

Candida pseudorugosa sp. nov., a Novel Yeast Species from Sputum[▽]

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Two yeast strains, strains XH 1026 and XH 1164, isolated from the sputum of an intensive care unit patient with acute pneumonia, were originally identified as *Candida albicans* and *C. tropicalis*, respectively. Sequence analysis of the 26S rRNA gene D1/D2 domain and the internal transcribed spacer (ITS) region indicated that the two strains represent a novel yeast species closely related to *C. rugosa*. The name *Candida pseudorugosa* sp. nov. is therefore proposed (type strain, AS 2.3107 [CBS 10433]). The new species is able to grow at 42°C and is resistant or insusceptible to amphotericin B (MIC, 2 µg/ml), caspofungin (MIC, 64 µg/ml), itraconazole (MIC, 1 µg/ml), and nystatin (MIC, 16 µg/ml); dose-dependent susceptible to fluconazole (MIC, 16 µg/ml); and susceptible to flucytosine (MIC, 0.125 µg/ml) and voriconazole (MIC, 0.125 to 0.25 µg/ml). The code for *C. pseudorugosa* sp. nov. provided by the API 20C AUX system is identical to that for *C. rugosa*. The colonies of the new species on CHROMagar Candida appear blue-green, similar to those of *C. albicans*. In addition to the molecular method based on D1/D2 domain or ITS region sequencing, use of the combination of the API system and CHROMagar Candida is helpful for the correct identification of *C. pseudorugosa* sp. nov.

Developments in medical and surgical therapy over the past two decades have changed the type of inpatient populations. Newer technologies and therapies, such as bone marrow or solid-organ transplantations, chemotherapy, monitoring with invasive devices, parenteral nutrition, broad-spectrum antimicrobial treatment, and assisted ventilation, have helped to treat patients with previously devastating or fatal diseases. However, these successes have resulted in the admission of increasing numbers of critically ill or immunocompromised patients to hospitals (4). Furthermore, the AIDS epidemic has added to the growing population of immunocompromised individuals (16). These patients are highly susceptible to opportunistic fungal pathogens (4, 7, 13). Candidiasis is most frequent in immunocompromised hosts, and *Candida albicans* is responsible for the majority of the infections (20, 21). However, data obtained over the past two decades clearly indicate that invasive candidiasis in critically ill, nonimmunocompromised patients is of increasing importance (1, 8, 18) and that serious life-threatening infections due to new and emerging fungal pathogens, including non-*C. albicans* *Candida* species, have increased significantly (3, 13, 17, 29, 30, 40, 41, 43).

Triazole-based antifungal treatment and prophylaxis applied since the 1980s have reduced the proportion of cases of invasive candidiasis caused by *C. albicans*. In parallel, a marked increase in the proportion of non-*C. albicans* *Candida* bloodstream isolates has been reported in several countries or regions (30). This trend is evident in patients with cancer. Neutropenia in tumor patients and acute leukemia and antifungal prophylaxis in hematology patients were significantly associated with non-*C. albicans* candidemia (10, 39). Species other

than *C. albicans* have accounted for nearly 50% of all systemic *Candida* infections in many cancer centers since the late 1980s (2, 19, 34, 39, 42).

During a survey of the species and the genetic diversity of *Candida* strains from clinical sources in Beijing, China, two strains isolated from the sputum of an intensive care unit (ICU) patient who died of acute pulmonary infection after an esophageal tumor resection was revealed to represent a novel species closely related to *C. rugosa* by rRNA gene sequence comparison. The two strains were originally misidentified as *C. albicans* and *C. tropicalis*, respectively. The new *Candida* species is of special clinical importance because it is insusceptible to several commonly used antifungal agents and cannot be correctly identified by the methods commonly used in clinical laboratories.

CASE REPORT

The patient was a 66-year-old woman who used to live in Tianjin, China. On 19 February 2003, the patient was treated by an esophageal tumor resection in a local hospital. One day after the operation, she presented with an episode of hyperpyrexia associated with mass viscous expectoration. Chest X rays indicated double-lung pneumonia. Laboratory test results did not reveal tuberculosis. Empirical treatment with a combination of antibiotics failed to control the infection. Beginning on 24 February, the patient was placed on mechanical ventilation, with a few short intervals of voluntary respiration. On 18 March 2003 the patient was transferred to the ICU of a hospital in Beijing. Repeated aerobic and anaerobic microbiological cultures of blood were negative. Examination and cultivation of a lung tissue sample, performed on 21 March, were negative. The following bacteria were detected from sputum cultures: *Acinetobacter baumannii* (8 and 18 March), *Escherichia coli* (21 March), and *Staphylococcus aureus* and *Stenotrophomonas maltophilia* (24 March). Yeasts were detected from

