

# *Agrobacterium tumefaciens* Integrates Transfer DNA into Single Chromosomal Sites of Dimorphic Fungi and Yields Homokaryotic Progeny from Multinucleate Yeast

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Received 15 April 2002/Accepted 12 September 2002

**The dimorphic fungi *Blastomyces dermatitidis* and *Histoplasma capsulatum* cause systemic mycoses in humans and other animals. Forward genetic approaches to generating and screening mutants for biologically important phenotypes have been underutilized for these pathogens. The plant-transforming bacterium *Agrobacterium tumefaciens* was tested to determine whether it could transform these fungi and if the fate of transforming DNA was suited for use as an insertional mutagen. Yeast cells from both fungi and germinating conidia from *B. dermatitidis* were transformed via *A. tumefaciens* by using hygromycin resistance for selection. Transformation frequencies up to 1 per 100 yeast cells were obtained at high effector-to-target ratios of 3,000:1. *B. dermatitidis* and *H. capsulatum* *ura5* lines were complemented with transfer DNA vectors expressing *URA5* at efficiencies 5 to 10 times greater than those obtained using hygromycin selection. Southern blot analyses indicated that in 80% of transformants the transferred DNA was integrated into chromosomal DNA at single, unique sites in the genome. Progeny of *B. dermatitidis* transformants unexpectedly showed that a single round of colony growth under hygromycin selection or visible selection of transformants by *lacZ* expression generated homokaryotic progeny from multinucleate yeast. Theoretical analysis of random organelle sorting suggests that the majority of *B. dermatitidis* cells would be homokaryons after the ca. 20 generations necessary for colony formation. Taken together, the results demonstrate that *A. tumefaciens* efficiently transfers DNA into *B. dermatitidis* and *H. capsulatum* and has the properties necessary for use as an insertional mutagen in these fungi.**

The systemic dimorphic fungi *Blastomyces dermatitidis* and *Histoplasma capsulatum* are animal pathogens that inhabit moist, high-organic-matter environmental niches as filamentous, sporulating molds (2, 21). In the lungs of a host, inhaled spores germinate into budding yeast, and infection can spread to other tissues of the body (9, 19). The phase transition from mold to yeast, and back, can be accomplished in the lab by shifting the temperature between 22 and 37°C, respectively. Although immunocompetent hosts are susceptible to infection, immunodeficient hosts such as AIDS patients are particularly at risk.

The genetic tools for studying these pathogenic fungi have been accumulating over the past decade (5, 24), and important virulence factors for both organisms have been identified. BAD1, an adhesin and immune modulator, is essential for the virulence of *B. dermatitidis* (6, 16). CBP1, a calcium binding protein of *H. capsulatum*, is essential for macrophage killing and virulence (33). Both of these virulence factors were identified by the reverse genetic approach of candidate gene disruption. A forward genetic approach of mutagenesis and phenotypic screening would be valuable for identifying other virulence factors or factors involved in regulation of other processes such as phase transition. This can be a powerful approach when mutagenesis involves random insertions of a known DNA sequence into the genome. The insertion se-

quence tag can then be used to clone the adjoining DNA for analysis. One highly successful insertional mutagenesis approach in plants utilizes the *Agrobacterium tumefaciens* transfer DNA (T-DNA)-transferring type IV secretion system (23, 36, 37).

The plant pathogen *A. tumefaciens* carries a ~200-kbp tumor-inducing (Ti) plasmid, within which is a portion referred to as T-DNA. Upon infection of plants, the T-DNA is randomly inserted into the plant genome and transforms the plant cells to a tumorous growth called a crown gall, which serves as host tissue for the growth of the bacterium (17, 38). Plant biologists have modified the Ti plasmid to remove tumor-causing and superfluous genes but keep the genes necessary for T-DNA transfer and integration into nuclear DNA (3). In addition, binary vectors have been developed whereby the T-DNA region is harbored in *trans* from the rest of the Ti plasmid (4). The binary vectors are smaller, can replicate in *Escherichia coli*, have selectable markers for growth in *E. coli* or plants, and provide cloning sites for addition of foreign DNA within the T-DNA. These binary vectors have been put to great use as insertional mutagens in plants and have been shown, with modification, to transfer T-DNA into *Saccharomyces cerevisiae* yeast (7), filamentous fungi (12), and, recently, the dimorphic fungus *Coccidioides immitis* (1). Changes necessary for use in fungi include addition of fungal selectable markers to the T-DNA and induction of the *A. tumefaciens* *vir* genes by special culture conditions. The medium used to induce the *vir* genes mimics the composition of wounded plant cell exudates, with a low pH, a high monosaccharide concentration, and the chemical acetosyringone (AS). One crucial property that has

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made this method useful for subsequent analysis of the tagged gene in plants is that most often only a single site of insertion is generated per transformant (12). This feature greatly simplifies the demonstration that the tagged gene represents the mutation responsible for the phenotype.

*B. dermatitidis* presents one potential obstacle to the use of a mutagenesis–phenotypic-screen approach for identifying fungal genes in a pathway: it is multinucleate. One study indicated an average of 3 to 4 nuclei per yeast for five different strains (11). Insertion mutations that result in a recessive phenotype would not be expressed if only one nucleus out of four is transformed. This problem can be circumvented by transformation of uninucleate conidia (12) or by performing multiple rounds of colonial growth under selection, which has been shown to result in the production of homokaryotic transformants for some multinucleate fungi (15). Since *H. capsulatum* yeast poses a single haploid nucleus, expression of recessive phenotypes should be possible (8).

Although previous experiments have shown that it is possible to transform *B. dermatitidis* and *H. capsulatum* via electroporation, this technique is not ideal for mutagenesis. Transforming DNA integrates randomly in the genome, but often at multiple sites in *B. dermatitidis* (18; unpublished data). In the present work, we developed the tools necessary for *A. tumefaciens* T-DNA transfer into *B. dermatitidis* and *H. capsulatum*, and we demonstrate here that this methodology generates the required fate of integrated DNA and the additional features necessary for the use of *A. tumefaciens* T-DNA as an insertional mutagen in these dimorphic fungi. One essential feature is that *B. dermatitidis* multinucleate yeast gives rise to homokaryotic progeny during outgrowth of transformed cells.

