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Discordant Influence of *Blastomyces dermatitidis* Yeast-Phase-Specific Gene *BYS1* on Morphogenesis and Virulence

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*Blastomyces dermatitidis* is a thermally induced dimorphic fungus capable of causing lung and systemic infections in immunocompetent animal hosts. With the publication of genomic sequences from three different strains of *B. dermatitidis* and the development of RNA interference as a gene-silencing tool, it has become possible to easily ascertain the virulence and morphological effects of knocking down the expression of candidate genes of interest. *BYS1* (*Blastomyces* yeast-phase-specific 1), first identified by Burg and Smith, is expressed at high levels in yeast cells and is undetectable in mold. The deduced protein sequence of *BYS1* has a putative signal sequence at its N terminus, opening the possibility that the *BYS1*-encoded protein is associated with the yeast cell wall. Herein, strains of *B. dermatitidis* with silenced expression of *BYS1* were engineered and tested for morphology and virulence. The silenced strains produced rough-surfaced cultures on agar medium and demonstrated a propensity to form pseudohyphal cells on prolonged culture *in vitro* and *in vivo*, as measured in the mouse lung. Tests using a mouse model of blastomycosis with either yeast or spore inocula showed that the *bys1*-silenced strains were as virulent as control strains. Thus, although silencing of *BYS1* alters morphology at 37°C, it does not appear to impair the pathogenicity of *B. dermatitidis*.

The endemic dimorphic fungi are primary pathogens with worldwide distribution and include *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Coccidioides posadasii*, *Paracoccidioides brasiliensis*, *Sporothrix schenckii*, and *Penicillium marneffei*. Infections caused by these fungi can cause severe disease in those immunocompromised by AIDS, malignancy, and pharmacologic immunosuppression (22). Preventive and therapeutic measures to combat these infections are required, and for this purpose, it is necessary to improve our understanding of the pathogenesis of these fungi.

The dimorphic fungi can undergo a temperature-dependent morphological change from conidium or mycelium at environmental temperatures (22°C) to pathogenic yeast at body temperature (37°C) (1, 12, 19–21, 24). *B. dermatitidis* is endemic to the Ohio and Mississippi River valleys and causes pneumonia following the inhalation of conidia from the soil and subsequent conversion to budding yeast cells in animal hosts. Recent research efforts have focused on identifying genes associated with this phase transition, based on a hypothesis that such genes may play important roles in pathogenesis.

Several yeast-phase-specific genes have been identified. In *B. dermatitidis*, *Blastomyces adhesin 1* (*BAD1*) (5, 6, 13) encodes a 120-kDa major surface protein that is expressed only in the yeast phase and functions as a host cell adhesin and immunomodulating factor. Deletion of *BAD1* attenuates the pathogen’s virulence (7). A *bad1*-null strain has been used as a live attenuated vaccine (26). *H. capsulatum* contains two well-studied yeast-phase-specific genes with pronounced roles in pathogenesis, i.e., that for calcium binding protein 1 (*CBP1*) (21) and that for yeast-phase-specific protein 3 (*YPS3*) (2, 3). *Coccidioides* spherule outer wall glycoprotein (SOWgp) is an immunodominant protein encoded by a parasitic-phase-specific gene that also has a role in adhesion and virulence (12). These examples strongly implicate yeast-phase-specific genes as crucial for virulence in the endemic dimorphic fungi.

In addition to *BAD1* in *B. dermatitidis*, a second yeast-phase-specific gene, that for *Blastomyces* yeast-phase-specific protein 1 (*BYS1*), has been identified by differential cDNA library screening (4, 9). The 0.9-kb-long *BYS1* transcript is highly expressed in yeast cells but is absent from mold (9). The function of *BYS1* remains unknown.