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the sputum cultures on 14, 24, and 25 March and were identified as *C. albicans*, a *Candida* sp., and *C. tropicalis*, respectively, by using CHROMagar Candida (28). The antifungal agent fluconazole (200 mg/day) had been administered since 18 March; and antibiotic treatments were adjusted beginning on 20 March so that they included impenem-cilastatin sodium (1,500 mg/day), vancomycin (1,500 mg/day), and metronidazole (1,000 mg/day), according to the results of susceptibility testing of the bacteria detected. No clinical improvements were achieved. Chest X rays and a computed tomography scan showed a progressive infection with tiny nodes and multiple patchy infiltrates in both lungs. On 25 March 2003, the patient died of respiratory failure, septic shock, and deterioration.

MATERIALS AND METHODS

Yeast strains and phenotypic characterization. Strains XH 1026 and XH 1164 were isolated from the sputum of the patient on 14 and 25 March 2003, respectively. The morphological, physiological, and biochemical characteristics were examined by standard methods commonly used in yeast taxonomy (45). Chromogenic testing of the colony was performed on CHROMagar Candida (CHROMagar Company, Paris, France), according to the manufacturer's instructions. The API 20C AUX kit (bioMérieux, Lyon, France) was used for identification of the yeast strains, according to the manufacturer's instruction.

Sequencing and molecular phylogenetic analysis. Nuclear DNA was extracted by the method of Makimura et al. (25). The internal transcribed spacer (ITS) region (including the 5.8S rRNA gene) and the 26S rRNA gene D1/D2 domain were amplified with primer pair ITS1 (5'-GTC GTA ACA AGG TTT CCG TAG GTG-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and primer pair NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3'), respectively. The PCR was performed for 36 cycles, with denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min. The PCR products were purified by using SUPREC-02 centrifugal filter tubes (TaKaRa Bio, Shiga, Japan), according to the instructions of the manufacturer. After purification, the PCR products were directly sequenced with forward primer ITS1 or NL1 and reverse primer ITS4 or NL4 by using the ABI BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). The sequences of the D1/D2 domains or ITS regions of the strains determined in this study and those of the reference strains obtained from GenBank were aligned by use of the Clustal X program (38). Phylogenetic analysis was performed by the neighbor-joining method, as described previously (5). The accession numbers of the reference sequences were indicated in the tree shown in Fig. 2.

Antifungal susceptibility. Antifungal susceptibility testing was performed by the broth dilution method, according to the guidelines outlined in Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards) document M27-A2 (26).

Nucleotide sequence accession numbers. The D1/D2 domain and ITS region sequences of strain XH 1164 have been released in GenBank with accession numbers DQ234791 and DQ234792, respectively.

RESULTS

Phenotypic characteristics. Strains XH 1026 and XH 1164 were morphologically and physiologically very similar. They grew well at 42°C. In yeast extract-malt extract broth (45), after 3 days at 30°C, the cells were ovoid, ellipsoid, or cylindrical. Budding was multilateral. On YM agar (45), after 10 days at 30°C, the steak culture was butyrous, cream colored, raised, semiglossy, and wrinkled; the margin was slightly undulating. Abundant pseudohyphae were formed in Dalmau plate culture on cornmeal agar. The carbon and nitrogen assimilation patterns of the two strains were similar to those of *C. rugosa*. Sexual structures were not observed.

On CHROMagar Candida, the colonies of strains XH 1026 and XH 1164 appeared dark blue-green, similar to those of *C. albicans* and *C. dubliniensis* (Fig. 1). An API system code (code