#### MATERIALS AND METHODS

**Fungal strains.** *B. dermatitidis* strains 26199, 60915, and 60636 were obtained from the American Type Culture Collection (ATCC; Manassas, Va.). Strain ER-3 (2) was obtained from D. Baumgardner (Department of Family Medicine, University of Wisconsin Medical School, Milwaukee). Spontaneous (ER-3ura5-11) and UV-induced (ER-3ura5-v33) uracil auxotrophic lines were obtained via fluoroorotic acid selection of germinating conidia (unpublished data). Strains SLH1312 and SLH14081 are patient isolates obtained from the Wisconsin State Laboratory of Hygiene (SLH; Madison, Wis.). *H. capsulatum* strains G217B (ATCC 26032), G217Bura5-23 (26), G184AR (ATCC 26027), and G184AS (22) were provided by Jon Woods (University of Wisconsin, Madison); strain SLHc4 is a patient isolate from the SLH.

Prototrophic *B. dermatitidis* strains were maintained at 37°C as yeast on 7H10 agar slants supplemented with oleic acid-albumin-dextrose-catalase (Difco, Detroit, Mich.). *ura5* auxotrophic strains of *B. dermatitidis* were maintained on 3M agar medium (43) made without hemin but with an additional 10 μM FeSO<sub>4</sub> and 100 μg of uracil/ml. *H. capsulatum* was maintained at 37°C on HMM agar (43) supplemented with uracil at 100 μg/ml for *ura5* auxotrophic strains. *H. capsulatum* was incubated in the presence of 5% CO<sub>2</sub>–95% air. HMM and 3M agar media were made by using SeaKem LE Agarose (BioWhittaker Molecular Applications, Rockland, Maine).

*B. dermatitidis* strain ER-3 was converted to mold and sporulated by inoculating potato flake agar plates (29) with approximately 10<sup>6</sup> yeast cells and maintaining the cultures at room temperature (~22°C) for 2 to 3 weeks. Spores were harvested by flooding the plates with phosphate-buffered saline (PBS) and gently rubbing the surfaces with a glass rod. Released spores were centrifuged at 2,000 × g for 10 min and then resuspended to the desired concentration in HMM.

**Plasmid and bacterial manipulations.** The binary vector pBIN19 (4) (GenBank accession no. 520486) and *A. tumefaciens* strain LBA1100, harboring the Ti plasmid pAL1100 (3), were obtained from C. van den Hondel (Leiden University, Leiden, The Netherlands). Plasmids pBTS4 and pBTS92 have been described previously (30). pBTS13 was constructed by inserting the *SalI/HindIII* fragment containing the *H. capsulatum URA5* gene from plasmid pWU20 (42)

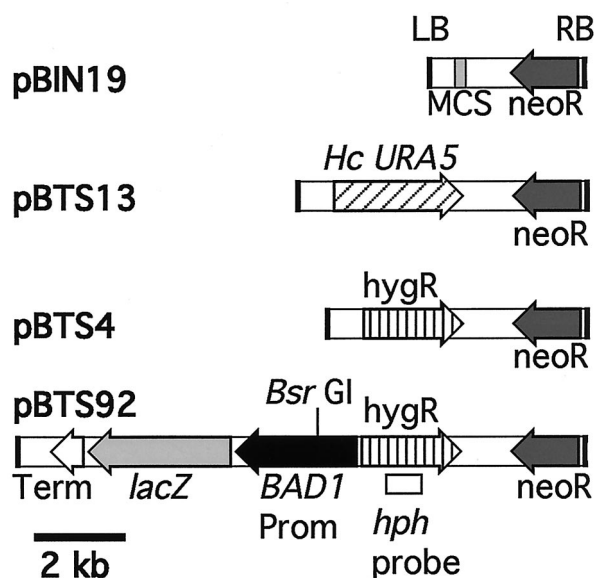


FIG. 1. Binary vectors used for these studies are based on pBIN19 (4). Only the T-DNA region, flanked by the right (RB) and left (LB) borders, is shown for each plasmid. Elements present in pBIN19 or cloned into the multiple cloning site (MCS) to produce the other plasmids are indicated: *neoR*, neomycin or G418 resistance, conferred by the *np1II* gene cassette; *Hc URA5*, the *H. capsulatum URA5* gene (42); *hygR*, hygromycin resistance, conferred by the *hph* gene cassette from pAN7-1 (28); *BAD1* Prom, 2.6 kb from *B. dermatitidis BAD1* 5'-flanking sequences, starting at the translation start codon; *lacZ*, *E. coli* β-galactosidase gene coding region; *Term*, *Aspergillus nidulans trpC* 3'-flanking sequence from pAN7-1. Arrows indicate transcriptional orientation for each fragment. The *Bsr*GI site and *hph* probe sequences used in Southern blot analyses of restricted DNA from pBTS92 transformants are indicated.

into the *SalI/HindIII* sites of pBIN19. These binary plasmids were introduced into *E. coli* strain XL1-Blue (Stratagene, La Jolla, Calif.) via electroporation (Gene Pulser; Bio-Rad, Hercules, Calif.) according to the manufacturer's instructions, and transformants were isolated by kanamycin selection (50 μg of kanamycin/ml in Luria-Bertani [LB] agar plates). Confirmed plasmids were introduced into *A. tumefaciens* strain LBA1100 via electroporation (14). *A. tumefaciens* transformants were isolated on LB agar plates supplemented with 0.1% glucose, 100 μg of kanamycin/ml, and 100 μg of spectinomycin/ml (to select for maintenance of the Ti plasmid). Figure 1 depicts the T-DNA portion of the plasmids used in this study.

