

Targeted Gene Disruption Reveals an Adhesin Indispensable for Pathogenicity of *Blastomyces dermatitidis*

By T. Tristan Brandhorst,* Marcel Wüthrich,* Thomas Warner,^{||} and Bruce Klein*^{‡§¶}

From the *Department of Pediatrics, the [‡]Department of Internal Medicine, the [§]Department of Medical Microbiology and Immunology, and the ^{||}Department of Pathology and Laboratory Medicine, and the [¶]Comprehensive Cancer Center, University of Wisconsin Medical School, Madison, Wisconsin 53792

Summary

Systemic fungal infections are becoming more common and difficult to treat, yet the pathogenesis of these infectious diseases remains poorly understood. In many cases, pathogenicity can be attributed to the ability of the fungi to adhere to target tissues, but the lack of tractable genetic systems has limited progress in understanding and interfering with the offending fungal products. In *Blastomyces dermatitidis*, the agent of blastomycosis, a respiratory and disseminated mycosis of people and animals worldwide, expression of the putative adhesin encoded by the WI-1 gene was investigated as a possible virulence factor. DNA-mediated gene transfer was used to disrupt the WI-1 locus by allelic replacement, resulting in impaired binding and entry of yeasts into macrophages, loss of adherence to lung tissue, and abolishment of virulence in mice; each of these properties was fully restored after reconstitution of WI-1 by means of gene transfer. These findings establish the pivotal role of WI-1 in adherence and virulence of *B. dermatitidis* yeasts. To our knowledge, they offer the first example of a genetically proven virulence determinant among systemic dimorphic fungi, and underscore the value of reverse genetics for studies of pathogenesis in these organisms.

Key words: dimorphic fungi • gene targeting • virulence factor • pathogenic mechanism • adhesin

The systemic dimorphic fungi *Blastomyces dermatitidis*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, and *Sporothrix schenckii* inhabit soil worldwide as saprophytic molds. They also cause hundreds of thousands of new fungal respiratory and deep tissue infections annually in healthy individuals (1, 2) and reactivate as opportunistic infections in up to 40% of AIDS patients in some geographic regions (1).

Despite the worldwide occurrence and growing incidence of these fungal infections, little is known about the factors that account for their pathogenicity. In many cases, pathogenicity can be attributed to the ability of fungi and other microbes to adhere to target tissues, but the lack of tractable genetic systems in systemic dimorphic fungi has severely limited our understanding of the responsible products. This stands in contrast to recent progress that has been made in genetically manipulating opportunistic fungi such as *Cryptococcus neoformans* (3) and *Candida albicans* (4), and to the well-established systems available for manipulating model fungi such as *Saccharomyces cerevisiae* (5). Nonetheless, there are theoretical and real advantages to performing genetic studies of pathogenicity in the pathogenic fungus itself, rather than in a more genetically manipulable, heterologous fungus.

The systemic dimorphic fungus *Blastomyces dermatitidis* produces a progressive pulmonary and disseminated infection and is one of the principal systemic mycoses of humans and animals worldwide. An immunodominant antigen and putative adhesin termed WI-1 decorates the fungus (6–8). Cell biological studies have shown that WI-1 binds the yeast to CD11b/CD18 and CD14 receptors on human macrophages (9). Fluorescence staining of the fungal surface and extractions of cell wall proteins have shown that WI-1 varies in expression on genetically related strains with reduced virulence (10). Together, these findings have pointed to a possible role for WI-1 in the pathogenesis of blastomycosis (11, 12), but formal proof of the role of WI-1 in either adherence or virulence demands additional genetic evidence.

We recently developed the ability to genetically manipulate *B. dermatitidis* (13) using DNA mediated gene transfer. This transformation system opens the way toward targeting genes, creating knockout strains, and formally testing the role of candidate factors such as WI-1 in adherence and virulence of *B. dermatitidis*. Such efforts rest on the feasibility of accomplishing homologous recombination and gene disruption at loci encoding putative virulence determinants, an achievement that has not yet been reported in a systemic

dimorphic fungus. Therefore, in this study we sought to achieve this goal.

We endeavored here to accomplish the following specific objectives: (a) to target and disrupt the WI-1 locus in *B. dermatitidis* by allelic replacement, thus creating WI-1 null strains, and to reconstitute WI-1 expression in knockout strains, also by means of gene transfer; (b) to analyze the adherence-promoting role of WI-1 in binding and phagocytosis assays using isogenic strains that do and do not display the putative WI-1 adhesin; and (c) to quantify the pathogenicity of these isogenic WI-1 null and reconstituted strains in an animal model of blastomycosis that closely mimics the human form of pulmonary disease. We report here successful homologous gene targeting and allelic replacement at the WI-1 locus in *B. dermatitidis*, and unambiguously establish the pivotal role of WI-1 in both adherence and virulence. To our knowledge, this work illustrates the first genetically proven virulence factor in *B. dermatitidis* and related systemic dimorphic fungi, and emphasizes the power of reverse genetics for studies of pathogenesis in these complex microorganisms.

Materials and Methods

Fungal Strains and Plasmids. *Blastomyces dermatitidis* ATCC strains 26199 and 60915 were used for gene disruption. The wild-type, parental strain 26199 was isolated originally from a human patient and is highly virulent in an experimental mouse model of infection (14). The genetically related strain 60915 was derived after repeated passage of strain 26199 in vitro and is reported to be 10,000-fold less virulent in mice (15).

The targeting vector pQWhph (see Fig. 1 A) was constructed as follows: pQE32/WI-1 (8.3 kb) was derived from a Qiagen[®] expression vector pQE32 (3.5 kb) and an AccIII fragment of the genomic WI-1 gene (4.8 kb) (8). A BamHI site in the 3' UTR of WI-1 was removed by HindIII digestion and religation. Another BamHI site in the 5' UTR was removed by EcoRI-NruI deletion. The resulting plasmid, pQWΔΔ, was digested with BamHI to excise 1.4 kb of WI-1 coding sequence. A 1.4-kb *hph* cassette (*E. coli hph* driven by 375 bp of WI-1 upstream sequence) was amplified from pWI-1P (13) using PCR primers TB No. 1 (5'-ATCGGATCCTCGAGGTTTTGGCTTAGGCTC-3') and TB No. 2 (5'-ATCGGATCCGGTCGGCATCTACTCTA-3'), which added BamHI sites (underlined). The *hph* cassette was ligated into the pQWΔΔ BamHI-digested vector, which was then linearized with HindIII, and the 1.4-kb HindIII fragment containing the 3'-untranslated region of WI-1 was ligated back into place. The orientations of the *hph* cassette and the HindIII fragment were verified by restriction analysis.

pCB1528, containing the sulfonil urea resistance gene of *Magnaporthe grisea*, was provided by Drs. James Sweigard (Dupont) and Paul Szanislo (University of Texas, Austin, TX) (16), and was used for reconstitution of WI-1 in knockout strains.