As a means of rapidly analyzing the role of *BYS1* in morphogenesis and virulence, we used a green fluorescent protein (GFP) sentinel gene-silencing strategy to effectively suppress *BYS1* expression (16). This strategy has been used to analyze the function of *B. dermatitidis* genes such as *BAD1* and *CDC11*, as well as the transgenically expressed *lacZ* gene (16). We cloned *BYS1* next to GFP in the GFP sentinel RNA interference (RNAi) vector to create a hairpin to simultaneously silence GFP and *BYS1* expression and allow diminished GFP to report the expression of *BYS1*. Strains with decreased expression of *BYS1* were thereby generated and used to study the role of *BYS1* in pathogenesis. Although reduced expression of *BYS1* promoted the growth of the organism as pseudohyphae after prolonged culture *in vitro* and to a lesser extent in the mouse lung, *bys1*-silenced strains showed no alterations in virulence *in vivo* in a murine model of infection.

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MATERIALS AND METHODS

Strains and growth conditions. B. dermatitidis strains 26199 (American Type Culture Collection) and SLH14083 (clinical isolate; referred to here as 14083) were used to generate the GFP reporter strains, 26199-GFP and 14083-GFP, used in this study. Both strains are virulent in a murine model of infection. Strain 55 is a derivative of 26199 in which the virulence factor gene BAD1 was knocked out by homologous recombination (7). Mycelia of strain 14083, but not 26199, are able to produce conidia. Yeast cells were grown at 37°C on Histoplasma macrophage medium (HMM) (25), Middlebrook 7H10 with oleic acid–salts–catalase–enrichment (Becton, Dickinson and Company, Franklin Lakes, NJ), or 3 M medium (25). Mycelial cultures were grown at 22°C on potato dextrose agar or 3 M medium. Use and maintenance of Agrobacterium tumefaciens strain LBA1100 were as previously reported (23).

RNA extraction, cDNA synthesis, and bys1 RNAi plasmid constructions. B. dermatitidis yeast cells were grown in liquid HMM at 37°C for 2 days and harvested. The yeast cell wall and membrane were disrupted with glass beads using a Mini-Beadbeater-8 (Biospec Products, Bartlesville, OK). Total RNA was extracted by using TRI Reagent RT RNA/DNA-Protein reagents according to the manufacturer’s protocol (Molecular Research Center, Inc., Cincinnati, OH). RNA was further purified using an RNAeasy Mini kit (Qiagen, Valencia, CA); the concentration was measured spectrophotometrically (Nanodrop Technologies, Wilmington, DE), and the integrity was evaluated by agarose gel electrophoresis. To prepare cDNA, 1 µg of total RNA was reverse transcribed using TaqMan reverse transcription reagents and the manufacturer’s protocol (Roche Molecular Systems, Branchburg, NJ).

An RNAi vector was constructed to silence GFP and BYS1. The B. dermatitidis target sequence was amplified from B. dermatitidis cDNA using gene-specific primers with the attB1 or attB2 Gateway (Invitrogen, Carlsbad, CA) sequences at the 5’ end (primers BdBys1-ΔAttB1 [5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTCTCACTGCA GCTACTCTCTGTGTG-3′] and BdBys1F-AttB2 [5′-GGGGACACATTGTGACAAAGGCTGGGTTCTCACTGCA GCTACTCTCTGTGTG-3′]). As previously described (16), the PCR product of the target gene was directionally cloned into the donor vector pDONR207 using the BP Clonase-mediated recombination reaction (Invitrogen) to create an entry vector. The entry vector was then subjected to an LR Clonase-mediated recombination reaction (Invitrogen) to introduce two copies of the target gene into the destination vector pFANTAi4 to make a GFP-bys1 gene RNAi vector (pFi-bys1) (16).

Transformation of B. dermatitidis. Agrobacterium-mediated gene transfer was used in all transformations as previously described (23). Transformants were selected on 3 M agar supplemented with 100 µg/ml hygromycin B (A. G. Scientific Inc., San Diego, CA) and 200 µM cetoxatime (Sigma-Aldrich).