**Fungal transformation.** The protocols for *A. tumefaciens*-mediated transformation of yeast and filamentous fungi (7, 12) were adapted for *B. dermatitidis* and *H. capsulatum* yeast. A heavy inoculum of *A. tumefaciens* strain LBA1100, carrying the desired binary plasmid, was incubated overnight (18 to 24 h) at 28°C in *Agrobacterium* minimal medium (MM) (20) supplemented with kanamycin and spectinomycin at 100 μg/ml. The culture was diluted to an *A*<sub>660</sub> of 0.05 in induction medium (IM) (7) supplemented with 200 μM AS (Sigma, St. Louis, Mo.) and again incubated overnight to achieve an *A*<sub>660</sub> of ~0.6. For some experiments, this second overnight culture was concentrated by centrifugation at 5,000 × g for 5 min, and the pellet was resuspended in IM to an *A*<sub>660</sub> of 10.0.

Yeast-phase cells of *B. dermatitidis* and *H. capsulatum* were harvested at 2 to 4 days of growth, counted by using a hemocytometer (individual cells as well as small clusters were counted as one), and centrifuged at 800 × g for 5 min. The supernatant was removed, and the cell pellet was resuspended in HMM to give up to 2 × 10<sup>8</sup> cells per ml for *B. dermatitidis* and 6 × 10<sup>9</sup> cells per ml for the smaller yeast, *H. capsulatum*. For cocultivation, 100 μl of *A. tumefaciens* in IM and 100 μl of yeast in HMM were mixed and spread onto a BiodyneA nylon membrane (Pall Gelman, Ann Arbor, Mich.) on an IM agarose plate containing 200 μM AS. Cocultivation plates were incubated at 28°C for 3 days. (For transformation of germinating spores, cocultivation with *A. tumefaciens* was done at 22°C.) Following cocultivation, the membranes were transferred to either HMM

or 3M selection medium containing 200  $\mu$ M cefotaxime (Sigma). Hygromycin B (Calbiochem, San Diego, Calif.) selection was done at 100  $\mu$ g/ml for *B. dermatitidis* and 200  $\mu$ g/ml for *H. capsulatum*. 3M plates (without uracil) were used to select uracil prototrophs. Selection plates were incubated at 37°C for 2 to 4 weeks to monitor production of colonies, which were enumerated and picked for further analysis. At least one of three types of negative-control experiments was performed for each transformation experiment: (i) cultivation of yeast without *A. tumefaciens*, (ii) cocultivation with *A. tumefaciens* but under noninducing (without AS) conditions, or (iii) cocultivation under inducing (with AS) conditions with *A. tumefaciens* harboring a binary vector plasmid lacking the selectable marker. In *B. dermatitidis* experiments, the frequency of spontaneous hygromycin resistance was zero but reversion of uracil auxotrophy was  $\sim 5 \times 10^{-8}$  per yeast cell. Therefore, for uracil selection experiments, the number of revertants was subtracted to arrive at the putative transformation frequency. For *H. capsulatum* transformed with the binary vector pBTS92, spontaneous hygromycin-resistant colonies appeared at a frequency of 0 to  $6 \times 10^{-7}$  per yeast, but the colonies all lacked *lacZ*; only *lacZ*<sup>+</sup> colonies were counted for transformation frequency. No spontaneous uracil prototrophs were obtained by using uracil selection for transformation of *H. capsulatum*.

**X-Gal stain for  $\beta$ -galactosidase activity.** To assess *lacZ* expression in putative transformants, either colonies were picked to an HMM plate containing 100  $\mu$ g of X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside; Promega, Madison, Wis.)/ml or the membrane containing intact colonies was moved to an X-Gal-containing plate. In both cases the plates were incubated overnight at 37°C and then inspected for cells containing a blue product indicating  $\beta$ -galactosidase activity. In some cases, after overnight staining, plates were stored at 4°C for several days to allow color development without further growth of colonies. For in situ photography of X-Gal-staining cells, membranes were placed overnight in neutral-buffered 10% formalin and then gently washed with PBS. In some cases, membranes were further processed by serial washes with increasing concentrations of ethanol (to 100%) and were air dried between layers of Whatman no. 1 paper. To detect transformants based on *lacZ* expression, cocultivations with  $\sim 10^4$  yeast cells and LBA1100::pBTS92 were performed as above for 3 days at 28°C. The membranes were transferred to 3M plates containing 200  $\mu$ M cefotaxime for 5 to 7 days at 37°C and then to X-Gal plates to allow the development of blue foci on a lawn of nonstaining cells. Yeast cells from blue foci were picked and spread on 3M plates to generate individual colonies. X-Gal staining identified transformed-cell colonies.

**Sytox Green nuclear stain.** Yeast cells from early-log-phase liquid cultures of *B. dermatitidis* ATCC 26199 in HMM were fixed overnight in neutral buffered 5% formalin, washed with 50 mM sodium citrate, pH 7.0, and treated for 90 min with 25 mg of RNase A/ml at 50°C. The yeast cells were washed three times with 50 mM sodium citrate and treated for >60 min at room temperature with 50  $\mu$ M Sytox Green (Molecular Probes, Inc., Eugene, Oreg.). Stained yeast cells were stored in the dark at 4°C before being viewed with an Olympus BX60 microscope in either bright-field or fluorescent mode with 460- to 490-nm excitation filters and 515- to 550-nm emission filters.

**Transformant PCR.** Putative transformants were tested for the presence of T-DNA by PCR amplification of either the *hph* gene (encoding hygromycin phosphotransferase, conferring Hyg<sup>r</sup>) or the *nptII* gene (encoding neomycin phosphotransferase, conferring Neo<sup>r</sup>). For *nptII*, a 542-bp fragment was amplified by using primers 5'-TCGGCAGGAGCAAGGTGAGAT and 5'-AGCCGC GGGTTTCTGGAGTTTAAT (Operon, Alameda, Calif.), Amplitaq DNA polymerase, and a reaction buffer containing 1.5 mM MgCl<sub>2</sub> (Perkin-Elmer Applied Biosystems, Foster City, Calif.) with a thermocycler program of 1 cycle at 94°C for 1 min, followed by 30 cycles of 1 min each at 94, 59, and 72°C, and 1 cycle of 72°C for 5 min. For *hph* PCR, an 822-bp fragment was amplified by using primers 5'-CGATGTAGGAGGGCGTGGATA and 5'-GCTTCTGCGGGCGATTTGT GT, 1.6 mM MgCl<sub>2</sub>, and 30 cycles as above, but with a 60°C annealing temperature.