Growth of Fungi. *B. dermatitidis* was maintained in the yeast form by growth on Middlebrook 7H10 agar medium containing oleic acid-albumin complex (OADC; Sigma Chemical Co.). Liquid cultures of yeast were grown in *Histoplasma* macrophage medium (HMM)¹ (17) on a rotary shaker at 200 rpm. All cultures were maintained at 37°C.

¹Abbreviation used in this paper: HMM, *Histoplasma* macrophage medium.

To measure the growth rate of yeasts, cells were grown synchronously in HMM and inoculated at a concentration of 2×10^4 per ml into 50 ml of fresh medium. Cultures were incubated at 37°C on a rotary shaker at 200 rpm for 72 h. Growth rates were monitored every 24 h by both hemacytometer cell count and OD₆₀₀. Doubling time was calculated from the results of hemacytometer cell counts according to the formula: No. of divisions = (Log Nt - Log No.)/Log 2, where Log No. is the number of yeasts at the starting point and Log Nt is the number of yeasts at each time point analyzed. The doubling time is then equal to 24 h divided by the number of divisions. Results are the mean ± SD of three replicates per time point.

Allelic Replacement of WI-1 and Its Reconstitution. *B. dermatitidis* yeast cells of strains 26199 and 60915 were transformed with 5–10 μg of XbaI-linearized pQWhph, using electroporation conditions previously described (13). Transformants were selected on HMM agar containing 200 μg/ml of hygromycin B. Replicates plated onto nitrocellulose membranes overlaying brain-heart infusion agar (Difco Labs.) were lysed with 0.2 M NaOH, 0.1% SDS and 0.5% mercaptoethanol as previously described (18). Membranes were probed with pooled anti-WI-1 mAbs DD5-CB4, AD3-BD6, BD6-BC4, and CA5-AA3 (19) using standard immunoblotting techniques (hybridomas were provided by Drs. Errol Reiss and Christine Morrison, Centers for Disease Control, Atlanta, GA).

The multinucleate nature of *B. dermatitidis* yeast had to be addressed to isolate cells with a WI-1-negative phenotype, as the presence of heterologous, "silent" nuclei might allow phenotypic reversion. To obtain genetic homogeneity at the WI-1 locus, each candidate isolate was taken through several rounds of single-cell isolation on selective medium with resultant colonies re-screened as above. This protocol has been shown to render transformants of multinucleate fungi homogenous for altered genes (20, 21).

Resulting candidates were screened for evidence of gene replacement by PCR. Primers internal to the WI-1 gene were used to determine if candidates contained an intact WI-1 locus. If an intact locus was not detectable, as in candidates 55 and 99 (described in Results), homologous recombination was assessed by amplifying the junction between the transformed *hph* gene and sequences 5' to the WI-1 promoter (not on the transforming vector) (see Fig. 1, A and B). Primers were 5'-TTGTTTGTCTCTGCCCGTTTTC-3' (forward) and 5'-CGTCGCGGTGAGTTCAGGCTTTTTC-3' (reverse). Knockouts were confirmed by Southern blot analysis as described below.

To restore the expression of WI-1 in knockout strains, yeast were cotransformed with pCB1528, together with WI-1 genomic clone 1 (8). After electroporation of 10^7 yeasts with a pool of 10 μg of each plasmid, transformants were selected on HMM plates containing 150 μg/ml of chlorimuron ethyl (Chem Service). Out of 19 total transformants, 18 were found to produce WI-1 in the colony immunoblot assay noted above. Transformants producing the most WI-1 were passed serially on HMM under selection as above to obtain a genetically homogeneous isolate. After repeated passage, strain 4/55 appeared closest to the parental strain 26199 in WI-1 production and was chosen for further study.

Detection of WI-1 Protein and DNA in *B. dermatitidis*. The presence of WI-1 protein was assayed by immunofluorescence staining, SDS-PAGE, and Western blotting. Immunofluorescence staining was performed as previously described (8–10). In brief, yeast (10^6 cells) were stained for WI-1 indirectly using 1 μg anti-WI-1 mAb DD5-CB4 followed by goat anti-mouse IgG-FITC. Stained cells were inspected for fluorescence microscopically us-

ing an Olympus BX60 fluorescent microscope or a FACScan® flow cytometer (Becton Dickinson). Cell-associated proteins were extracted by boiling yeast in treatment buffer containing 1.5% SDS, and 5.0% 2-ME for 3–5 min, followed by analysis of the cell-free material by SDS-PAGE and Western blotting using anti-WI-1 mAb DD5-CB4 as previously described (8). For Southern blot analysis, chromosomal DNA was prepared by grinding cells in liquid nitrogen and extracting them in detergent as previously described (13). Purified DNA was restricted with XbaI (Promega Corp.) at a ratio of 40 U/10 µg of DNA, incubated at 37°C overnight, and then separated on 1% agarose gel and transferred to nitrocellulose membrane. A 100-ng aliquot of probe was labeled with α -[³²P]dCTP to a specific activity of 10⁹ cpm/µg using random oligonucleotides as primers (Amersham Pharmacia Biotech). Washed Southern blots were used to expose Kodak XAR-5 film with intensifying screens at –80°C.

Binding and Phagocytosis Assays. Murine macrophage cell line J774.16 (22–25), provided by Dr. Arturo Casadevall (Yeshiva University, NY), was used in most in vitro binding and phagocytosis assays. Further experiments were done with resident peritoneal macrophages of BALB/c mice. Macrophages were grown in DMEM (GIBCO BRL) with 10% heat-inactivated fetal calf serum (HyClone Laboratories Inc.), 10% NCTC-109 medium, and 1% nonessential amino acids (GIBCO BRL), and plated at 2.5 × 10⁴ cells per well in 16-well tissue culture Chamber Slides (Nunc Inc.). Cells were stimulated with 500 U/ml of recombinant murine IFN-γ (Boehringer Mannheim). After overnight incubation at 37°C/8% CO₂, medium in each well was replaced with fresh medium containing 500 U of IFN-γ/ml, 3 µg/ml of LPS (Sigma Chemical Co.) and *B. dermatitidis* yeasts.

