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Species Identification and Virulence Attributes of *Saccharomyces boulardii* (nom. inval.)

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***Saccharomyces boulardii* (nom. inval.) has been used for the treatment of several types of diarrhea. Recent studies have confirmed that *S. boulardii* is effective in the treatment of diarrhea, in particular chronic or recurrent diarrhea, and furthermore that it is a safe and well-tolerated treatment. The aim of the present study was to identify strains of *S. boulardii* to the species level and assess their virulence in established murine models. Three strains of *S. boulardii* were obtained from commercially available products in France and Italy. The three *S. boulardii* strains did not form spores upon repeated testing. Therefore, classical methods used for the identification of *Saccharomyces* spp. could not be undertaken. Typing by using the restriction fragment length polymorphisms (RFLPs) of the PCR-amplified intergenic transcribed spacer regions (including the 5.8S ribosomal DNA) showed that the three isolates of *S. boulardii* were not separable from authentic isolates of *Saccharomyces cerevisiae* with any of the 10 restriction endonucleases assessed, whereas 9 of the 10 recognized species of *Saccharomyces* could be differentiated. RFLP analysis of cellular DNA with *Eco*RI showed that all three strains of *S. boulardii* had identical patterns and were similar to other authentic *S. cerevisiae* isolates tested. Therefore, the commercial strains of *S. boulardii* available to us cannot be genotypically distinguished from *S. cerevisiae*. Two *S. boulardii* strains were tested in CD-1 and DBA/2N mouse models of systemic disease and showed intermediate virulence compared with virulent and avirulent strains of *S. cerevisiae*. The results of the present study show that these *S. boulardii* strains are asporogenous strains of the species *S. cerevisiae*, not representatives of a distinct and separate species, and possess moderate virulence in murine models of systemic infection. Therefore, caution should be advised in the clinical use of these strains in immunocompromised patients until further study is undertaken.**

Saccharomyces boulardii (nom. inval. [19]) has been widely used in Europe to treat diarrhea. This organism was first isolated from litchi fruit in Indochina (33, 49). A lyophilized form has been studied for oral administration in the United States by Biocodex Inc. (Seattle, Wash.), and the patent strain is held in the American Type Culture Collection (ATCC).

S. boulardii has been used in Europe and has been proposed for use in the United States for the treatment of several types of diarrhea, either as a preventative agent for antibiotic-associated diarrhea or as a treatment for diarrhea in adults and children infected with *Clostridium difficile*, for diarrhea in human immunodeficiency virus-infected patients, and for acute diarrhea in children and adults. The efficacy of these treatments has been previously reviewed (33). Several recent studies have confirmed that *S. boulardii* is effective in the treatment of diarrhea, in particular chronic or recurrent diarrhea (17, 25, 34, 35), and in the prevention of antibiotic-associated diarrhea (51). Furthermore, a recent study, which assessed the effect of 3 weeks of oral administration of *S. boulardii* in humans, showed that there was an increase in the brush border enzyme activity of the duodenal mucosa as well as a positive effect on the maturation of enterocytes (22). Several studies have shown that *S. boulardii* is a safe and well-tolerated treatment (25, 35, 39). However, there are also several documented reports of *S. boulardii* fungemia and septicemia resulting from the oral administration of this organism in immunocompromised and immunocompetent patients (46, 55, 57).

The taxonomy of the genus *Saccharomyces* has undergone significant changes recently, with more importance being placed on genotypic rather than phenotypic methods for the identification of isolates to the species level. This has resulted in a change in the number of species of *Saccharomyces* from 41 (27) to 7 (3) or 10 (4, 42). Recent and ongoing research in our laboratories has investigated the genetics and virulence of clinical and wild-type isolates of *Saccharomyces cerevisiae* (8, 13–16, 30, 31). The aim of the present study was to attempt to identify isolates of *S. boulardii* to the species level and assess their virulence in established murine models.