**DNA extraction and Southern blot hybridization.** Genomic DNA for use in PCR and Southern blot analysis of *B. dermatitidis* and *H. capsulatum* samples was obtained by using published protocols (18, 39, 41). Southern blot hybridizations were performed using standard protocols (32). The primary probe used to analyze the fate of transforming DNA was an 822-bp *hph* fragment made by PCR (described above). Another T-DNA probe was the *trpC* terminator sequence, subcloned from pAN7-1 (28) by PCR amplification using primers 5'-AGAGCT CGGATCCACTTAACGTTACTGA and 5'-TGAATTCTCGAGTGGAGATG TGGAGTGG. A 770-bp fragment was cut from the clone by using *SacI* and *EcoRI*. Non-T-DNA pBIN19 probes included the 7.9-kb *BglII*, the 2.4-kb *BglII/NotI*, or the 5.5-kb *BglII/NotI* fragment of pBIN19. Fragments were gel purified (Qiaquick Gel Extraction kit; Qiagen, Valencia, Calif.) and labeled with

TABLE 1. *Agrobacterium*<sup>a</sup>-mediated transformation of *B. dermatitidis* by use of hygromycin selection

| Strain        | No. of target cells/<br>cocultivation | No. of Hyg <sup>r</sup> colonies/<br>cocultivation<br>± SD <sup>b</sup> | No. of Hyg <sup>r</sup> colonies/<br>10 <sup>6</sup> target<br>cells ± SD |
|---------------|---------------------------------------|---|---|
| <b>Yeast</b>  |                                       |   |   |
| Expt 1        |                                       |   |   |
| 60915         | 1.0 × 10 <sup>7</sup>                 | 211 ± 45  | 21.1 ± 4.5  |
| 26199         | 1.0 × 10 <sup>7</sup>                 | 44 ± 65   | 4.0 ± 6.5   |
| Expt 2        |                                       |   |   |
| 26199         | 2.0 × 10 <sup>7</sup>                 | 296 ± 118   | 14.8 ± 5.9  |
| ER-3          | 2.7 × 10 <sup>7</sup>                 | 84 ± 41   | 3.1 ± 1.5   |
| 60636         | 7.5 × 10 <sup>6</sup>                 | 2.0 ± 1.0   | 0.3 ± 0.2   |
| SLH1312       | 4.0 × 10 <sup>6</sup>                 | 0.7 ± 1.0   | 0.2 ± 0.3   |
| SLH14081      | 3.0 × 10 <sup>6</sup>                 | 0.3 ± 0.6   | 0.1 ± 0.2   |
| Expt 3        |                                       |   |   |
| 26199         | 1.4 × 10 <sup>7</sup>                 | 658 ± 210   | 7.0 ± 15  |
| ER-3          | 1.6 × 10 <sup>7</sup>                 | 24 ± 3.2  | 1.5 ± 0.2   |
| 60636         | 4.0 × 10 <sup>6</sup>                 | 1.2 ± 2.4   | 0.3 ± 0.6   |
| SLH1312       | 1.4 × 10 <sup>6</sup>                 | 0   | <0.7 <sup>c</sup>   |
| SLH14081      | 1.3 × 10 <sup>6</sup>                 | 0   | <0.8 <sup>c</sup>   |
| <b>Spores</b> |                                       |   |   |
| Expt 4, ER-3  | 7.6 × 10 <sup>6</sup>                 | 38 ± 15   | 5.0 ± 2.0   |
| Expt 5, ER-3  | 1.0 × 10 <sup>7</sup>                 | 165 ± 140   | 16.5 ± 14.0   |
| Expt 6, ER-3  | 1.8 × 10 <sup>6</sup>                 | 230 ± 32  | 128.0 ± 17.9  |

<sup>a</sup> *A. tumefaciens* harboring plasmid pBTS4 for experiment 1; plasmid pBTS92 for experiments 2 through 6.

<sup>b</sup> Mean ± standard deviation for three to five samples.

<sup>c</sup> Number given is based on <1 colony per target cell population.

[<sup>32</sup>P]dCTP by using the Oligolabeling kit from Amersham Pharmacia Biotech (Piscataway, N.J.).

## RESULTS

### *A. tumefaciens*-mediated transformation of *B. dermatitidis*.

One rationale for using *A. tumefaciens* for transformation was the desire for a simple fate of T-DNA integration in the genome. In addition, not all strains of *B. dermatitidis* are transformed by electroporation (unpublished data). Starting with yeast-phase organisms, T-DNA plasmid pBTS4 or pBTS92 (Fig. 1), and hygromycin selection, several strains of *B. dermatitidis* were successfully transformed. For some strains the protocol gave rise to hundreds of transformants per cocultivation (Table 1). However, the transformation frequency (transformants per 10<sup>6</sup> target yeast cells) ranged over 2 orders of magnitude among strains tested within a single experiment. There was between-experiment variation in transformation frequency, but the ranking between strains was preserved. In initial experiments, putative transformants were checked for the presence of the *hph* gene by PCR (data not shown). In experiments utilizing pBTS92, putative transformants were confirmed by demonstrating with X-Gal that they had acquired the *lacZ* gene (data not shown). Negative-control experiments indicated that addition of AS was essential for transformation.

