412590), respectively. There was no mismatch in the D1/D2 domain within the subgroups. Table 3 shows a summary of the sequence analysis. Other than the difference in sequence length, the two subgroups differed by three mismatches. Subgroup A isolates demonstrated 99.1% (four mismatches) nucleotide similarity with the *C. rugosa* ATCC 10571 type strain, whilst subgroup B isolates demonstrated 99.6% (two mismatches including an insertion) nucleotide

Table 3. Comparison of the D1/D2 domain and ITS gene regions of the isolates in this study with those of reference strains

Strain	Percentage similarity (D1/D2 domain/ITS gene region)			
	<i>C. rugosa</i> ATCC 10571	Subgroup A	Subgroup B	<i>C. pseudorugosa</i> XH1164
<i>C. rugosa</i> ATCC 10571	100/100	99.1/89.2	99.6/93.7	94.6/81.5
<i>C. rugosa</i> subgroup A	99.1/89.2	100/100	99.1/89.5	94.4/79.5
<i>C. rugosa</i> subgroup B	99.6/93.7	99.1/89.5	100/100	94.6/81.3
<i>C. pseudorugosa</i> XH1164	94.6/81.5	94.4/79.5	94.6/81.3	100/100

similarity with the ATCC strain. Subgroups A and B demonstrated 94.4% (27 mismatches) and 94.6% (25 mismatches) nucleotide similarity with *C. pseudorugosa* strain XH1164, the closest relative of *C. rugosa*. A search in GenBank showed that the closest match (100%) of subgroup A was with several environmental *C. rugosa* isolates, and subgroup B with a few unspciated *Candida* species from clinical sources (Table 2). The phylogenetic tree shown in Fig. 1(a) confirmed the clustering of the isolates with the *C. rugosa* ATCC 10571 strain and the close relatedness of our isolates with other *C. rugosa* isolates available in GenBank (Table 2).

Sequence analysis of the ITS sequence data

A total of 351 and 352 nt were determined for the ITS gene region of *C. rugosa* subgroup A (STC1) and B (STC4) isolates, respectively. There was no mismatch in the ITS region sequences within each subgroup. Other than the difference in sequence length, the two subgroups differed by 37 nt. The similarity among the two subgroups as determined by pairwise alignment was 90.1%. Sequence analysis of the ITS region sequences showed 89.2% (29 mismatches and one deletion) and 93.7% (20 mismatches) nucleotide similarity of subgroup A and B isolates with *C. rugosa* ATCC 10571, respectively (Table 3). Subgroups A and B demonstrated 79.5% (71 mismatches) and 81.3% (65 mismatches) nucleotide similarity with *C. pseudorugosa* XH1164, the closest relative of *C. rugosa* (Table 3).

Fig. 1(b) shows a dendrogram demonstrating the genetic relationship of our isolates based on ITS gene sequences of *Candida* reference strains and *C. rugosa* isolates documented in GenBank. The ITS sequences of *C. rugosa* subgroup A isolates showed 100% similarity only to the environmental isolates. The sequences of the subgroup B isolates matched (100%) that of a *C. rugosa* blood isolate (strain UOA/HCPF 2) and a series of clinical isolates (from blood and pericatheter and rectal swabs) from Brazil (Table 2). The two subgroups clustered at two different branches of the ITS phylogenetic tree with a high bootstrap value (100%).

Determination of planktonic and biofilm MICs

Table 1 shows the *in vitro* susceptibilities of the isolates. The MICs for ketoconazole and flucytosine ranged from 0.007 to 0.015 and from <0.125 to 8 µg ml⁻¹, respectively.

High amphotericin MICs (≥ 1 µg ml⁻¹) were noted for four isolates (two from each subgroup). A blood isolate (strain STC1) from subgroup A showed dose-dependent susceptibility to fluconazole (MIC 32 µg ml⁻¹). The sessile cells of all isolates displayed increased resistance to amphotericin (MIC ≥ 8 µg ml⁻¹). Five of the six isolates had biofilm MICs of ≥ 128 µg ml⁻¹ against fluconazole.