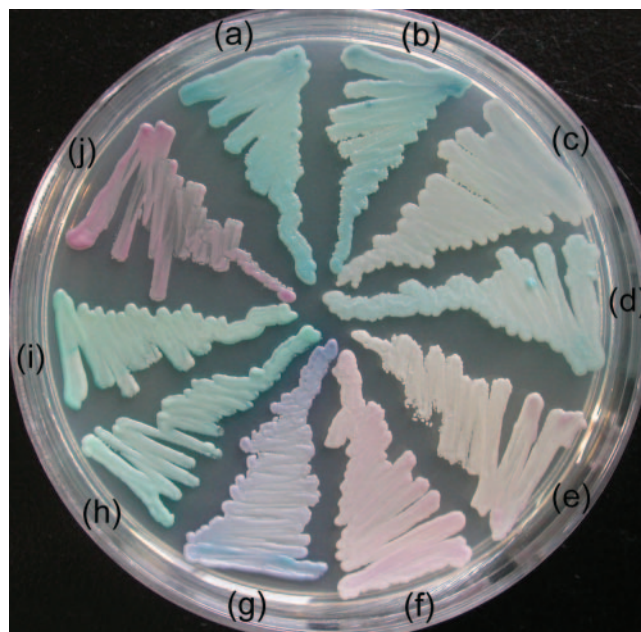


FIG. 1. Colonies grown on CHROMagar Candida for 48 h at 30°C: (a) *C. pseudorugosa* XH 1026, (b) *C. pseudorugosa* XH 1164, (c) *C. rugosa* CBS 613, (d) *C. rugosa* AS 2.1498, (e) *C. parapsilosis* ATCC 22019, (f) *C. krusei* AS 2.3194, (g) *C. tropicalis* AS 2.3195, (h) *C. dubliniensis* CBS 7988, (i) *C. albicans* ATCC 90028, and (j) *C. glabrata* ATCC 90030.

6442104) typical of that for *C. rugosa* was obtained for both strains by using the API 20C AUX system.

Sequence comparison. Strains XH 1026 and XH 1164 had identical D1/D2 domain and ITS region sequences, confirming their conspecificity. The results of a BLAST search of the sequences in GenBank by use of the D1/D2 domain and ITS region sequences of the two strains as the queries showed that the closest matches were the corresponding sequences of the species *C. rugosa*. In the phylogenetic tree drawn from the D1/D2 domain sequence alignment, the two strains clustered together with *C. rugosa* (Fig. 2). They differed from the type strain of *C. rugosa* by 23 (4.7%) mismatches (21 substitutions and 2 indels) in the D1/D2 domain and by 64 (20.4%) mismatches (61 substitutions and 3 indels) in the ITS region (including the 5.8S rRNA gene).

Antifungal susceptibilities. The in vitro susceptibilities of strains XH 1026 and XH 1164 to the following nine antifungal agents were the same: amphotericin B (MIC, 2 µg/ml), caspofungin (MIC, 64 µg/ml), clotrimoxazole (MIC, 1 µg/ml), fluconazole (MIC, 16 µg/ml), flucytosine (MIC, 0.125 µg/ml), itraconazole (MIC, 1 µg/ml), miconazole (MIC, 2 µg/ml), nystatin (MIC, 16 µg/ml), and terbinafine (MIC, ≥16 µg/ml). The MICs of voriconazole for strains XH 1026 and XH 1164 were 0.25 and 0.125 µg/ml, respectively.

DISCUSSION

Previous studies have shown that yeast strains with a greater than 1% substitution in the D1/D2 domain or ITS region usually represent separate species (15, 23, 24, 35, 36). The sequence comparison performed in the present study clearly

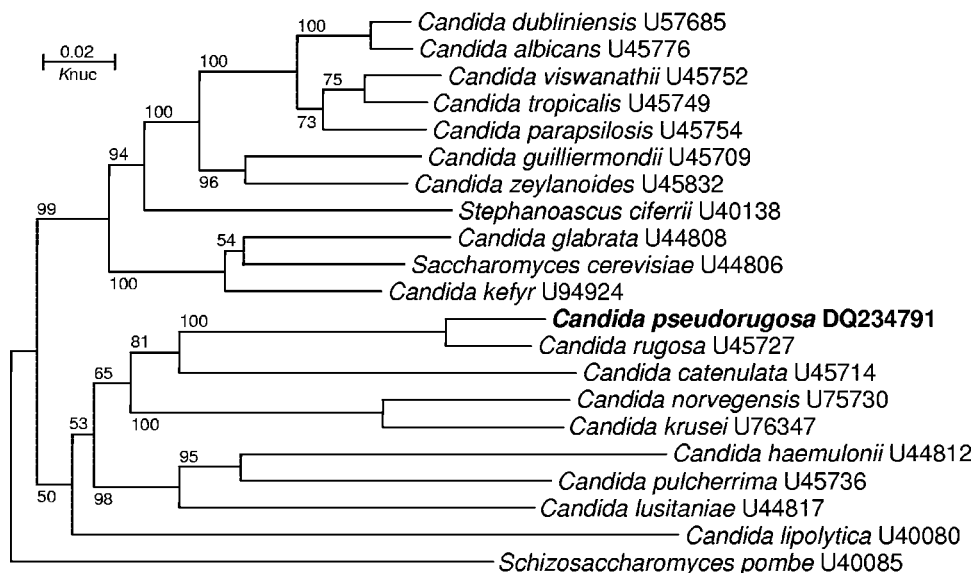


FIG. 2. Phylogenetic tree drawn from neighbor-joining analysis of 26S rRNA gene D1/D2 domain sequences depicting the relationships of *Candida pseudorugosa* sp. nov. with closely related yeast taxa. Bootstrap percentages over 50% from 1,000 bootstrap replicates are shown. Reference sequences were from the type strains of the species and were retrieved from GenBank under the accession numbers indicated.

indicated that strains XH 1026 and XH 1164 represent a distinct novel species with a close phylogenetic relationship to *C. rugosa*. The name *Candida pseudorugosa* sp. nov. is therefore proposed for the new species.