Because of the potential problem of multinucleate yeast producing heterokaryotic transformants, experiments were undertaken with germinating conidia of the sporulating strain



TABLE 2. High effector-to-target ratio during cocultivation dramatically increases the transformation frequency

| Strain       | No. of yeast cells/cocultivation | <i>A. tumefaciens</i> / <i>B. dermatitidis</i> ratio <sup>a</sup> | No. of Hyg <sup>r</sup> colonies/cocultivation ± SD <sup>b</sup> | No. of Hyg <sup>r</sup> colonies/10 <sup>6</sup> yeast cells ± SD |
|--------------|----------------------------------|---|--|---|
| Expt 1, ER-3 | 2 × 10 <sup>7</sup>              | 3   | 215  | 11  |
|              | 2 × 10 <sup>6</sup>              | 30  | 141  | 71  |
|              | 2 × 10 <sup>5</sup>              | 300   | 147  | 735   |
|              | 2 × 10 <sup>4</sup>              | 3,000   | 146  | 7,150   |
| Expt 2       |                                  |   |  |   |
|              | 26199 (A) <sup>c</sup>           | 3   | >650 ± 99  | >65 ± 9.9   |
|              | 1 × 10 <sup>4</sup>              | 3,000   | 27 ± 7   | 2,767 ± 723   |
|              | 26199 (B)                        | 3   | 17 ± 1.5   | 1.7 ± 0.15  |
|              | 1 × 10 <sup>4</sup>              | 3,000   | 100 ± 9  | 10,033 ± 907  |

<sup>a</sup> *A. tumefaciens* harboring pBTS92 was used at 6 × 10<sup>7</sup> bacteria per cocultivation in experiment 1 and at 3 × 10<sup>7</sup> bacteria per cocultivation in experiment 2.

<sup>b</sup> There were no replicates in experiment 1; in experiment 2, cocultivations were done in triplicate, so means ± standard deviations are given.

<sup>c</sup> (A) and (B) are two different stocks of ATCC 26199.

ER-3. As with yeast, the conidia were successfully transformed by *A. tumefaciens* (Table 1). Although there was between-experiment variation here also, transformation frequency appears to be higher for ER-3 germinating conidia than for yeast (Table 1).

**Cocultivation ratio of *A. tumefaciens* to *B. dermatitidis*.** To optimize transformation frequency, the effector-to-target ratio was altered. First, the number of *A. tumefaciens* cells in a cocultivation was increased or decreased while the amount of yeast was held constant. For *C. immitis* as a target, 500-fold changes in the amount of *A. tumefaciens* (up to 500 bacteria per target) produced a 10-fold change in transformation frequency (1). In contrast, with *B. dermatitidis* yeast and spores, 100-fold changes in the amount of *A. tumefaciens* (up to 180 bacteria per target) had no effect on the transformation frequency (data not shown). However, when the amount of *A. tumefaciens* was held constant (at 3 × 10<sup>7</sup> or 6 × 10<sup>7</sup> bacteria per cocultivation), and the amount of yeast was serially decreased to give up to 3,000 bacteria per target yeast, transformation frequencies increased dramatically (Table 2). In the extreme case, the transformation frequency was ~10<sup>4</sup> transformants per 10<sup>6</sup> yeast cells, or about 1 per 100 targets (experiment 2, 26199 B).

***A. tumefaciens*-mediated transformation of *H. capsulatum*.** Several strains of *H. capsulatum* yeast were tested for *A. tumefaciens*-mediated transformation using the pBTS92 plasmid and hygromycin selection. All strains generated transformants, and although the transformation frequency was lower than for some *B. dermatitidis* strains, hundreds of transformants were produced per cocultivation. As with *B. dermatitidis*, the transformation efficiency of *H. capsulatum* varied between strains and experiments (Table 3).

**Comparison of hygromycin versus uracil selection.** The availability of uracil auxotrophic lines of *B. dermatitidis* and *H. capsulatum* allowed comparisons of the efficacy of uracil versus hygromycin selection in the *A. tumefaciens* transformation system. Direct comparisons were made by using the two binary plasmids pBTS4 and pBTS13 (Fig. 1). Table 4 shows two significant trends. First, the transformation frequency was 5- to

TABLE 3. *A. tumefaciens*-mediated transformation of *H. capsulatum* yeast by use of hygromycin selection

| Strain              | No. of yeast cells/cocultivation       | No. of Hyg <sup>r</sup> colonies/cocultivation ± SD <sup>a</sup> | No. of Hyg <sup>r</sup> colonies/10 <sup>6</sup> yeast cells ± SD |           |
|---------------------|--|--|---|-----------|
| Expt 1              |  |  |   |           |
|                     | G217B                                  | 2.7 × 10 <sup>8</sup>  | 729 ± 54  | 2.7 ± 0.2 |
|                     | SLHc4                                  | 5.7 × 10 <sup>8</sup>  | 741 ± 171   | 1.3 ± 0.3 |
| Expt 2              |  |  |   |           |
|                     | G217B                                  | 1.9 × 10 <sup>8</sup>  | 266 ± 57  | 1.4 ± 0.3 |
|                     |  | 5.7 × 10 <sup>7</sup>  | 279 ± 29  | 4.9 ± 0.5 |
|                     |  | 1.7 × 10 <sup>7</sup>  | 158 ± 26  | 9.3 ± 1.5 |
|                     | G217B <sub>Bura5-23</sub> <sup>b</sup> | 2.9 × 10 <sup>8</sup>  | 406 ± 58  | 1.4 ± 0.2 |
|                     | SLHc4                                  | 3.4 × 10 <sup>8</sup>  | 272 ± 204   | 0.8 ± 0.6 |
|                     | G184AS                                 | 3.8 × 10 <sup>8</sup>  | 494 ± 38  | 1.3 ± 0.1 |
| G184AR <sup>c</sup> | 4.5 × 10 <sup>7</sup>                  | 283 ± 77   | 6.3 ± 1.7   |           |

<sup>a</sup> *A. tumefaciens* harboring pBTS92 was used; only lacZ<sup>+</sup> colonies were counted; means ± standard deviations for three (experiment 2) or four (experiment 1) samples are given.

<sup>b</sup> The selection medium was supplemented with 100 µg of uracil/ml.

<sup>c</sup> G184AR (rough) forms large aggregates of cells, each counted as a single cell. This experiment was done with a mass of cells approximately equal to those of the other strains.