Binding and phagocytosis of yeasts was analyzed in vitro as previously described (22–25). In brief, yeasts were heat-killed for 45 min at 65°C and stained with rhodamine isothiocyanate (RITC) (10 µg/ml). Assays done in the presence and absence of complement used 10% normal mouse serum (NMS) and heat-inactivated NMS, respectively. Complement was inactivated by heating NMS at 56°C for 30 min. Yeasts and macrophages were incubated at an E/T ratio of 1:4 for varying periods at 37°C/8% CO₂. Unattached yeasts were removed by washing wells three times with PBS. Attached but uningested yeasts were stained with 0.1% Uvitex 2B (Specialty Chemicals for Medical Diagnostics, Germany) for 30 s. Cells were fixed in 1% paraformaldehyde for 15 min. After fixation, glycerol was added to the slide. To quantify binding and phagocytosis, the number of yeasts attached to and ingested by 100 macrophages was counted at ×1,000 using a U-MWU fluorescence cube in an Olympus BX60 microscope (Leeds Precision Instruments, Inc.). The association index is defined as the number of attached and ingested yeasts divided by the number of macrophages counted. The ingestion index is defined as the number of yeasts ingested per macrophage. Results are expressed as the mean ± SEM of at least three experiments.

Binding of yeast to lung tissue was analyzed using modifications to an ex vivo assay described previously (26). Lung tissue from a healthy mouse was embedded in O.C.T. compound (Miles Inc.), frozen in liquid isopentane at –80°C, and sliced into thin, 6-µm sections in a cryostat at –20°C. Sections were applied to Superfast/plus®-coated glass slides (Fisherbrand; Fisher Scientific Co.) and air dried. RITC-stained yeasts (10⁶) in 0.1 ml HBSS containing 20 mM HEPES, 0.25% BSA, and 3 mM CaCl₂, were added to a wax-inscribed circle of the tissue section. Slides were incubated for 60 min at 37°C, washed three times with HBSS to remove unattached yeasts, and fixed with 1.25% glutaraldehyde for 35 min at room temperature. Binding of yeasts

was enumerated by counting the number that adhered to a 0.01 mm² area of the slide viewed at ×600 using an Olympus IX50 fluorescent microscope (Leeds Precision Instruments). Results are expressed as the mean ± SEM of at least six experiments.

Experimental Model of *B. dermatitidis* Infection. Male BALB/c mice ~5–6 wk of age (Harlan Sprague Dawley) were infected intranasally with *B. dermatitidis* yeasts as previously described (27). In brief, mice were anesthetized with inhaled Metafane® (Malinckrodt Veterinary Inc.) to administer a 25-µl suspension of yeast cells dropwise into their nares. The minimum number of yeasts needed to achieve a lethal infection was established in preliminary work as 10² intranasally for ATCC 26199, and 10⁶ for ATCC 60915. Mice were housed according to guidelines of the University of Wisconsin Animal Care Committee, which approved this work.

Statistical Analysis. Kaplan Meier (28) survival curves were generated for mice that received a lethal infection. Survival times of mice that were alive by the end of the study were regarded as censored. Time data were analyzed by the log rank statistic and exact *P* values were computed using the statistical package Stat Xact-3 by CYTEL Software Corporation. The survival of two groups are considered to be significantly different if the two-sided *P* value is <0.05. When multiple comparisons were made simultaneously, *P* values were adjusted according to Bonferroni's correction to protect the overall significance level of 0.05.

Differences between *B. dermatitidis* strains in the number of CFU in tissue were analyzed statistically using the Wilcoxon Rank test for nonparametric data (28). Differences between strains in binding and phagocytosis were analyzed using methods for standard analysis of variance (29). *P* < 0.05 was considered significant.

Results

Allelic Replacement of WI-1 and Its Reconstitution. Our gene targeting efforts capitalized on the preferred fate of incoming DNA in *B. dermatitidis*, which is integrative transformation (13). Substantial WI-1 DNA flanking the *hph* selectable marker was used to target the knockout vector pQWhph and achieve the desired crossover event (Fig. 1 A).

WI-1 was disrupted by allelic replacement in ATCC strains 26199 and 60915. Presumptive evidence of homologous recombination was sought by PCR amplification of the junction between the *hph* gene on the transforming vector and the upstream sequences 5' to the WI-1 promoter (not on the transforming vector) (Fig. 1, A and B). Either of two possible products could signal such an event. A joint fragment of 1.6 kb would be amplified if chromosomal DNA had recombined at the 1 kb of WI-1 flanking sequence on pQWhph, whereas a joint fragment of 675 bp would be amplified if chromosomal DNA had recombined within the WI-1 minipromoter (375 bp) that directs expression of the *hph* gene. In each positive candidate (strains 55 and 99) the amplified joint fragment was 675 bp (Fig. 1 B). To verify the amplified product's authenticity, it was digested with EcoRV and AatII (Fig. 1 B); the resulting DNA fragments corresponded in size to predictions based on the genomic sequence of WI-1 (8). Southern blot analyses described below confirmed the initial PCR results and demonstrated allelic replacement of WI-1 after screening