MATERIALS AND METHODS

Isolates. Three isolates of *S. boulardii* were obtained from commercially available products in France (Ultralevure; two different batches, no. 48 and 49 [Sb 48 and Sb 49]) and Italy (Codex; Sb It). The company distributing the product in France is the parent company of the U.S. subsidiary clinically studying the product. The isolate of *S. boulardii* deposited at the ATCC (no. 74012) was requested; however, access was denied by the company (Biocodex Inc.) holding the patent on this isolate. Other isolates of *Saccharomyces* used (8, 14) are listed in Table 1 and the legend for Fig. 1.

Genetic tests and phenotyping. Standard yeast genetic techniques were attempted (50) to assess spore viability and to test for species (40). These included an assay of *S. boulardii* isolates for the ability to mate with known mating-

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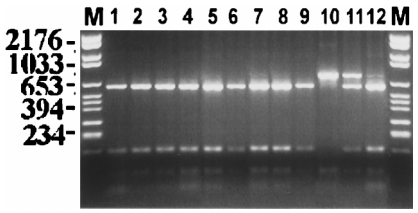

FIG. 1. Photograph of the ethidium bromide-stained, UV-transilluminated, *Mae*I-digested PCR products after electrophoresis within a 3% agarose gel. The DNA from the PCR had been first purified by the Wizard PCR Preps purification system prior to overnight digestion with the 10 U of restriction endonuclease *Mae*I. Molecular size markers are in lanes M, and their corresponding sizes (in base pairs) are given on the left of the figure. Lanes: 1, *S. boulardii* Sb 48; 2, *S. boulardii* Sb 49; 3, *S. boulardii* Sb It; 4, *S. cerevisiae* ATCC 52530; 5, *S. cerevisiae* ATCC 26108; 6, *S. cerevisiae* YJM128, a clinical isolate (14, 30); 7, *S. cerevisiae* ItB9, a clinical isolate (28); 8, *S. cerevisiae* ItB8, a clinical isolate (28); 9, *S. bayanus* ATCC 76515; 10, *S. paradoxus* ATCC 76856; 11, *S. paradoxus*/*S. cerevisiae* hybrid YJM508 (29); 12, *S. bayanus*/*S. cerevisiae* hybrid YJM334 (29).

competent *S. cerevisiae* laboratory strains. A complementation test was used to assess the mating competence of *S. boulardii*. Ethidium bromide-induced petite (mitochondrial respiration deficient) derivatives of *S. boulardii* were made (50) and were mixed with mating-competent MAT**a** Lys2 and MATα Lys2 *S. cerevisiae* strains on a YEPD (1% [wt/vol] yeast extract, 2% [wt/vol] Bacto Peptone, 2% [wt/vol] dextrose, and 2% [wt/vol] agar) plate. The mixed strains were grown overnight and then replica plated to minimal medium containing ethanol and glycerol as the sole carbon sources, on which neither the petite *S. boulardii* strains nor the auxotrophic *S. cerevisiae* strains can grow. If the *S. boulardii* strains were able to mate, the *S. boulardii*/*S. cerevisiae* hybrids would grow on the minimal medium containing ethanol and glycerol.

The *S. boulardii* isolates were tested for their abilities to ferment galactose, maltose, and raffinose and for their abilities to grow on minimal medium with dextrose (50). Sugar fermentation was tested by replicate plating from a YEPD plate to plates with galactose, maltose, or raffinose as the carbon source and with antimycin A to block respiration. An isolate showing abundant growth is fermenting and would be designated "+" (e.g., Gal+ equals galactose fermenting).

Genotyping. Cellular DNA was isolated by previously described methods (16, 45, 48).

Intergenic transcribed spacer region PCR (ITS-PCR) ribotyping is a method that utilizes restriction fragments of PCR products derived from the 5.8S ribosomal DNA (rDNA) and associated intergenic spacer regions (ITS1 and ITS2). Primers for the amplification of the 5.8S rRNA genes were ITS1 (5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′). DNA was amplified, and the amplicons were purified as described previously (29). For the restriction fragment length polymorphism (RFLP) analysis of the amplicons, the purified PCR products were digested with one of the following restriction enzymes: *Mae*I, *Hae*III, *Cfo*I, *Dde*I, *Bgl*II, *Bam*HI, *Hin*dIII, *Eco*RI, *Sma*I, or *Pst*I (Boehringer Mannheim, Indianapolis, Ind.) and the PCR products, with and without endonuclease digestion, were then analyzed by electrophoresis and visualized by staining and transillumination (29).