10-fold higher with uracil selection for both fungi, and second, transformed colonies appeared earlier under uracil selection. For most experiments, more than half the eventual number of colonies arose within the first 2 weeks of uracil selection. In contrast, more than 80% of the colonies developed after the first 2 weeks with hygromycin selection.

**Fate of transforming DNA.** For both *B. dermatitidis* and *H. capsulatum*, the fate of T-DNA transferred from *A. tumefaciens* was analyzed by Southern blot hybridization. In both cases, analysis of undigested DNA indicated that the T-DNA had consistently inserted into chromosomal DNA and was not present as episomal plasmids, as is sometimes the case for *H. capsulatum* when the DNA is introduced by other means (39; data not shown). For transformants generated from the pBTS92 binary plasmid, genomic DNA was digested with *Bsr*GI, which cuts once within the T-DNA, and blots were probed with a fragment of the *hph* gene so that only *Bsr*GI fragments on the *hph* side of the T-DNA would be detected (Fig. 1). Under these conditions, each different site of T-DNA integration in the genome gives a single, unique hybridizing band, whose size is determined by the next site of *Bsr*GI digestion in the adjoining DNA. If head-to-tail concatemers of transforming DNA are formed, as can be the case with *B. dermatitidis* electroporation (18), digestion at a single site in the sequence would give rise to an additional fragment that is equal to the unit length of the repeated DNA. Since either the T-DNA (defined by border sequence repeats) or the entire sequence of a binary vector can be transferred during *A. tumefaciens*-mediated transformation, the unit length of a concatemer repeat depends on the sequence transferred. For pBTS92 the predicted concatemer bands for T-DNA versus the entire plasmid would be 11.9 versus 20.3 kbp. Further analysis of the digested genomic DNA blots entailed hybridizing with probes for the non-T-DNA portions of the plasmid or with probes from the other side of the *Bsr*GI site. These probes were used to confirm that some fragments represented transfer

TABLE 4. *A. tumefaciens*-mediated transformation with hygromycin versus uracil selection

| Strain   | T-DNA plasmid | Selection         | No. of transformants/10 <sup>6</sup> target cells ± SD <sup>a</sup> at: |                    |
|--|---------------|-------------------|---|--------------------|
|  |               |                   | 14 days   | 32 days            |
| <i>B. dermatitidis</i> yeast                         |               |                   |   |                    |
| Expt 1   |               |                   |   |                    |
| ER-3ura5-s11   | pBTS4         | H100 <sup>b</sup> | 0.04 ± 0.08   | 0.4 ± 0.5          |
|  | pBTS13        | URA <sup>c</sup>  | 4.4 ± 1.8   | 5.0 ± 1.6          |
| ER-3ura5-v33   | pBTS4         | H100              | 0.03 ± 0.05   | 0.05 ± 0.05        |
|  | pBTS13        | URA               | 0.4 ± 0.4   | 0.6 ± 0.4          |
| Expt 2, ER-3ura5-s11                                 |               |                   |   |                    |
|  | pBTS4         | H100              | 1.2 ± 0.4   | 6.8 ± 1.2          |
|  | pBTS13        | URA               | 17.0 ± 10.7   | 29.0 ± 0.6         |
| <i>B. dermatitidis</i> spores, ER-3ura5-s11 (expt 3) |               |                   |   |                    |
|  | pBTS4         | H100              | 0.4 ± 0.7   | 5.0 ± 1.8          |
|  | pBTS13        | URA               | 25.4 ± 3.1  | 41.7 ± 3.1         |
| <i>H. capsulatum</i> yeast, G217Bura5-23 (expt 4)    |               |                   |   |                    |
|  | pBTS4         | H200 <sup>d</sup> | <0.03 <sup>e</sup>  | <0.03 <sup>e</sup> |
|  | pBTS13        | URA               | 0.4 ± 0.4   | 1.1 ± 0.2          |

<sup>a</sup> Means ± standard deviations for duplicate (experiment 4) or triplicate (experiments 1 to 3) samples.

<sup>b</sup> H100, hygromycin selection at 100 µg/ml; plates were supplemented with 100 µg of uracil/ml.

<sup>c</sup> 3M medium without uracil supplement.

<sup>d</sup> H200, hygromycin selection at 200 µg/ml; plates were supplemented with 100 µg of uracil/ml.

<sup>e</sup> Value for <1 colony per target population (3.2 × 10<sup>7</sup>).

of the entire plasmid (i.e., hybridization with the non-T-DNA probes) and to confirm that the suspected concatemer band represents a unit length of transferred DNA.

Figure 2 shows Southern blots of 20 independent pBTS92 transformants of *B. dermatitidis* strain 26199 (Fig. 2A and B), and 15 transformants of *H. capsulatum* strain G217B (Fig. 2C). The transformants were generated by using *A. tumefaciens* at an  $A_{660}$  of 0.1 for Fig. 3A and an  $A_{660}$  of 10 for Fig. 3B, so that the ratio of *A. tumefaciens* to target yeast was 50 or 0.5, respectively. In all three panels the majority of lanes contain either a single, unique hybridizing band or a unique band plus an additional band representing the entire plasmid unit length, indicating a concatemer at the site of insertion. For 29 *B. dermatitidis* transformants analyzed in this way (Fig. 2A and B; also data not shown), there were 24 (83%) with single sites of insertion, 27 (93%) with a concatemeric band, and 26 (90%) in which the whole plasmid was transferred. Although the sample size was small (10 each), there were some differences in the fate of DNA between transformants obtained with large versus small amounts of bacteria during cocultivation. At high density ( $A_{660}$ , 10; *A. tumefaciens*-to-yeast ratio, 50), the transformants all appeared to have single sites of insertion, although they all had multiple DNA copies at the site, giving rise to a concatemeric band equal to the full length of the plasmid (Fig. 2B). In contrast, in some of the cases where a low density of bacteria was used ( $A_{660}$ , 0.1; *A. tumefaciens*-to-yeast ratio, 0.5), there is no apparent concatemeric band (Fig. 2A, lanes 1, 4, 5, and 9), but there are more cases of multiple sites of insertion (Fig. 2A, lanes 8 and 10). For 15 *H. capsulatum* transformants (Fig. 2C), 12 (80%) had single sites of integration, 6 (40%) had concatemeric bands, and 7 (47%) had the whole plasmid transferred.