Silencing of B. dermatitidis yeast-phase-specific gene BYS1. Strains 26199-GFP and 14083-GFP were transformed with control vectors pH2AB-GFPi, containing sequences (15). The PCR product of the target gene was directionally cloned into the donor vector pDONR207 using the BP Clonase-mediated recombination reaction (Invitrogen) to create an entry vector. The entry vector was then subjected to an LR Clonase-mediated recombination reaction (Invitrogen) to introduce two copies of the target gene into the destination vector pFANTAi4 to make a GFP-bys1 gene RNAi vector (pFi-bys1) (16)

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Northern blot analysis. RNA blot hybridization used standard protocols (8). Formaldehyde-agarose gels for Northern blot analysis were loaded with 15 µg of total RNA per lane. The probe for B. dermatitidis GAPDH was a 0.75-kb EcoRI fragment from pCR2.1-GAPDH#1 (provided by Laurenz Smith, Idaho State University). A 421-bp fragment probe for B. dermatitidis BAD1 was amplified by PCR from pFANTAi4 (the GFP-bys1 RNAi vector used in this study) using primers BD(bys1)F (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTCTCACTGCA GCTACTCTCTGTGTG-3′) and BD(bys1)R (5′-GGGGACACATTGTGACAAAGGCTGGGTTCTCACTGCA GCTACTCTCTGTGTG-3′). Fractions for probes were gel purified using a QIAquick gel extraction kit (Qiagen) and radiolabeled with [32P]dCTP using a Prime-a-Gene probe-labeling kit (Promega, Madison, WI). Radioactive signals from probed and washed membranes were detected using a PhosphorImager (Storm860; Molecular Dynamics, Sunnyvale, CA). The PhosphorImager signal levels were quantified using Quantity One Software (version 4.6.8) from Bio-Rad Laboratories.

Assessment of virulence in experimental pulmonary infection. C57BL/6 mice were infected with either yeast cells from strain 26199 or spores from strain 14081 of three independent isogenic GFP-bys1 silenced isolates or controls (including the GFP sentinel strain without any RNAi and the sentinel strain that had been transformed with the non-RNAi vector or the GFP-only RNAi vector). Mice (n = 8 to 10/group) received 500 yeast cells or spores intratracheally. Signs of disease during daily monitoring, survival over 14 days, and burden of infection (CFU count) in harvested lungs at day 14 after infection were analyzed. Statistically significant differences between CFU counts were determined using the Wilcoxon rank test for nonparametric data (10).

Fungal cell morphology in infected lungs. Sections from paraaffin-embedded, formalin-fixed lungs from up to 10 different infected mice for each strain tested were stained with hematoxylin-eosin and Gomori’s methenamine silver stain. Organisms were counted and evaluated for yeast or pseudohyphal morphology at a magnification of ×400 on a monitor after digital photography by a trained pathologist who was blinded to the identity of the samples. Counts were evaluated in 10 random fields of 0.67 mm² and expressed as percent pseudohyphae (of the total count per field). The significance of differences between experimental and control groups was based on logistic regression models.

RESULTS

BYS1 and BYS1-related genes. Analysis of the deduced amino acid sequence of BYS1 revealed a putative signal peptide, indicating that the protein may be targeted to the endoplasmic reticulum-Golgi apparatus and possibly secreted. To see if homologues with known activities or sequence motifs could help to predict the BYS1 gene function, BLAST analyses were performed using the NCBI database or the Broad Institute Fungal Genome Initiative (http://www.broadinstitute.org /science/projects/fungal-genome-initiative/). From these analyses, a possible paralog of BYS1 with only 34% amino acid identity (expected value = 9e-14) was identified in the B. dermatitidis genome. In addition, a BYS1 superfamily conserved domain (cc04669) was identified in the Conserved Domain Database. Within this superfamily are members of other fungal species, including the closely related species H. capsulatum and Coccioidioides spp., but the identified sequences encode proteins more closely related to the BYS1 paralog (by both amino acid identity and reciprocal BLAST) than to BYS1 itself. None of these predicted proteins has known functions or conserved protein motifs, other than being placed in the BYS1 superfamily (9; data not shown). Therefore, from the BYS1 sequence information alone, it is not possible to predict BYS1 gene function.