DISCUSSION

The six Malaysian isolates appeared to be identical phenotypically based on carbohydrate assimilation profiles (API 20C AUX and ID 32 C kits), reactions on chromogenic agar (with one subgroup exhibiting more intense pigmentation), growth temperatures and enzymic characterization. Different colonial morphologies have been described for *C. rugosa* on CHROMagar, which include the description of rough, dry, flat colonies with a pale border resembling *Candida krusei* (Hospenthal *et al.*, 2002) and small, dry colonies of a brilliant blue colour with a pale or white border indistinguishable from those of *Candida albicans* (Horvath *et al.*, 2003). In this study, only one of the six isolates produced morphologically dry colonies on both potato dextrose agar and OCCA (Table 1). Based on the growth morphology and their ability to grow at 42 °C, these isolates (particularly subgroup B) may be mistaken for *C. albicans* if the results of germ tube and other biochemical tests are not available.

Data on the epidemiology and antifungal susceptibilities of this emerging yeast pathogen are scanty. *C. rugosa* has been isolated most frequently from blood in outbreak situations (Behera *et al.*, 2010; Colombo *et al.*, 2003; Dubé *et al.*, 1994; Singh *et al.*, 2011) and other specimens including respiratory, skin, soft tissue and genital specimens in case reports of single cases (Minces *et al.*, 2009; Pfaller *et al.*, 2006). As the six isolates investigated in this study were isolated only once from our patients, this may not be sufficient for definite confirmation of their pathogenic roles, particularly for those obtained from non-sterile body sites. The origin of four of the six isolates (two from each subgroup) from blood and urine specimens in this study suggests a similarity to previous findings where *C. rugosa* was isolated most often from bloodstream and urinary tract infections (Minces *et al.*, 2009; Pfaller *et al.*, 2006). Interestingly, five of the six isolates were obtained from elderly patients (>60 years old) in this study. This finding is similar to that reported by Colombo *et al.* (2003), where

Fig. 1. Neighbour-joining tree based on the D1/D2 domain (a) or the ITS gene region (b) depicting the relationship of *C. rugosa* subgroups A and B with other yeasts. The estimated confidence levels, expressed as percentages, were determined by bootstrap analysis with 1000 replications. Reference sequences were retrieved from GenBank (Table 2).

the patients in five out of six *C. rugosa* cases in Brazil were >65 years old, suggesting that older individuals are at higher risk of infection with *C. rugosa*.

The high level ($\geq 1 \mu\text{g ml}^{-1}$) of MICs of amphotericin for four of our isolates is a typical feature of *C. rugosa* (Mincez *et al.*, 2009). In addition, treatment failures with polyene agents have been reported, with isolates demonstrating *in vitro* susceptibility to amphotericin B (Behera *et al.*, 2010; Colombo *et al.*, 2003). Decreased susceptibility to fluconazole (40.5% susceptible) was observed particularly among Asian-Pacific and Latin American isolates (Pfaller *et al.*, 2006). Singh *et al.* (2011) reported that 16% of their *C. rugosa* isolates were resistant to fluconazole compared with 12.5% of *Candida glabrata* isolates and <10% of *Candida tropicalis* and *Candida parapsilosis* isolates.

Bloodstream isolates have been reported to be the least susceptible to fluconazole (Pfaller *et al.*, 2006). In this study, two *C. rugosa* blood isolates, STC1 and STC2, were

considerably less susceptible to fluconazole (MICs of 32 and $4 \mu\text{g ml}^{-1}$, respectively) than those from other specimen types (Table 1). The susceptibility pattern of our *C. rugosa* isolates was therefore similar to that of *C. glabrata* and *C. krusei*, which are less susceptible to fluconazole and amphotericin B than most other species of *Candida* (Pfaller & Diekema, 2004). Hence, accurate identification and antifungal susceptibility testing of the yeast may have a significant impact on the antifungal therapy of *C. rugosa* infections.