**Homokaryotic progeny following *B. dermatitidis* transformation.** For *A. tumefaciens*-mediated transformation to be useful in insertional mutagenesis of multinucleate *B. dermatitidis* yeast, transformed cells must yield homokaryotic progeny so that recessive mutations can be detected in phenotypic screens;

this is not an issue for the uninucleate yeast form of *H. capsulatum*. One approach to generation of homokaryotic progeny has been to subject multinucleate fungi to multiple rounds of selection (15). A mutant screen involving thousands of transformants would be greatly complicated by the need for multiple rounds of colony selection for each transformant. Therefore, we tested whether a transformed colony produced with a single round of selection (i.e., selection only during primary colony growth) contained mostly homokaryotic cells.

The relative proportion of transformed or parental nuclei (as a measure of the degree of homokaryosis in the colony) was determined by converting the yeast to mold and sporulating the mold to trap a single haploid nucleus in each spore. The genotype of the nucleus in a spore can be determined by growing individual yeast colonies from each spore and testing for the transformed or parental phenotype. If most of the cells in a colony are homokaryotic, when the yeast cells convert to mold and sporulate, most of the haploid spores would contain transformed nuclei and give rise to transformed colonies. If, on the other hand, most of the cells in a colony remain heterokaryotic, two kinds of spores would be produced, harboring either a transformed or a parental nucleus. When these spores are germinated and grown as yeast, the resulting colonies would display either the transformed or the parental phenotype.

*B. dermatitidis* strain ER-3 was transformed with pBTS92 via *A. tumefaciens*, and hygromycin-resistant transformants were selected, plated for single-colony isolation without selection, and amplified to produce yeast to inoculate sporulation plates. Spores were harvested and plated to generate isolated yeast colonies at 37°C. Resulting colonies were stained with X-Gal to determine if the spore that gave rise to the colony harbored a transformed nucleus that could express the *lacZ* gene from the pBTS92 T-DNA.

The progeny of five independent transformants were tested in this manner. Over 600 progeny colonies were scored for

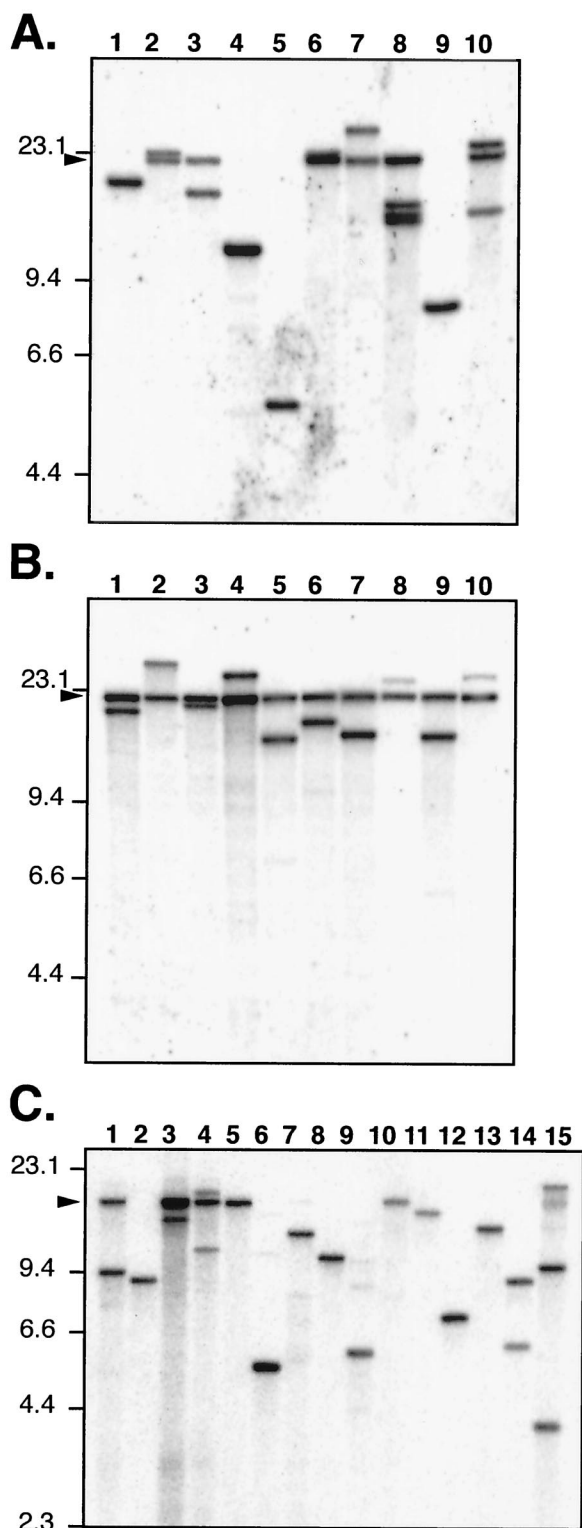


FIG. 2. Southern blot analyses indicate a simple fate of transforming DNA. A 5- $\mu$ g portion of genomic DNA from yeast transformed with LBA1100::pBTS92 was digested with *Bsr*GI and probed with *hph* (Fig. 1). Each lane represents an independent transformant. Molecular mass standards are indicated in kilobase pairs on the left. Arrowheads on the left show the migration position of linearized pBTS92 plasmid. (A) *B. dermatitidis*, cocultivations done with *A. tumefaciens* at an  $A_{660}$  of 0.1. (B) *B. dermatitidis*, cocultivations done at an  $A_{660}$  of 10. (C) *H. capsulatum*, cocultivations done at an  $A_{660}$  of 0.7.

each transformant, and all colonies were  $\beta$ -galactosidase positive, indicating that, at the time of sporulation, each of the transformants was homokaryotic. These results suggest that it is not necessary to go through multiple rounds of colonial selection to achieve homokaryosis for the majority of cells.