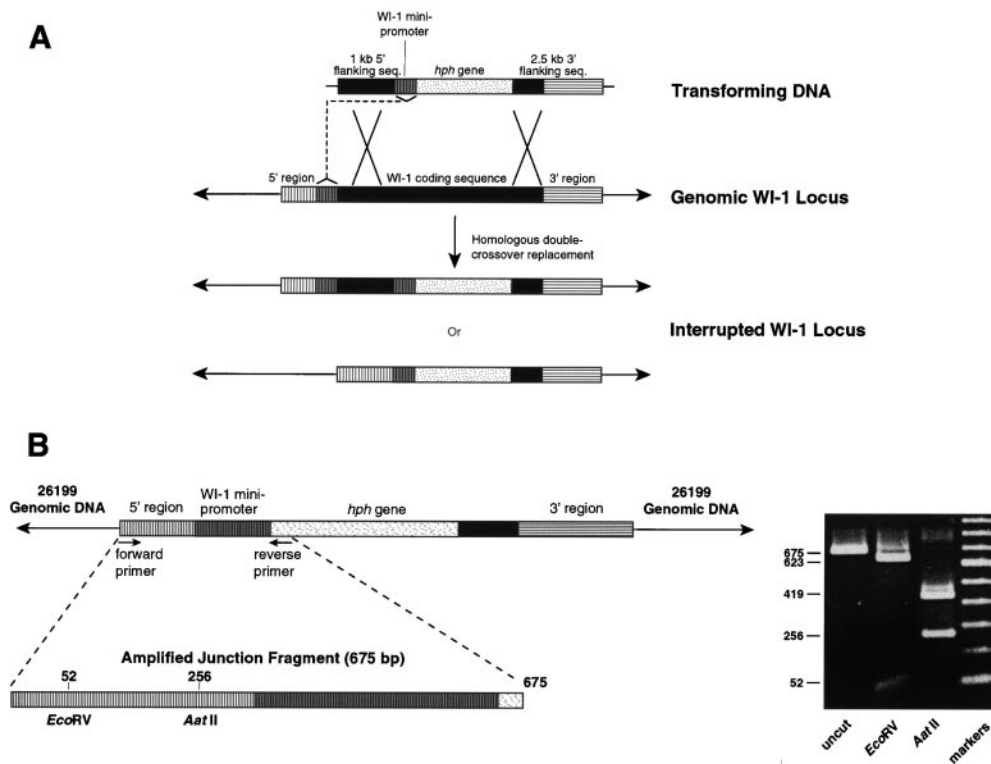


Figure 1. (A) Strategy for targeting and replacing the WI-1 locus in *B. dermatitidis*. Anticipated homologous crossover leading to a gene replacement event at the WI-1 locus. Linearized pQWhph can direct the *hph* gene to the WI-1 locus through homologous crossover in two ways. X's depict crossing over at 1 kb of WI-1 5' flanking sequence (all WI-1 coding, designated in black) and at 2.5 kb of WI-1 3' flanking sequence (881 bp of WI-1 coding in black and 1395 bp of noncoding in horizontal lines). Alternatively, the broken line depicts crossing over at a short region of the 375-bp WI-1 mini-promoter in front of the *hph* gene. Either crossover event would replace most of the WI-1 coding region with the *hph* cassette. (B) PCR analysis for targeted gene replacement of WI-1. Candidates isolated in colony screening for a WI-1 null phenotype as described (Materials and Methods) were further analyzed for targeted recombination by PCR amplification of a novel junction fragment spanning

the WI-1 5' region (not present on the targeting vector) and the *hph* gene. The forward primer (5'-TTGTTTGTCTCTGCCCGTTTTTC-3' at -644 in the WI-1 5' region) is present only in the endogenous locus, whereas the reverse primer (5'-CGTCGCGGTGAGTTTCAGGCTTTTTTC-3' at +31) is present in the *hph* gene on transforming DNA. The resulting 675-bp fragment is amplified if WI-1 sequences have been interrupted by *hph* in the manner depicted by a dashed line in A, yielding an interrupted locus shown at the bottom of A and in B. The product's authenticity is confirmed by the presence of EcoRV and AatII restriction sites in the WI-1 upstream segment of the product, as shown in an accompanying agarose gel, and in accord with published WI-1 genomic sequence (8).

60 transformants of ATCC 26199 and 100 transformants of ATCC 60915. The frequency of homologous targeting at the WI-1 locus was 1–2%.

WI-1 expression was restored in one of the knockout strains, 55, by means of gene transfer involving cotransformation with pCB1528 and genomic clone 1 containing WI-1 (as described in Materials and Methods), yielding strain 4/55.

Phenotypic and Genotypic Analyses of Recombinant Strains. The phenotype and genotype of the knockouts and the reconstituted strain were established by anti-WI-1 mAb fluorescence staining, Western blots of extracted protein, and Southern blot analysis (Fig. 2, A–C). Surface WI-1 was not detectable on knockout strain 55 by either FACS[®] analysis or Western blotting of extracted cell wall proteins (Fig. 2, A and B). In contrast, the amount of surface WI-1 on the reconstituted strain 4/55 was comparable to, if not greater than, the amount detected on the parental strain. Similar results were observed in isogenic strains ATCC 60915 and knockout strain 99 (data not shown).

To confirm that WI-1 had been disrupted by allelic replacement, and to analyze the nature of the transforming event, Southern blot analyses were performed (Fig. 2 C). The upstream WI-1 probe hybridized to a 9.3-kb XbaI fragment in DNA from parental strains 26199 and 60915, but not knockout strains 55 and 99. Instead, the WI-1 probe hybridized to a novel fragment of ~8.3 kb in each of these

knockout strains. The *hph* gene probe also hybridized with this same 8.3-kb fragment in the knockouts, indicating that the residual, upstream sequence for WI-1 and the *hph* sequence were on the same DNA fragment. For each knockout strain, the presence of a single band hybridizing with the WI-1 probe, which differs in size from the respective one in the parental strain, indicates a disrupted WI-1 locus without other ectopic integrations. The size of this fragment in knockout strains (~8.3 kb; Fig. 2 C), together with the size of the joint fragment amplified in PCR (675 bp; Fig. 1 B), suggests that transforming DNA crossed over at the 375-bp stretch of homology (mini-promoter) with the 5' region of WI-1, excising ~1 kb of coding sequence. This event probably placed the full 5' region and the complete, endogenous WI-1 promoter upstream of *E. coli hph* (Fig. 1 B), perhaps transcribing *hph* more efficiently.

Southern blot analysis of reconstituted strain 4/55 demonstrated that the WI-1 transgene was located on a single XbaI fragment that differed in size from the retained 8.3-kb fragment harboring an *hph*-disrupted copy of WI-1 (Fig. 2 C). This indicated that the WI-1 transgene had integrated ectopically, rather than homologously, into the chromosome in a single copy.

Thus, phenotypic and genotypic analyses demonstrated that isogenic strains differing in the expression of WI-1 had been created: wild-type parental strain 26199, with a high