For RFLP analysis of the cellular DNA, approximately 3 μg of the cellular DNA was digested with 20 U of restriction endonuclease *Eco*RI for 6 h at 37°C. The DNA fragments were separated through a 0.7% (wt/vol) agarose gel in TAE buffer (40 mM Tris-acetate, 0.2 mM EDTA [pH 8.3]) for 20 h at 2 V/cm and visualized by UV transillumination at 302 nm after ethidium bromide staining.

In vivo studies. For the in vivo study of virulence, two experimental murine models of systemic infection were studied to assess the relative virulence of *S. boulardii*, as described previously (8, 14). *S. cerevisiae* YJM145 and Y55 (14, 30) and two of our isolates of *S. boulardii*, randomly selected, Sb 48 and Sb 49, were grown overnight at ambient temperature in YPD broth on a gyratory shaker. In the nonimmunocompromised model, 6-week-old male CD-1 mice (Charles River Laboratories, Hollister, Calif.) were used. In the immunocompromised model, DBA/2N mice (Taconic, Germantown, N.Y.) were used; however, these were 6-week-old mice instead of the previously studied 4-week-old animals (8). Infection was initiated for groups of 10 mice by intravenous inoculation with 2×10^7 CFU of yeast. Two weeks postinfection, all mice were euthanized, and the number of viable yeast cells in the brain of each animal was determined by quantitative plating of serially diluted organ homogenates (14). The mean burdens of fungi were compared by using the nonparametric Mann-Whitney U test (14).

RESULTS

Genetics, genotyping, and phenotyping. None of the three strains of *S. boulardii* produced any spores upon repeated testing at 16, 20, 24, and 30°C. The *S. boulardii* strains did not mate with known mating-competent *S. cerevisiae* strains. Therefore, the classical genetic methods for identification to the species level (40) could not be undertaken for these strains. All three isolates were Gal+ Mal+ Raf+. All three grew on minimal medium, showing that they do not have an auxotrophic requirement for any amino acid or base.

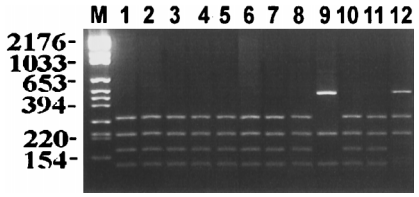

FIG. 2. Photograph of the ethidium bromide-stained, UV-transilluminated, *Hae*III-digested PCR products after electrophoresis within a 3% agarose gel. The DNA from the PCR had been first purified by the Wizard PCR Preps purification system prior to overnight digestion with the 10 U of restriction endonuclease *Hae*III. Molecular size markers are in lane M, and their corresponding sizes (in base pairs) are given on the left of the figure. Lane assignments are the same as those for Fig. 1.

Typing using RFLPs of the PCR-amplified intergenic transcribed spacer regions (including the 5.8S rDNA) showed that the three isolates of *S. boulardii* were not separable from the well-authenticated strains of *S. cerevisiae* with any of the 10 restriction endonucleases assessed, whereas 9 of the 10 recognized species of *Saccharomyces* could be differentiated. *Saccharomyces bayanus* and *Saccharomyces pastorianus* could not be differentiated; these two species were previously postulated to be conspecific (29). Figures 1 and 2 show representative examples of the two most discriminatory restriction endonuclease (*Mae*I [Fig. 1] and *Hae*III [Fig. 2]) digests of the PCR products. In each instance, the PCR product size was approximately 1,000 bp and the product gave a single amplicon, which was subsequently digested into smaller fragments. The interspecific hybrid species gave a PCR product that was the same size as that of the parental species. However, restriction of that PCR product revealed two separate amplicons. Thus, in Fig. 1 (lane 11) and Fig. 2 (lane 12), the hybrids share the ITS-PCR ribotyping pattern of the parental species.