Identification of bys1-silenced strains. As a first step in the functional analysis of BYS1, we sought to generate strains with reduced BYS1 expression and determine if they displayed altered morphological or pathogenic properties. The GFP sentinel RNAi tracking strategy was used to rapidly identify B. dermatitidis bys1-silenced strains (16). We constructed an RNAi plasmid for BYS1 by using a 421-bp fragment of the B. dermatitidis BYS1 coding sequence from the cDNA of strain 26199. The 421-bp BYS1 sequence was cloned into the GFP sentinel RNAi vector pFANTAi4 (16) to create the GFP-bys1 RNAi vector (pFi-bys1). The B. dermatitidis 26199-GFP reporter strain was then transformed with pFi-bys1. We screened GFP-bys1 RNAi transformants for a low GFP signal level as a sentinel of reduced BYS1 expression. Thirty-two clones of GFP-bys1 RNAi were randomly selected for GFP signal quantification (Fig. 1A). As controls, eight transformants each from
FIG. 1. Silencing GFP-bys1 in *B. dermatitidis* GFP sentinel strains. (A) GFP signal levels of individual colonies of 8 each of the control transformants (non-RNAi and GFP-only RNAi) and 32 randomly selected GFP-bys1 RNAi transformants detected by FluorImager. (B) The GFP signal level of cells from individual colonies was measured by flow cytometry, and the mean fluorescence intensities are shown. Control strains: non-RNAi, *n* = 2; GFP-only RNAi, *n* = 2. GFP-bys1 RNAi strains, *n* = 32. The number designation for each of the GFP-bys1 RNAi strains is indicated. (C) Northern blot analysis to confirm BYS1 silencing in GFP-bys1 RNAi strains. Each lane represents 15 μg of total RNA. The same blot was probed for BYS1, exposed to the Phosphorimager screen, stripped, and reprobed with GAPDH (see Materials and Methods). Shown is a composite image of the two probes, with only the specific hybridizing regions shown for each. The relative BYS1 expression of each strain is indicated below and was obtained by dividing the BYS1 signal level by the GAPDH signal level (to correct for loading differences). GFP-expressing strain 26199-GFP, two control strains (non-RNAi and GFP-only RNAi), and two GFP-bys1 RNAi strains are shown.
FIG. 2. Rough colony phenotype correlates with sentinel GFP silencing. 7H10 slants were inoculated with the indicated strains and incubated at 37°C for 3 weeks. The mean fluorescence intensity from the flow cytometry analyses in Fig. 1B is shown below each image. There is a strong correlation between a low GFP value and a rough colony appearance. Note that for GFP-bys1 RNAi strain 27, silencing failed and the colony phenotype is smooth.

the GFP reporter (26199-GFP) transformed with a non-RNAi or a GFP-only RNAi vector were also randomly selected (Fig. 1A). Non-RNAi transformants showed GFP signal levels as high as that of the reporter strain 26199-GFP, while GFP-only RNAi transformants showed low GFP signal levels equivalent to that of untransformed wild-type strain 26199 (data not shown). GFP-bys1 RNAi transformants showed a wide range of GFP signal levels (Fig. 1B). Compared to the non-RNAi control, about 50% of the GFP-bys1 transformants showed an ~10-fold GFP signal level reduction (Fig. 1B). We analyzed these low-GFP transformants for mRNA abundance and phenotypic alterations.