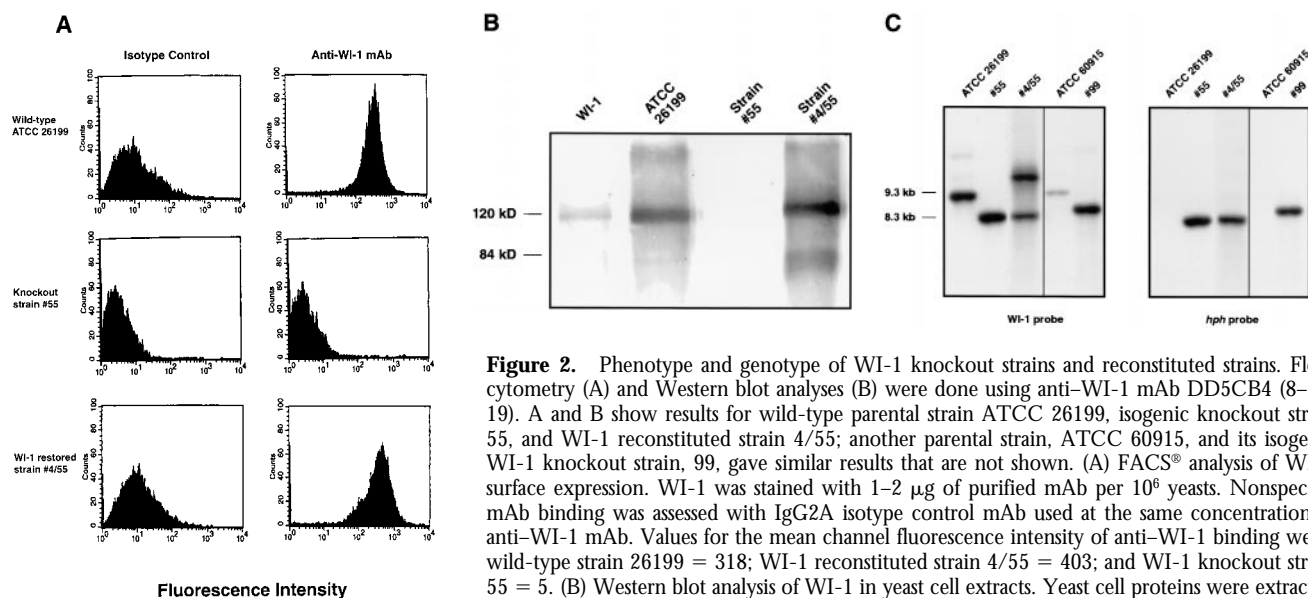


Figure 2. Phenotype and genotype of WI-1 knockout strains and reconstituted strains. Flow cytometry (A) and Western blot analyses (B) were done using anti-WI-1 mAb DD5CB4 (8–10, 19). A and B show results for wild-type parental strain ATCC 26199, isogenic knockout strain 55, and WI-1 reconstituted strain 4/55; another parental strain, ATCC 60915, and its isogenic WI-1 knockout strain, 99, gave similar results that are not shown. (A) FACS[®] analysis of WI-1 surface expression. WI-1 was stained with 1–2 μg of purified mAb per 10^6 yeasts. Nonspecific mAb binding was assessed with IgG2A isotype control mAb used at the same concentration as anti-WI-1 mAb. Values for the mean channel fluorescence intensity of anti-WI-1 binding were: wild-type strain 26199 = 318; WI-1 reconstituted strain 4/55 = 403; and WI-1 knockout strain 55 = 5. (B) Western blot analysis of WI-1 in yeast cell extracts. Yeast cell proteins were extracted as described in Materials and Methods, using 10^7 yeasts per strain, and loading an equivalent volume of cell-free extract per lane. Lane 1 is a positive control with 4 μg of purified WI-1. (C) Southern blot analysis of WI-1 targeting and replacement. Genomic DNAs were restricted with XbaI. Strains are wild-type parent strain ATCC 26199, isogenic knockout strain 55, and WI-1 reconstituted strain 4/55. ATCC 60915 and isogenic WI-1 knockout strain 99 are included to illustrate targeted gene replacement of WI-1 in a second strain of *B. dermatitidis*. The *hph* probe contains only *hph* coding sequence. The WI-1 probe is an AatII/EcoRI fragment from an upstream region of the gene (nucleotides –1091 to –388), which is not carried on the knockout vector QWhph.

level of expression; strain 55, devoid of WI-1; and strain 4/55, with expression restored to the level of wild-type, or perhaps higher. In addition, parental strain 60915 containing WI-1 and its isogenic knockout strain (99) lacking WI-1 were also developed (Fig. 2 C).

Defective Binding and Entry of WI-1 Knockout Yeast. Because WI-1 is believed to bind yeasts to macrophages (8–10), we investigated whether WI-1 knockout yeasts were capable of binding. Knockout yeasts bound and entered murine macrophages poorly (Fig. 3 A). The number of knockout yeasts bound to or inside of macrophages in vitro was far lower than that for wild-type yeasts at all time points between 15 min and 6 h of incubation. At 6 h, the association index for the knockout yeast was one-sixth the value for the wild-type yeast, and the ingestion index was one-eighth as high. These yeast strains bound resident mouse peritoneal macrophages in a manner similar to that observed for the macrophage cell line J774.16 (data not shown). The defect in binding and entering macrophages was restored fully in the knockout when 10% serum was added as a source of complement (Fig. 3 B) or after WI-1 was reexpressed in strain 4/55 (Fig. 3 C). These findings imply that WI-1 mediates binding and entry of yeast into macrophages in the opsonin-poor environment of the lung early in the course of infection, before tissues are inflamed and serum exudes into the alveolus.

To determine whether knockout yeasts bound poorly within the noninflamed lung itself, we quantified the number of yeasts that adhered to thin sections of lung in an ex vivo binding assay. Again, the knockout bound poorly, whereas both the wild-type isolate and the WI-1 reconstituted isolate 4/55 bound avidly (Fig. 3 D), emphasizing the

key role of WI-1 in promoting interactions directly with constituents of the lung alveolus.

Indispensable Role of WI-1 in Virulence. We hypothesized that yeasts unable to bind lung tissues and enter macrophages during infection would be much less virulent, and tested this in a previously described murine model of lethal pulmonary blastomycosis (27). All mice infected with the wild-type strain ATCC 26199 at a dose of 10^2 yeasts died from an overwhelming pulmonary and disseminated infection several weeks after inoculation, whereas all mice infected with the same dose of isogenic, WI-1 knockout strain 55 lived and appeared healthy during observation over 72 d (Fig. 4 A). Mice infected with either 10^3 or 10^4 yeasts of knockout strain 55, which are respectively 10 and 100 times the lethal dose of wild-type yeast, also survived and appeared healthy during the 72-d observation period. Finally, lethality studies comparing parental strain ATCC 60915 (15) and its isogenic WI-1 knockout strain 99 yielded similar results (data not shown).

Representative mice infected with knockout strain 55 were killed 3 wk after they were infected with 10^3 yeasts. They had <600 organisms in the lungs and none in the liver or spleen, suggesting that strain 55 produces a self-limited infection. To analyze the evolution and clearance of infection with the knockout and wild-type strains over time, groups of mice were tested serially for the burden of infection 1–14 d after they were infected intranasally with 10^4 yeasts. The number of yeasts in the lungs of mice infected with the knockout remained low throughout the study interval, whereas the number for the wild-type strain 26199 grew steadily; a sharp difference in the burden of infection between the strains was evident by 3 d and became statistically significant after 1 wk of infection (Table I). On