RFLP analysis of cellular DNA with *Eco*RI showed that all three isolates of *S. boulardii* had identical patterns. These RFLP patterns were similar to those of other genotype A (16) *S. cerevisiae* isolates and strains used (Fig. 3).

In vivo studies. The results of the in vivo testing of *S. boulardii* isolates for virulence are presented in Table 1. In CD-1

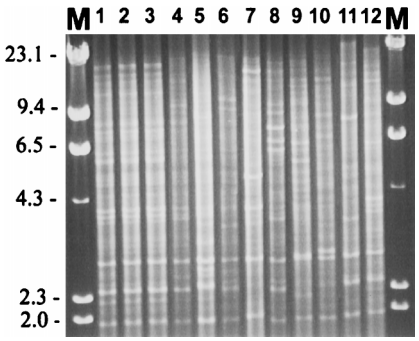

FIG. 3. Representative photograph of UV-transilluminated, ethidium bromide-stained agarose gel. RFLPs generated by *Eco*RI digestion of *S. cerevisiae* DNA are shown. Molecular size markers are in lanes M, and their corresponding sizes (in kilobases) are given on the left of the figure. Lane assignments are the same as those for Fig. 1.

TABLE 1. Recovery of *S. boulardii* and *S. cerevisiae* from the brain in murine models of systemic infection

Isolate[a]	Log_{10} geometric mean CFU/brain (95% CI[b]) for murine model:	
	CD-1	DBA/2N
YJM145	4.51 (4.4–4.6)	4.73 (4.5–4.9)
Sb 48	3.73 (3.6–3.9)	4.42 (4.3–4.6)
Sb 49	3.22 (3.1–3.3)	3.57 (3.5–3.6)
Y55	1.64 (1.3–2.0)	4.51 (4.4–4.6)

[a] Sb 48 and Sb 49 are commercial isolates of *S. boulardii*; the others are *S. cerevisiae* isolates (14).
[b] CI, confidence interval.

mice, comparison with strains YJM145 and Y55 of *S. cerevisiae* showed that both isolates of *S. boulardii* were significantly ($P < 0.001$) more virulent than Y55 but were significantly less virulent than YJM145 ($P < 0.001$) (Table 1). In comparison with each other, Sb 48 was more virulent than Sb 49 ($P < 0.001$). In CD-1 mice, YJM145 is intermediate (and above the median) in virulence and Y55 is avirulent (14). Thus, in this model the rank order of virulence was YJM145 > Sb 48 > Sb 49 > Y55. In comparison with previous work (14), the *S. boulardii* isolates would also be ranked more virulent than some clinical and nonclinical isolates. In the DBA/2N mice the *S. boulardii* isolates proved to be different from each other (Table 1). YJM145 was again significantly more virulent than the other strains ($P < 0.01$ to 0.001). Y55 was equivalent to Sb 48 in virulence ($P > 0.05$), and both were significantly more virulent than Sb 49 ($P < 0.001$). Differences in relative virulence between the two models for individual isolates have been previously described (8). In DBA/2N mice, YJM145 has intermediate virulence and Y55 is less virulent (but is not avirulent) (8). Overall, the two isolates of *S. boulardii* showed some capacity for virulence in these models and are considered to be of intermediate virulence.

DISCUSSION

The commercial strains of *S. boulardii* available to us could not be distinguished by the typing methods used in this study. The results of the present study show that these strains are asporogenous subtypes within the circumscription of *S. cerevisiae* and are not representatives of a separate species.