Northern analysis of BYS1 transcript. To prove that the sentinel reporter and the phenotypes accurately signaled BYS1 silencing, we determined relative mRNA levels by Northern blot analysis for representative transformants of wild-type 26199, non-RNAi control, GFP-only RNAi control, and GFP-bys1 RNAi lines. The blotted membrane was probed sequentially with BYS1 and GAPDH probes (Fig. 1C). The wild type, non-RNAi, and GFP-only RNAi controls had a strong BYS1 signal. The BYS1 transcript was sharply reduced in the GFP-bys1 RNAi transformants to nearly background levels (Fig. 1C). The GAPDH transcript signal level was used to correct for RNA loading differences, and the BYS1 mRNA levels in the silenced strains were found to be reduced by more than 3% of the control levels (Fig. 1C).

bys1-silenced strains exhibit a defect in morphogenesis. Changes in yeast cell surface components (11, 14) or cell morphology (i.e., pseudohyphae [17]) often result in a macroscopic change from a smooth-colony phenotype to a rough colony surface. GFP-bys1 RNAi transformants with a low GFP signal level showed a rough-colony phenotype on 7H10 agar medium that was not apparent after the initial passage of the cells but appeared as the cultures were incubated beyond 2 weeks. In contrast, representative GFP-bys1 RNAi strains with a normal GFP signal level, non-RNAi controls (normal GFP signal level), and GFP-only controls (low GFP signal level) all retained smooth colony surfaces during early and extended culture, as expected for normal yeast cells (Fig. 2). Microscopically, the GFP-bys1 RNAi strains with a low GFP signal level and a rough colony appearance also exhibited pseudohyphal morphology, whereas the controls strains, including the non-RNAi (normal GFP signal level) and GFP-only RNAi (low GFP signal level), demonstrated a homogeneous, round budding-yeast appearance (Fig. 3). Despite the underlying defect in the GFP-bys1 RNAi strains, there was no effect on the growth rate, as these strains (and the control strains) grew at the same rate as wild-type cells based on optical density measurements of liquid cultures. Growth rates were measured for freshly subcultured strains in early- to mid-log-phase liquid cultures. Under these conditions, both the bys1-silenced and control stains exhibit budding-yeast morphology (data not shown). The cells grown in liquid culture did not adopt a pseudohyphal morphology during short-term growth. The growth rates of pseudohyphal-form cells after extended culture were not quantified.

Measurements of cell surface and cell wall intactness. Because of the changes in colony appearance and morphology resulting from BYS1 silencing, we sought to probe for perturbations of yeast cell wall content and chemical sensitivity that may be associated with these altered morphologies. Alteration of the cell wall α,1,3-glucan content of B. dermatitidis grown at 37°C results in a rough colony appearance (11). Therefore, the GFP-bys1 RNAi strains were tested for loss of α,1,3-glucan on
the cell surface using a fluorescent-antibody microscopic assay (11). There was no difference in the intensity of fluorescence detected in the GFP-bys1-silenced strains compared to that of the controls (data not shown). Immunostaining and flow cytometry were used to determine if the morphological effect of BYS1 silencing alters the localization of the yeast-phase-specific virulence factor BAD1 on the cell surface. As expected, BAD1 knockout strain 55 (n = 1) lacked surface BAD1 (and GFP), whereas the non-RNAi controls (n = 8) expressed surface BAD1 and GFP and the GFP-only RNAi controls (n = 8) expressed surface BAD1 but had reduced GFP. For the GFP-bys1 strains, the results were similar to those of the GFP-only RNAi control (n = 32), indicating no alteration in the expression of surface BAD1 (data not shown).

Some mutant strains of fungi with altered cell walls are more sensitive than their wild-type progenitors to the inhibiting effect of the cell wall-binding compounds Congo Red and Calcofluor White (17). Therefore, we tested whether a bys1 RNAi strain had increased growth sensitivity to these compounds by culturing them in liquid HMM supplemented with increasing amounts of either inhibitor (0.2 to 20 μg/ml). We did not find increased sensitivity of the bys1 RNAi strain for either Congo Red or Calcofluor White compared to the GFP-only RNAi control (data not shown).