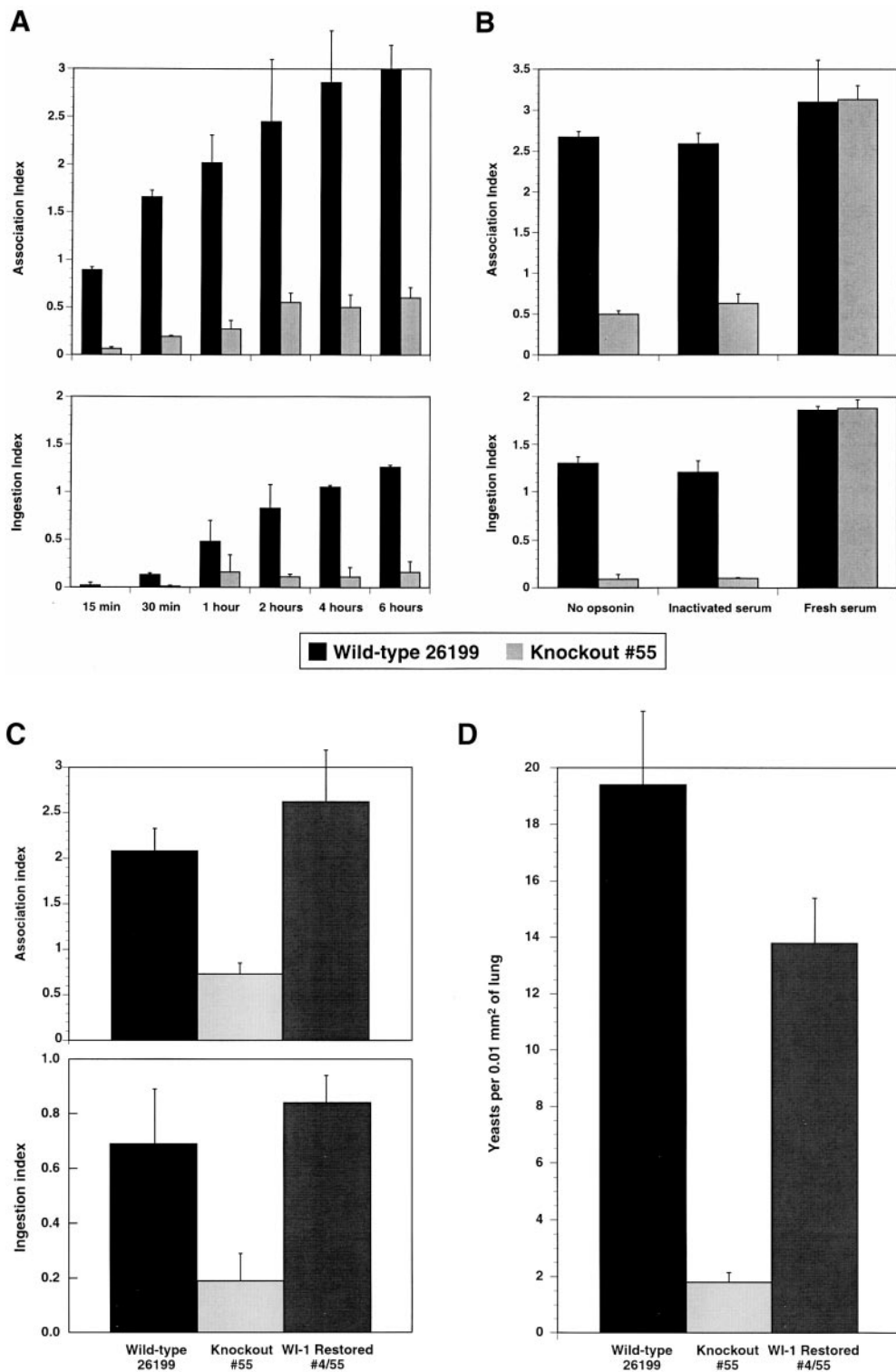


Figure 3. WI-1 mediated binding of *B. dermatitidis* to macrophages and lung tissue. (A) Binding and entry of macrophages by *B. dermatitidis* wild-type and knockout yeast. The upper portion of the panel shows binding of yeasts to macrophage J774.16 cells. The lower portion shows entry of yeasts into the macrophages. Binding and entry were quantified after incubation for varying intervals as shown. Unopsonized yeasts were added to wells of chamber slides containing 2.5×10^4 macrophages at a ratio of four yeast cells per macrophage. The association index is defined as the number of attached and ingested yeasts per macrophage. The ingestion index is defined as the number of yeasts internalized per macrophage. Results are mean \pm SEM of three independent experiments. The two strains differ significantly in binding and entry of macrophages for all time points after 1 h of incubation ($P < 0.01$). (B) Complement reverses defects in binding and entry in the WI-1 knockout strain. Assays were done as in A, except that they included 10% fresh mouse serum (as a source of complement) or heat-inactivated mouse serum (as a control). Results are the mean \pm SEM for three separate experiments. Wild-type and knockout yeast differ significantly from each other in binding and ingestion both in the absence of serum and in the presence of heat-inactivated serum ($P < 0.001$ for each comparison), but not in the presence of fresh serum. (C) Defective binding and entry of macrophages by WI-1 knockout yeasts is reversed after reexpression of WI-1. Unopsonized yeasts were incubated with macrophages for 6 h; results are the mean \pm SEM of three separate experiments. Wild-type yeasts and WI-1 reconstituted yeasts bind and enter macrophages significantly better than do knockout yeasts ($P < 0.001$ for each comparison). (D) Binding of yeasts to lung tissue ex vivo. Lungs of normal mice were removed and cryopre-

served and 6- μ m sections were applied to glass slides as in Materials and Methods. The number of unopsonized yeasts attached to lung tissue sections was quantified. The extent of binding is defined as the number of yeasts attached per 0.01 mm² of lung tissue, which was determined by inspecting a high power field at $\times 600$ and counting 30 fields per strain in each experiment. Results are the mean \pm SEM of six independent experiments. Wild-type yeasts and WI-1 reconstituted yeasts bind to lung tissue significantly better than do knockout yeasts ($P < 0.001$ for each comparison).

postmortem examination, the lungs of mice infected with the knockout were macroscopically normal, but contained a small number of well-formed granulomas with sequestered organisms (Fig. 4 B). The lungs of mice infected with

the wild-type strain were filled with organisms and widespread inflammation. Taken together, these findings suggest that eliminating WI-1 expression greatly reduces the pathogenicity of the yeast.