Detection of the proteinase activity from the isolates in this study suggests the potential of the isolates to cause infection, as proteinase is generally known to facilitate microbial adhesion and tissue invasion and has been identified as one of the major pathogenic determinants (Naglik *et al.*, 2003). Identical enzymic profiles by API ZYM analysis were found for the two subgroups in this study. The enzymes detected include alkaline phosphatase,

esterase, leucine arylamidase, acid phosphatase, esterase lipase and lipase. Of these enzymes, lipase has been postulated to facilitate proliferation of the yeast in lipid-rich environments such as total parenteral nutrition (Minces *et al.*, 2009).

Infections caused by *C. rugosa* are usually seen in patients within medical and surgical inpatient services and are associated with the use of central venous catheters and parenteral nutrition (Minces *et al.*, 2009). Our finding of the ability of the isolates to form biofilms *in vitro* on plastic surfaces is probably correlated with previous reports of the association of *C. rugosa* with catheter-related infections and resistance against therapy (Colombo *et al.*, 2003; Minces *et al.*, 2009; Rosas *et al.*, 2004). The increased resistance to amphotericin (MIC $\geq 8 \mu\text{g ml}^{-1}$) and fluconazole (MIC $\geq 128 \mu\text{g ml}^{-1}$) of the sessile cells compared with planktonic cells is similar to the observation for other *Candida* species at the biofilm stage (Ramage *et al.*, 2001).

The organisms identified in this study are among a number of novel yeast species in clinical specimens that cannot be distinguished phenotypically from an existing species and rely on a molecular approach for identification (Ciardo *et al.*, 2006). The D1/D2 domain and ITS sequences of our isolates do not match any known yeast species in GenBank or the CBS yeast sequence database (<http://www.cbs.knaw.nl/collections/>). Kurtzman & Robnett (1997) predicted that yeast strains showing >1% substitutions in the ~600 nt D1/D2 domain are likely to be different species and that strains with 0–3 nt mismatches are either conspecific or sister species. Hence, based on this correlation, subgroup B (with two mismatches compared with the type strain) is most probably conspecific with *C. rugosa*.

Although subgroup A had only 0.9% substitutions in its D1/D2 domain (four mismatches) compared with the type strain, the greater ITS sequence variability (10.8%) of this subgroup supports the suggestion that the yeast be recognized as a distinct species, as the percentage intraspecies similarity reported previously for *C. rugosa* is 94.5–100% (Ciardo *et al.*, 2006). However, the predicted conspecificity and proposal of a novel *Candida* species will require more detailed taxonomic reassessment using DNA–DNA hybridization assays (Kurtzman & Robnett, 1997) or other more sophisticated molecular approaches.

The source of *C. rugosa* is unknown. The yeast is frequently isolated from the milk of mastitic cows (Crawshaw *et al.*, 2005) and its lipase has been used for food production (Benjamin & Pandey, 1998). Hence, contaminated milk or food products have been speculated as a source of infection (Barton, 2011). Sequence comparison in this study demonstrated the association of subgroup A isolates (only reported from environmental sources so far) with human infections for the first time, following identification of the isolates from different clinical specimens (blood, urine and bronchoalveolar lavage) in this study. Isolation of the subgroup B isolates from various clinical specimens in this and other studies (as documented in GenBank) is an

indication of the emergence of this yeast as a human pathogen in various geographical regions.

Over the past few years, several new and emerging yeast pathogens misidentified using conventional chemotaxonomic criteria have been reported by sequence analysis of the D1/D2 domain and ITS sequences. Molecular techniques have assisted in the identification of *C. pseudorugosa* (Li *et al.*, 2006) and *Candida pararugosa* (Ciardo *et al.*, 2006), which were misidentified previously as *C. rugosa* using phenotypic methods. In this and the above-mentioned studies, the ITS region has been shown to be a valuable region for differentiation of closely related subgroups of *C. rugosa* (tentatively referred to as the *C. rugosa* complex). Further clarification of the complex taxonomy of *C. rugosa* will assist in our understanding of the epidemiology and clinical significance of this yeast.

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