The classical genetic methods for species assignation cannot be utilized for these organisms as they do not produce spores. The method of ITS-PCR ribotyping used here for species designation is a relatively new technique. This method assesses the DNA that encodes the rRNA genes (rDNA) and has been utilized by many investigators for the determination of species of a wide variety of fungi (1, 5, 18, 20, 24, 26, 43, 56). This methodology has been applied also to *Saccharomyces* species for the authentication of strains in the *S. cerevisiae* "complex" (21). Messner and Prillinger (36) used a similar, yet more thorough method for the differentiation of 10 genotypically distinct *Saccharomyces* species. A recent investigation showed that ITS-PCR ribotyping is a simple method that can distinguish all *Saccharomyces* species, not just *S. bayanus* and *S. pastorianus* (29). Previous research has shown the latter two species to be very closely related (2, 21, 37, 44, 53). Furthermore, the findings of this recent study showed that interspecific hybrids resulting from the mating of two closely related yet distinct species of *Saccharomyces* shared the ITS-PCR ribotyping pattern of both parental species (29). This research (29) therefore allowed for the differentiation of isolates for which differentiation was previously not possible (40). It was anticipated that this technique would be able to differentiate the commercial strains of *S. boulardii* from other species of *Saccharomyces* and would therefore confirm previous reports that these strains belonged to a separate species. However, the findings of the present study showed that the commercial strains designated *S. boulardii* are not representatives of a separate species and should be reidentified as isolates of *S. cerevisiae.*

The results of the *Eco*RI-generated RFLPs of cellular DNA showed that these three strains, obtained from two regions of Europe, are genotypically indistinguishable. These results also support the above contention that these strains, purportedly *S. boulardii*, do not belong to a separate species as the RFLP patterns appear similar to those of the *S. cerevisiae* isolates studied here. The RFLP pattern of the *S. boulardii* isolates appears similar to those of over 80 *S. cerevisiae* isolates we have studied in previous research (16, 28).

A recent communication (32) from the company which holds the patent on this strain emphatically states that "*S. boulardii* is a completely different species of yeast from baker's, brewer's or wine yeast," implying that the commercial isolates (and that deposited at ATCC) are derived directly from the originally described (49) isolate. The author supports the statement about species with seven pieces of evidence, which are analyzed as follows. (i) The strain has a separate designation by the ATCC. This designation would have been done only at the request of the patent-holding supplier; it does not confer species status on the organism, and the strain has not been made available for study. (ii) The quoted reference for phenotypic data which reportedly can separate this strain from *S. cerevisiae* is an unpublished personal communication and cannot therefore be assessed. Furthermore, the author states that *S. boulardii* does not use galactose as a carbon source as do wild-type *S. cerevisiae* strains. This is an unacceptable marker for species identification as the utilization of galactose has been shown to be variable among *S. cerevisiae* strains (3); indeed, sugar utilization is not useful for species delimitation or as a marker for DNA homology (41). Moreover, many *S. cerevisiae* isolates are Gal− in our hands and in the literature; these include well-authenticated laboratory isolates related to strain S288c (38) and strain Y55 and clinical isolates YJM436 and YJM522 (14). In addition, many clinical isolates are heterozygous for mutations affecting galactose fermentation ability (30). At least one group of *S. cerevisiae* is entirely or predominantly Gal− (41). Fermentation tests for galactose for all three of the *S. boulardii* strains in the present study were positive. This may be explained in part by differences in the methodology used for this test; the previous communication (32) gave no indication as to how the author arrived at the conclusion that *S. boulardii* is unable to utilize galactose as a carbon source. (iii) Three separate genetic studies which reportedly can distinguish this organism from *S. cerevisiae* are quoted; however, all of these are again unpublished data and cannot therefore be assessed. (iv) The final two references which are cited to support the distinct status of *S. boulardii* are the most interesting as they are published and can be studied. One (20) assessed the four sibling species of the genus *Saccharomyces* (*S. bayanus*, *S. cerevisiae*, *S. paradoxus*, and *S. pastorianus*) for variation by restriction analysis of mitochondrial DNA. Isolates of *S. boulardii* were not studied, nor was this organism mentioned in that article. The last study quoted is that by Cardinali and Martini (9), who investigated the electrophoretic karotypes of authentic strains representative of the genus *Saccharomyces* sensu stricto. This careful study used a principal-component analysis of the molecular weights of the bands of the electrophoretic karyotypes of 32 certified authentic strains to develop a matrix in which