Experimental infection of bys1-silenced strains in mice. As a test for altered virulence in the GFP-bys1 RNAi strains, we used a murine model of infection (26). For experimental infection, inocula of 500 cells of the 26199-GFP reporter, a representative non-RNAi transformant, a representative GFP-RNAi transformant, and three independent GFP-bys1 RNAi strains with the lowest GFP signal level, all grown at 37°C, were introduced intratracheally into mice (8 to 10/group). In order to avoid differences in the efficiency of intratracheal infection using morphologically different cells, we used cells from fresh cultures for the silenced and control strains that were similar in yeast morphology. To investigate whether the decreased BYS1 expression impairs initial lung colonization, 2 or 3 mice from each group were sacrificed for lung CFU counting at day 1 postinfection. The average lung CFU counts of the groups were as follows: 26199-GFP reporter, 716 (n = 3); non-RNAi transformant, 854 (n = 2); GFP-RNAi transformant, 894 (n = 2); the 3 GFP-bys1 transformants, 216 (n = 3), 334 (n = 2), and 683 (n = 3). The rest of the mice were monitored for the onset of disease symptoms, which appeared at similar times in all of the groups. At 14 days, all of the animals in all of the groups appeared moribund and some of the mice in both the control and bys1 RNAi groups had died. At this time, lungs were harvested and the burdens of infection were determined (Fig. 4A). In all of the groups, the mice had a burden of infection in the range of 10^6 CFU. None of the GFP-bys1 RNAi strains were significantly different (P < 0.05) from the GFP-only RNAi strain (Fig. 4). Analysis of the yeast colonies obtained from mouse lungs indicated that GFP expression was still silenced in the RNAi strains (data not shown). These results indicate that silencing induced by bys1 RNAi did not lead to decreased virulence.

BYS1 does not regulate phase transition or impair conidiation. To extend the analysis of the effects of BYS1 silencing to phase conversion and conidiation, we generated a second set of bys1 RNAi lines in B. dermatitidis strain 14081, since this strain produces more abundant conidia than strain 26199. Both the 26199 and 14081 GFP-bys1-silenced strains converted to mycelia following a temperature shift from 37°C to 22°C on 3 M agar. Analysis using light microscopy revealed normal mycelial morphology and conidiation. Conidia harvested from 14081 GFP-bys1-silenced strains and controls were enumerated, and
dilutions were plated on 3 M agar and incubated at 37°C to determine if the GFP-\textit{bys1}-silenced strains were defective in spore germination or conversion back to the yeast form. Based on colony counts, the silenced and control strains germinated and gave rise to yeast colonies with the same efficiency \textit{in vitro} (data not shown).

To investigate if \textit{bys1} RNAi affects morphological conversion from spores to yeast and virulence \textit{in vivo}, the murine model of pulmonary infection (above) was used. Here, C57BL/6 mice were each infected intratracheally with an inoculum of 500 conidia (instead of yeast cells) with the 14081 control and GFP-\textit{bys1} RNAi strains. Three weeks postinfection, mice infected with the control and GFP-\textit{bys1}-silenced strains appeared moribund. All of the GFP-\textit{bys1}-silenced strains had lung CFU levels similar to or, in the case of \textit{bys1} RNAi strain 10, higher than those of the controls (Fig. 4B). Colonies that emerged from lung cultures were analyzed for GFP fluorescence (a total of 50 colonies, from up to 10 lungs per strain). In the three \textit{bys1} RNAi strains, 10, 20, and 36, there were 3.3-, 7-, and 6-fold reductions in fluorescence, respectively, compared to non-RNAi control strains, demonstrating that the \textit{bys1} RNAi strains maintained silencing in the lungs.