The original clinical records, which contained no histopathologic evidence, are not sufficient to definitely ascribe the lung infection of the patient to the new *Candida* species. There is no more evidence at present to indicate that this organism is a human pathogen. However, the origin and special properties of the species suggest that it is highly possibly a new opportunistic fungus worthy of note. It is common to isolate *C. albicans* strains from the sputum of patients or even healthy people, for this species is a commensal organism found frequently in healthy humans (27). However, it is uncommon to isolate strains of a new *Candida* species repeatedly from the sputum of an ICU patient with acute pneumonia. Although a few pathogenic bacterial species were also isolated from the sputum of the patient, the susceptibilities of the bacteria to antibiotics were tested in vitro and the antibacterial treatments were adjusted accordingly, but the lung infection failed to be controlled.

The resistance or insusceptibility of *C. pseudorugosa* sp. nov. to multiple antifungal agents is noticeable. If the breakpoints for *Candida* species tentatively adopted in NCCLS document M27-A2 (26) and other literature (4, 6, 9, 13, 22, 29, 32, 33, 44) are used as references, the susceptibility of the new species to the antifungal agents tested can be interpreted as resistance to amphotericin B, caspofungin, itraconazole, and nystatin; dose-dependent susceptibility to fluconazole; and susceptibility to flucytosine and voriconazole.

The antifungal-insusceptible property of the new species is most probably intrinsic, since strain XH 1026 was isolated before the use of fluconazole and other antifungals were not used during the whole course of treatment. Among the opportunistic non-*C. albicans* *Candida* species, *C. glabrata*, *C. krusei*,

C. lusitaniae, and *C. rugosa* are well-known species that may exhibit innate or acquired resistance to one or more established antifungal agents (32). An amphotericin B- and azole-resistant species, *C. pseudohaemulonii*, isolated from the blood of a patient was reported recently (37). *C. rugosa*, the closest relative of the new species, is an emerging recognized opportunistic pathogen for humans. A cluster of six episodes of candidemia caused by *C. rugosa* was recently reported from Brazil (11). Fifteen episodes of candidemia due to *C. rugosa* in burn patients receiving topical nystatin treatment were reported in 1994 (12). The *C. rugosa* isolates were all shown to be resistant to nystatin (12). *C. rugosa* also has decreased susceptibilities to amphotericin B and fluconazole (12, 14). As a close relative of *C. rugosa* with a broader antifungal resistance spectrum, *C. pseudorugosa* sp. nov. is worthy of note because of its possible ability to infect humans.

Because of the resistance or insusceptibility of *C. pseudorugosa* sp. nov. to commonly used antifungal agents, the correct and rapid identification of the new species is clinically important. However, *C. pseudorugosa* sp. nov. tends to be easily misidentified by the methods commonly used in clinical laboratories. In agreement with the close phylogenetic relationship to *C. rugosa*, the new species is similar to *C. rugosa* physiologically. By using the present kit and database of the API 20C AUX system, it can be unambiguously identified as *C. rugosa*. However, on CHROMagar *Candida*, the color of the colonies of the new species is more similar to that of *C. albicans* than to that of *C. rugosa* (Fig. 1). This was the reason that strain XH 1026 was originally misidentified as *C. albicans*. The original identification of strain XH 1164 as *C. tropicalis* was probably misguided by its blue tinge, but *C. pseudorugosa* sp. nov. is greener than *C. tropicalis* and does not produce a dark halo, as *C. tropicalis* usually does on CHROMagar *Candida* (28). Although a side-by-side comparison showed slight differences in colors between *C. pseudorugosa* sp. nov. and *C. albicans* or *C.*

dublinskiensis, without the awareness of this new species, isolates of the new species may easily be misidentified as the most common species *C. albicans* on CHROMagar Candida. If the maximum growth temperature is tested, the isolates of the new species may be misidentified as *C. dublinskiensis* (31), since *C. pseudorugosa* sp. nov. grew well at 42°C but not at 45°C.