strains of the four species studied were clustered. This established a reference for assessing the localization of electrophoretic karyotype profiles obtained from unknown, noncertified yeast. Utilizing this model these investigators assessed the statistical distance (residual standard deviation [RSD]) of an isolate of *S. boulardii* (isolate DBVPG 6699; see Table 3 in reference 9) as 0.3730 compared to that of a standard *S. cerevisiae* isolate. The reference RSD value for *S. cerevisiae* in this model was given as 0.3952, and according to this analysis, an unknown strain belongs to a given species when its RSD value falls below the calculated reference RSD for the universe representing that species (9). Therefore, the only possible conclusion is that *S. boulardii* is a subtype of the species *S. cerevisiae*, a conclusion supported by our findings.

A recent study using the random amplified polymorphic DNA analysis methodology also concluded that *S. boulardii* is a member of the genotypic species *S. cerevisiae* (37). DNA reassociation data have recently shown 95% homology of *S. boulardii* and *S. cerevisiae* (54). Strains with >80% of their base sequences in common have been considered to belong to the same species (3, 52).

The name *S. boulardii* is invalid per the International Code of Botanical Nomenclature (ICBN) (19), because it was not effectively published in a readily available forum and lacks a description in Latin and the designation of accessible dried or lyophilized material (ICBN Articles 29.1, 32.1, 36, and 37). Taxonomic manuals and reviews (3, 4, 54) consider *S. boulardii* an abolished term and synonymous with *S. cerevisiae*. On the basis of this review of the literature and the results of the current investigation, it is not possible to support the previously made contention that the strain previously designated *S. boulardii* is a separate species from *S. cerevisiae*. Moreover, Biodiphar, the Biocodex subsidiary marketing the product in Belgium, also describes *S. boulardii* as *S. cerevisiae* (11). If indeed, as indicated previously (32, 33), the commercial isolates are direct descendants of the originally described isolate (49), then the latter may also be correctly described as *S. cerevisiae*, a conclusion that may be supported by the work of others (9, 37, 54).

There have been an increasing number of reports of human infections due to *S. cerevisiae*. For example, McCusker et al. (31) cited 12 such publications in 1994, 11 of these dealing with systemic infections, and that was not an exhaustive bibliography at the time. Many previously unpublished descriptions of clinical isolates appear in a study by Clemons et al. (14). Although immunocompromised patients are at risk, infection in healthy hosts also occurs (14, 31). Systemic infection can occur after oral ingestion (23). To assess the pathogenic potential of *S. boulardii*, two of the strains were used as the infecting organisms in murine models. Our previous work (8, 14) with these models has demonstrated that isolates of *S. cerevisiae* display a continuum of virulence potential and that virulence, as previously defined, in mice (14) was significantly associated with clinical isolates. In comparison with virulent and avirulent isolates of *S. cerevisiae* (8, 14), the two isolates of *S. boulardii* proved to have an intermediate degree of virulence. Mortality in the DBA/2N model used in this study was not seen, likely due to the increased resistance to fungal infection of (even slightly) older mice (7). While the level of virulence of *S. boulardii* is modest, it raises the question of the wisdom of using a potential pathogen as a therapeutic agent, especially in immunocompromised patients, in light of the demonstration that uptake of this organism from the gut lumen and translocation across the mucosa may occur (10) and reports of fungemia caused by *S. boulardii* administered orally (46, 55, 57). In addition, two isolates from different *S. boulardii* batches significantly differed in virulence in the same direction in two different models. This suggests that different batches of the preparation may not be uniform and may differ in a gene or genes that determine virulence. Possibly also relevant to strain variation is that the initial publication (49) described a sporulating yeast, whereas our isolates are asporogenous, as shown by repeated testing.

It would appear then that this strain of *S. cerevisiae*, which has been previously arbitrarily designated *S. boulardii*, has been shown to be useful in a range of clinical situations, particularly in the preventive treatment of diarrhea. It is likely that some other strains of *S. cerevisiae* also have these properties. In fact, animal model studies (6) and at least two clinical studies (12, 47) have shown that other *S. cerevisiae* strains do have such therapeutic properties.

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