At the time mice were analyzed for the burden of lung infection, a portion of lung tissue was resected and fixed in formaldehyde for microscopic analysis. The lung sections were stained, and the fungal cells were assessed for yeast or pseudohyphal morphology. For the 14081-GFP and non-RNAi control strains, pseudohyphal cells were rarely observed (<0.6%). In the \textit{bys1}-silenced strains, there was an increase in pseudohyphal morphology up to an average of 4.2% of the cells of \textit{bys1} RNAi strain 36 (individual counts from mouse lungs ranged from 0% to 7%, with a mean of 3.2%, averaged from 30 microscopic fields analyzed, representing three different \textit{bys1} RNAi strains and a total of 1,541 fungal cells counted). The proportion of pseudohyphal cells of the \textit{bys1}-silenced strains \textit{in vivo} was significantly higher than that in the control groups (\(P < 0.004\)).

**DISCUSSION**

Previous work has shown that \textit{BYS1} mRNA is expressed at high levels in yeast cells but rapidly diminishes and disappears as yeast-to-mycelial conversion occurs following a temperature shift to 25°C (4, 9). Incubation of mycelia at 37°C leads to the reappearance of \textit{BYS1} within 12 h. \textit{BYS1} is postulated to encode an 18.6-kDa protein that contains multiple putative phosphorylation sites, two 34-amino-acid domains with similarly spaced 9-amino-acid degenerative repeating motifs, and a hydrophobic N terminus, which may act as a signal sequence for endoplasmic reticulum-Golgi apparatus localization and secretion (9). BLAST analyses of the predicted \textit{BYS1}-encoded amino acid sequence against the NCBI and Broad Institute databases revealed that several fungal species have weakly matching hypothetical proteins. The identified sequences have higher identity to a paralog of \textit{BYS1} than to \textit{BYS1} itself. This was true even for closely related species, including \textit{H. capsulatum}, \textit{P. brasiliensis}, and \textit{C. immitis}. While there is no predicted function for either \textit{BYS1} or the \textit{BYS1} paralogs, these analyses suggest that \textit{BYS1} has diverged in sequence from the others and therefore may also have diverged in function.

Gene silencing has become a useful tool for investigating gene function in eukaryotes. However, one limitation to RNAi is the range, from very low to very high, of the efficiency of silencing of a target gene among sampled RNAi transformants. Therefore, it is necessary to monitor the efficiency of gene silencing by measuring decreases in target gene mRNA in individual transformants by a time-consuming and labor-invasive assay such as real-time PCR or Northern blot analysis. Recently, a GFP sentinel RNAi system was developed to allow easy and rapid screening for the most efficiently silenced transformants (16, 18). The GFP signal level is an indicator of the level of silencing in transformants, and the level of GFP fluorescence accurately reports the level of target gene silencing (16).

The GFP sentinel RNAi system was applied for loss-of-function analysis of \textit{BYS1} by transforming a GFP reporter strain using an \textit{A. tumefaciens} binary plasmid engineered to express hairpin RNA containing both GFP and \textit{BYS1}. Thirty-two clones of \textit{GFP-bys1} RNAi were picked for further analysis. In these, the degree of GFP silencing varied, and about half of the transformants had an at least 10-fold reduction in GFP fluorescence (Fig. 1A and B). RNA blot hybridization showed nearly background levels of \textit{BYS1} mRNA transcript in \textit{GFP-bys1} RNAi strains with low GFP intensity. In contrast, the control strains maintained close-to-wild-type levels of \textit{BYS1} transcripts (Fig. 1C). Several of the \textit{GFP-bys1} RNAi strains (with low GFP intensity) were analyzed for phenotypes that would affect the pathogenic life cycle of this organism.