The special antifungal resistance and physiological and morphological characteristics of *C. pseudorugosa* sp. nov. suggest that the clinical yeast strains identified as *C. albicans*, *C. dublinskiensis*, or *C. tropicalis* by chromogenic testing or as *C. rugosa* by the methods based on physiological reactions, such as those in the API 20C AUX system or a similar system, but that were refractory to amphotericin B, fluconazole, or other antifungal therapy should be reidentified. The combination of the testing results obtained from the API system and CHROMagar Candida is helpful for the correct recognition of *C. pseudorugosa* sp. nov. The new species can be distinguished from *C. rugosa* on CHROMagar Candida by its deeper color (Fig. 1). The lengths of the D1/D2 domain (484 bp) and the ITS1-5.8S rRNA-ITS2 region (310 bp) of *C. pseudorugosa* sp. nov. are remarkably shorter than those of *C. albicans*, *C. dublinskiensis*, and *C. tropicalis* (570 to 571 bp for the D1/D2 domain, 440 to 450 bp for the ITS region). Direct comparison of the lengths of the PCR products of the D1/D2 domain or the ITS region by agarose gel electrophoresis may clearly separate the new species from the last three ones. The identification of the new species should be confirmed by sequencing of the D1/D2 domain or the ITS region.

Latin description of *Candida pseudorugosa* Bai & Li sp. nov.

In medio liquido YM post dies 3 ad 30°C, cellulae elipsoideae, 2.5–4.5 × 3.0–6.0 μm, singulae, binae et adhaerents. Per gemmatum multipolarem reproducentur. Post 1 mensem sedimentum et annulus formatur. In agaro YM post 1 mensem ad 30°C, butyrose, cremaea, infimo-convexa, seminitida, et margo undulato. Fermentatio nulla. Glucosum, galactosum, L-sorbosum (lente), maltosum, melibiosum (infirmum), D-xylosum, ethanolum, glycerolum, D-mannitolum, D-glucitolium, methyl-α-D-glucosidum, salicinum, acidum DL-lacticum, acidum succinum et hexadecanum assimilantur non sucrosus, cellobiosum, trehalosum, lactosum, raffinum, melezitiosum, inulinum, amyllum solubile, L-arabinosum, D-arabinosum, D-ribosum, L-rhamnosum, D-glucosaminum, methanolum, erythritolum, acidum citricum nec inositolium. Ethylaminum, L-lysinum, cadaverinum, ammonium sulfatum assimilantur non natrum nitrosus nec kalium nitricum. Vitaminae externae ad crescentiam necessaria sunt. Maxima temperatura crescentiae: 42°C. Materiae ammyloidea iodophila non formantur. Diazonium caeruleum B non respondens. Ureum non hydrolysat. Typus: isolatus ex saliva, XH 1164, depositus in collectione China General Microbiological Culture Collection Centre, Academia Sinica (AS 2.3107).

Description of *Candida pseudorugosa* Bai & Li sp. nov. In YM broth, after 3 days at 30°C, the cells are ovoid, ellipsoid, or cylindrical and 2.5 to 4.5 by 3.0 to 6.0 μm and occur singly, in pairs, or in chains. Budding is multilateral. After 1 month at 30°C, sediment and a thin ring are present. On YM agar, after 10 days at 30°C, the streak culture is butyrous, cream colored, raised, semiglossy, and wrinkled; the margin is slightly undulating. In Dalmau plate culture on cornmeal agar, abundant pseudohyphae are formed. Sporulation was not observed. Fer-

mentation is negative. Glucose, galactose, L-sorbose (delayed), maltose, melibiose (weak), D-xylose, ethanol, glycerol, D-mannitol, D-glucitol, methyl α-D-glucoside, salicin, DL-lactic acid, succinic acid, and hexadecane are assimilated. Sucrose, cellobiose, trehalose, lactose, raffinose, melezitose, inulin, soluble starch, L-arabinose, D-arabinose, D-ribose, L-rhamnose, D-glucosamine, methanol, erythritol hydrochloride, citric acid, and inositol are not assimilated. Ethylamine hydrochloride, L-lysine, cadaverine dihydrochloride, and ammonium sulfate are assimilated. Sodium nitrate and potassium nitrate are not assimilated. Growth in vitamin-free medium is negative. The maximum growth temperature is 42°C. Starch-like free compounds are not produced. The diazonium blue B reaction is negative. Urease activity is negative. The type strain, XH 1164, was isolated from the sputum of an ICU patient with acute pneumonia in Beijing, China, on 25 March 2003. This strain has been deposited in the China General Microbiological Culture Collection Center (CGMCC), Academia Sinica, Beijing, China, as AS 2.3107 and in the Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, as CBS 10433. The specific epithet *Candida pseudorugosa* refers to the close relationship of the species to *Candida rugosa*.

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