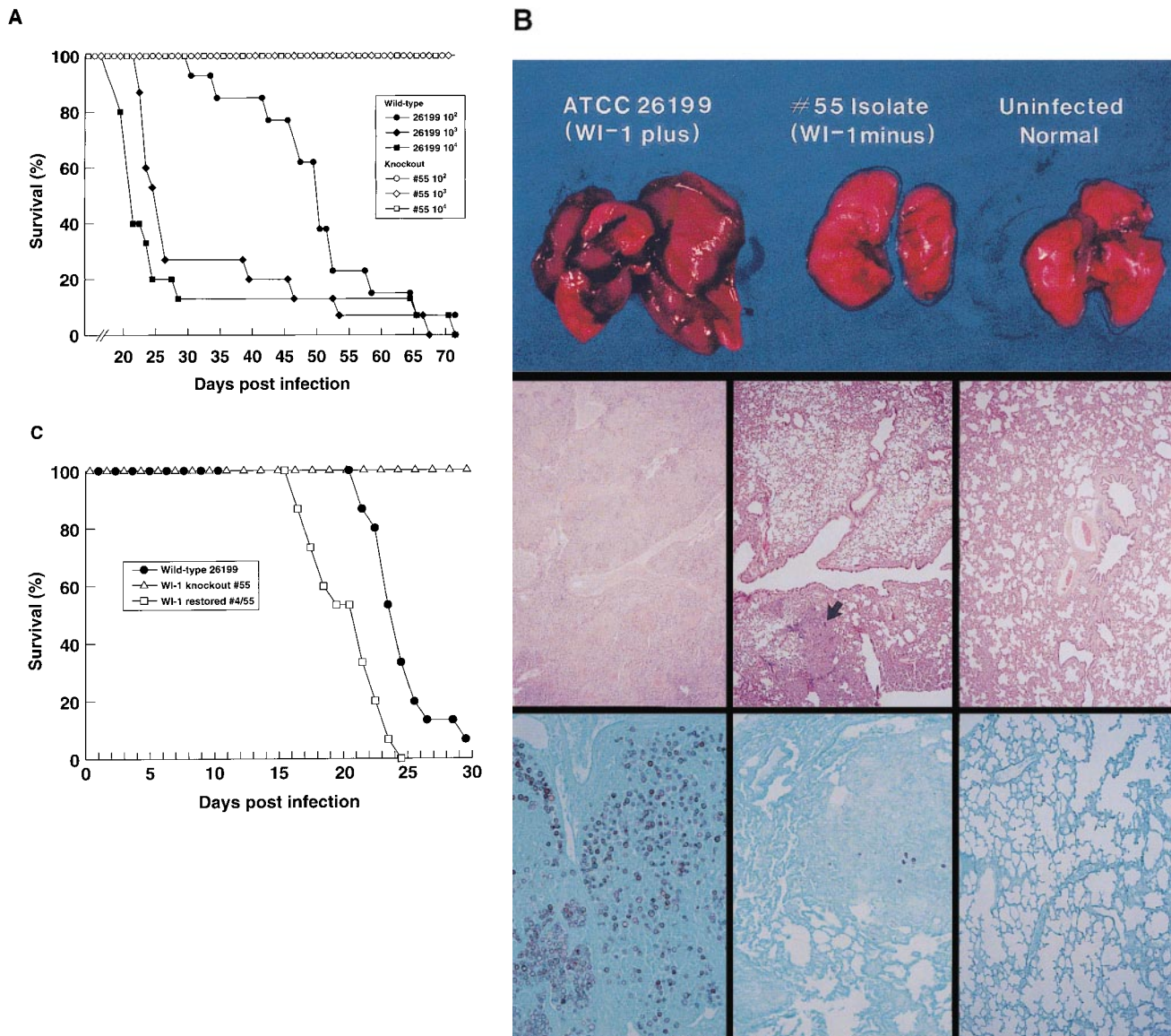


Figure 4. Targeted gene replacement of WI-1 reduces the pathogenicity of *B. dermatitidis*. (A) Survival after infection with wild-type strain 26199 and WI-1 knockout strain 55. Male BALB/c mice ($n = 15$ mice/group) were infected intranasally with yeast cells of each strain, in varied doses. Survival was monitored for 72 d after infection. The two groups differ significantly ($P < 0.001$) at each infectious dose tested. The experiment shown is representative of three independent experiments. The phenotype of knockout yeasts was stable; no revertants were identified among yeasts grown from mice infected with strain 55. (B) Gross and microscopic pathology of mice infected with ATCC 26199 wild-type yeasts or WI-1 knockout yeasts. Mice were analyzed 3 wk after infection. Lungs were stained with hematoxylin and eosin to assess inflammation, and with Gomori methenamine silver to visualize yeasts. The arrow denotes an isolated granuloma surrounded by normal lung tissue in a mouse infected with the WI-1 knockout strain 55. (C) Survival after infection with wild-type strain 26199, WI-1 knockout strain 55, and WI-1 reconstituted strain 4/55. Survival experiments were done as in A, using a dose of 10^4 yeasts to establish infection. The wild-type and WI-1 reconstituted strains were significantly different from the WI-1 knockout strain ($P < 0.001$ for each comparison).

We found no evidence for a growth defect in strain 55 knockout yeasts, as an alternative explanation for its reduced pathogenicity. Doubling times in liquid culture were calculated to be 17.5 h for the knockout strain and 17.1 h for the wild-type strain at 48 h after inoculation into liquid, which represented the peak log phase of growth. Wild-type parental yeasts and strain 55 knockout yeast appeared morphologically similar on agar and on microscopic examination. However, knocking out WI-1 in strain 99 led to excessive elongation into pseudohyphae, with few round

yeast cells like those seen in parental ATCC strain 60915 (data not shown). This could be due to accumulated defects in the cell wall, as parental strain 60915 also has diminished α -(1,3)-glucan (30).

Restoration of Virulence in the Knockout Strain. We next confirmed that loss of WI-1 is directly responsible for reduced virulence of strain 55 in vivo, by showing that restored expression of WI-1 conferred pathogenicity. The WI-1 reconstituted strain 4/55 killed 100% of infected mice, as did the wild-type strain 26199 (Fig. 4 C). Strain

Table I. Burden of Yeast in Lungs after Mice Were Infected with *B. dermatitidis**

Strain	Time of death				
	3 h	24 h	3 d	7 d [†]	14 d [§]
Knockout strain 55	750 ± 150	850 ± 150	750 ± 250	500 ± 100	1,250 ± 300
ATCC 26199	600 ± 150	500 ± 150	2,300 ± 750	4,600 ± 1,350	60,450 ± 15,550

*Mean CFU/lung ± SEM, $n = 6$ mice per time point for each yeast strain.

[†] $P = 0.06$ when comparing the burden of infection between the strains at this time point.

[§] $P = 0.005$ when comparing the burden of infection between the strains at this time point.

4/55 accelerated the time to death and thus appeared more virulent than the parental strain 26199. Strain 4/55 yeast also displayed larger amounts of WI-1 than did the parent strain, as observed in FACS[®] and in Western blots of yeast cell extracts (Fig. 2, A and B), suggesting a possible dose effect on virulence. To assess the appearance and extent of infection with strain 4/55, mice were analyzed at the time of death. These mice demonstrated severe pulmonary and disseminated disease with $>10^7$ yeasts in the lungs and $>2.5 \times 10^4$ yeasts in the liver, indicating that they died from an overwhelming infection, similar to that observed with wild-type yeast.

Discussion

Formal proof of the importance of a virulence determinant requires fulfillment of a “molecular” Koch’s postulate: loss of virulence upon gene disruption, and restoration of virulence upon gene reconstitution (31). Prior studies of systemic dimorphic fungi have implicated candidate virulence factors through correlative and other means (32), but none of the observations have been substantiated by genetic tests, leaving their validity open to debate. Past studies that have implicated WI-1 in adherence, pathogenesis, and virulence are one such example, using exclusively non-genetic methods. Genetic intractability of *B. dermatitidis* and related fungi has, until very recently, prevented definitive studies. In addition to the transformation system recently described for *B. dermatitidis* (13), high efficiency transformation can be accomplished in the dimorphic fungus *H. capsulatum* (33), and the molecular and genetic tools for manipulation of that pathogen are now in hand (33–35).