One striking phenotype observed in \textit{bys1}-silenced strains was the rough appearance of colonies grown on agar surfaces. This phenotype is not apparent immediately but develops as the cultures mature. Microscopically, \textit{GFP-bys1}-silenced strains at this stage showed an increased proportion of pseudohyphal types. Since \textit{B. dermatitidis} can develop pseudohyphal morphology when grown under stressful conditions (such as hyperosmotic medium; data not shown), we felt it essential to show that the altered morphology of \textit{GFP-bys1} RNAi strains is due to the silencing of \textit{BYS1} and not to a general stress response.

We took great care in generating control strains with which to compare cell morphology and culture surface phenotypes. At the same time as for the \textit{GFP-bys1} RNAi strains, several types of control strains were produced by \textit{Agrobacterium}-mediated transformation. These included strains in which a non-RNAi vector was introduced, and strains harboring an introduced RNAi vector that silences only GFP. These strains were grown in parallel with the \textit{GFP-bys1} RNAi strains, and none of them had the morphological phenotypes found by silencing \textit{BYS1}. Several independently generated \textit{bys1}-silenced strains were analyzed (Fig. 2, strains 29, 32, 35, and 47), as was a strain that failed at silencing \textit{BYS1} (Fig. 2, strain 27). The strains with strong \textit{GFP-bys1} silencing all show the rough surface phenotype, and the strain with weak \textit{GFP-bys1} silencing had morphological and surface characteristics of wild-type \textit{B. dermatitidis}. Following the infection of mice, \textit{bys1}-silenced cells had a mild but significant increase in the proportion of pseudohyphal cells in the lungs of these animals. The visualization of pseudohyphal forms of \textit{B. dermatitidis in vivo} is remarkable in itself, which is underscored by their absence in the control strains used to infect mice.

These phenotypes suggest that \textit{BYS1} may play a role in cell wall formation or morphogenesis in \textit{B. dermatitidis}. Since the
BYS1 gene sequence appears to encode a signal peptide at its amino terminus, it is enticing to speculate that the BYSI-encoded protein is secreted and localized on the cell surface, where it may impact cell surface structure and perhaps overall cell morphology. Since the bysl-silencing phenotype only develops as the cells mature, it may be that the loss of BYSI affects the cell wall in a way that is conditional on cell density, nutrient availability, or another property inherent to older cultures.

BAD1 is a surface protein of B. dermatitidis that is an essential virulence factor whose distribution on the yeast cell surface can be affected in strains with altered yeast cell morphology and surface structure (11, 17). Therefore, we tested whether bysl silencing results in altered BAD1 distribution on the cell surface. However, this is not the case, as immunological detection of BAD1 on the surface of bysl-silenced strains was similar to that on control cells (data not shown).

Several types of experimental results point to connections between the yeast cell surface, cell morphology, and virulence. These include the need for BAD1 on the surface for virulence but also observations that changes in cell wall glucan composition can decrease virulence (11) and that deletion of DRK1, which results in a failure to form yeast cells at 37°C, also ablates virulence (17). These published findings suggest the possibility that bysl-silenced strains, with their conditional morphological defect, could also have an effect on virulence. However, we found no significant reduction in virulence in bysl-silenced strains compared to the controls. As the proportion of pseudohyphal cells in the lungs of mice infected with the bysl-silenced strains only amounted to a small fraction of the total fungal cells, we cannot formally exclude the possibility that the bysl-silenced cells with a pseudohyphal phenotype have altered virulence compared to those with budding yeast morphology. In contrast to other well-characterized yeast-phase-specific genes (BAD1, CBP1, YPS3, and SOWgp) which are essential for virulence but not morphogenesis, the yeast-phase-specific BYSI gene of B. dermatitidis has a role in morphogenesis but not virulence.

We have provided additional evidence that the GFP sentinel RNAi system (16) is useful for quickly identifying phenotypes after disruption of the expression of a target gene in the dimorphic fungus B. dermatitidis. As more whole-genome sequences of dimorphic fungi become available, this will be a valuable tool in determining which genes may have a role in dimorphism and virulence.

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