In this study, we analyzed the pathogenetic role of WI-1 in *B. dermatitidis* by reverse genetics, overcoming past limitations in virulence studies of systemic dimorphic fungi. Here, the WI-1 locus was targeted and disrupted by allelic replacement using the dominant selectable marker encoding resistance to hygromycin B. Evidence of homologous gene targeting and elimination of WI-1 expression was provided at both the DNA and protein levels. The frequency of homologous gene targeting at the WI-1 locus ranged from 1 to 2% in the two independent strains studied, 26199 and 60915, which compares favorably with rates observed in a study of *H. capsulatum*. Woods et al. (35) re-

ported that homologous gene targeting is a rare event in that pathogen, occurring at a frequency of $<10^{-3}$ at the *URA5* locus. Several reasons could account for the higher frequency of targeting in the current study of *B. dermatitidis*, including the differences in pathogen, genetic locus, and gene targeting strategy.

We suspect that the gene targeting strategy used here was a critical feature and increased the rate of homologous recombination. Our targeting plasmid allowed a crossover event to occur in either of two locations in the 5′ region, as illustrated in Fig. 1 A: a long, 1-kb stretch of WI-1 coding sequence, or a shorter 375-nucleotide stretch of WI-1 minipromoter that served to express *E. coli hph* in transforming DNA. The plasmid had been designed so that the long stretch of homologous sequence in the 5′ (and 3′) region might enhance the frequency of gene targeting. Surprisingly, *B. dermatitidis* handled the transforming DNA in an unanticipated way in both instances in which homologous recombination was observed. Crossing over in the 5′ region occurred preferentially at the shorter stretch of homologous sequence rather than at the longer stretch. Evidence for the nature of the crossover event includes the size of the joint fragment PCR-amplified from the knockout strains (Fig. 1 B), and the size of the hybridizing band detected in Southern blot analysis (Fig. 2 C). This type of crossover event may have had the effect of “trapping” a more active WI-1 promoter in front of the *hph* coding sequence. Gene targeting by “promoter trapping” has been described in mammalian embryonic stem cells (36), where the expression of selection genes was made dependent on acquisition of transcriptional start signals from host DNA, thereby enriching for homologous recombination among preponderantly random integrations. This sort of targeting strategy may have broader application to other genes and dimorphic fungi if it renders them more amenable to allelic replacement and gene disruption by enhancing low frequencies of homologous recombination.

The isogenic strain pairs created here were used to investigate the role of WI-1 in the pathogenesis of blastomycosis. Prior work using cell biological approaches suggested that WI-1 promotes binding to human macrophages (8–10). Genetically related strains of *B. dermatitidis* that vary in surface expression of WI-1 showed concordant differences in binding to macrophages in vitro: Fab fragments of anti-

WI-1 mAbs blocked a portion of binding by yeast to macrophages, and purified WI-1 on polystyrene microspheres greatly enhanced their binding to the macrophages. Taken together, these findings suggested an adhesive property of WI-1. They could not address whether WI-1 is necessary or simply sufficient for binding yeast to cells, and to what extent binding by yeast would be impaired or eliminated in the absence of WI-1. Our study confirms and extends prior observations by demonstrating that WI-1 is necessary for binding the yeast to macrophages in vitro, as well to lung tissue ex vivo. Wild-type yeast bound avidly, in a time-dependent manner, whereas knockout yeast were impaired profoundly in their binding, and binding was largely restored in the WI-1 reconstituted strain. In addition, cells that bound poorly did not enter macrophages, as evidenced by low ingestion indices during the course of the incubation. In addition to data generated with the J774 macrophage-like cell line and lung tissue sections, similar trends were observed with peritoneal macrophages from mice. These observations suggest that WI-1 serves as the principal ligand mediating attachment of yeast to macrophages and host tissue, and that other ligands play a lesser role.

The isogenic strains also were used to address the role of WI-1 in virulence. In a prior study (10), altered WI-1 expression in genetically related wild-type and variant strains correlated with differences in virulence. Variant strains with the lowest reported virulence surprisingly had the highest level of surface WI-1 (and binding to macrophages). Thus, a possible interpretation of that study could be that WI-1 interferes with virulence of the yeast. However, those variants also had multiple coexisting defects, including impaired secretion of WI-1 and reduced or absent surface expression of the polymer α -(1,3)-glucan. The multiple defects impeded a definite conclusion about an independent role of WI-1 or any other single component, and underscored the need for reverse genetics to allow a definitive conclusion.

This study tackles this issue and demonstrates firmly that WI-1 is indispensable for the pathogenicity of *B. dermatitidis*. Knocking out WI-1 in wild-type strain 26199 rendered these yeast unable to establish a lethal pulmonary infection, even when administered at 100 times the minimal lethal in-

oculum. Similar results were observed after knocking out WI-1 in a second strain, 60915, further strengthening our conclusions about the importance of WI-1. Analyses of organ load and distribution of infection showed that wild-type yeast grew steadily in the lung and increased sharply in number by 7–14 d after infection. Although the number of knockout yeast that entered the lung was comparable to that of the wild-type immediately after infection, the number of knockout yeast in the lung remained low throughout the observation period. The persistently low number was probably not due to a growth defect, since knockout and wild-type yeast demonstrated a similar doubling time in vitro. After primary lung infection, wild-type yeast also disseminated to liver and spleen, whereas knockout yeast did not disseminate. This could reflect the ability of wild-type yeast to enter macrophages and disseminate while inside them, or possibly a burden of yeast beyond the lung's capacity. On histological examination, the pulmonary alveoli of mice infected with the wild-type were obliterated with inflammation and yeast, whereas the lungs of mice that received the knockout appeared mostly normal, except for focal well-formed granulomas with small numbers of sequestered organisms. These findings suggest that cell-mediated immunity remained intact and facilitated clearance of knockout yeasts. If this is true, administration of WI-1 knockout yeasts might vaccinate against *B. dermatitidis* infection due to wild-type yeasts.

The indispensable role of WI-1 in pathogenicity is substantiated by this study, but the mechanism(s) whereby the molecule exerts its effect requires more investigation. A deeper understanding of WI-1 and other fungal virulence mechanisms will be important in developing new pharmacological and immunological approaches to management of systemic fungal infections. Moreover, insight into where and when fungal virulence determinants are expressed in vivo and how virulence genes are regulated should provide new information about how pathogenic fungi sense the inimical environment of the host and adapt to it successfully. Our study marks a significant first advance toward these goals by establishing WI-1 as a key virulence determinant of *B. dermatitidis* worthy of further investigation.

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Address correspondence to Bruce S. Klein, University of Wisconsin-Madison, 600 Highland Ave., Rm. K4/434, Madison, WI 53792. Phone: 608-263-9217; Fax: 608-263-0440; E-mail: bsklein@facstaff.wisc.edu

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