**Homokaryons generated without antibiotic selection.** The previous result could be explained by several models: selective growth advantage of homokaryons over heterokaryons in hygromycin, yeast bud formation with a single nucleus, or nuclear sorting to form homokaryons independently of selection. Organelle sorting is seen for segregating chloroplasts in plant cells with a heteroplasmic constitution. Following rounds of non-selective division, homoplasmic progeny arise with one or the other of the chloroplastic types (25, 31). Further experiments were performed to test predicted aspects of these models.

If homokaryons arise under antibiotic selection because of a growth advantage of homokaryotic cells over heterokaryons, then transformants obtained in the absence of antibiotic selection would be predicted to maintain a heterokaryotic condition. If, however, the homokaryotic cells arose as a result of nuclear sorting, independent of antibiotic, then they should still arise in the absence of antibiotics. To test these predictions, and to differentiate between the selection and sorting models, transformations were performed without antibiotic selection. Transformants were identified by *lacZ* gene expression from transforming DNA. This approach was feasible with the high frequency of transformation obtained at high *A. tumefaciens*-to-*B. dermatitidis* ratios (Table 2).

ER-3 yeast cells were transformed with pBTS92, by using  $\sim 10^4$  target yeast cells per plate, and transformants were identified by staining with X-Gal. Foci of blue-staining cells represented transformed cell clones developed within the yeast lawn (Fig. 3A).  $\beta$ -Galactosidase-positive cells from the blue foci were colony purified and sporulated, and progeny were tested for homokaryosis, as was done above for hygromycin-selected transformants. For each of 9 independent clones (i.e., from separate blue foci), over 300 progeny were tested and all were *lacZ*<sup>+</sup>, indicating that the vast majority of sporulating cells harbored only transformed nuclei. An additional clone was progeny tested and gave rise to 1 colony lacking *lacZ* and 260 *lacZ*<sup>+</sup> colonies, suggesting that, for this line, the population that was sporulated was not completely homokaryotic.

The sorting model predicts that, in the absence of antibiotic selection, both parental and transformed homokaryotic progeny should form in the generation of a colony derived from a yeast with one transformed nucleus and several nontransformed, parental nuclei. The resulting colony should then have sectors with *lacZ*<sup>+</sup> cells and cells lacking *lacZ*. Several colonies with this phenotype were generated in experiments performed under high-frequency transformation conditions; one such colony can be seen in Fig. 3B.

**Budding in multinucleate *B. dermatitidis* yeast.** Our results suggest that, following transformation of a single nucleus in a multinucleate yeast cell, cycles of budding give rise to homokaryotic progeny which eventually outnumber heterokaryons. This would be the obvious result if only a single nucleus segregated into each bud. Once a bud forms with a single nucleus, then that bud's progeny would be homokaryotic in all subsequent generations. If more than one nucleus segregates into a bud, other mechanisms for generating homokaryons



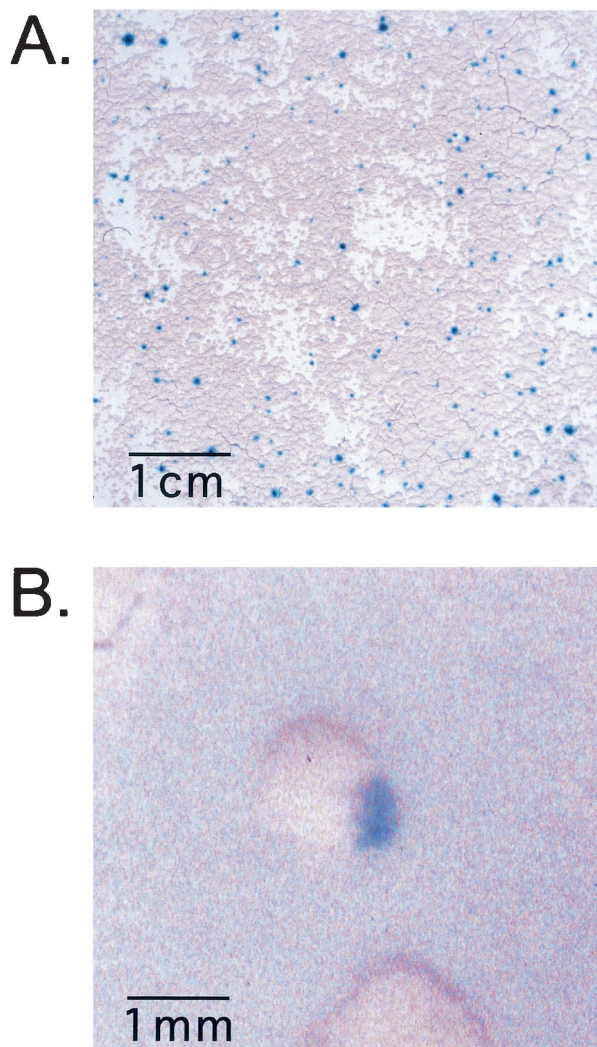


FIG. 3. High-frequency transformation permits visible selection of transformants with *lacZ* expression. *B. dermatitidis* yeast was cocultivated with LBA1100::pBTS92 at a high ratio of bacteria to yeast. After 5 to 7 days of unselected growth, the yeast cells were stained in situ with X-Gal and processed for photography. (A) A total of  $2 \times 10^4$  ER-3 yeast cells were cocultivated with  $\sim 6 \times 10^7$  bacteria. Individual blue-staining foci of *lacZ*<sup>+</sup> cells are seen on a tan background of untransformed *B. dermatitidis*. Note 1-cm scale bar. (B) A total of  $10^3$  strain 26199 yeast cells were cocultivated with  $3 \times 10^7$  bacteria. A close-up image of a single blue-sectored colony is shown. Note 1-mm scale bar.

must be invoked (see Discussion). To analyze the nuclear composition of buds, we stained the DNA in budding cells and looked for uni- or multinucleate bud formation. During this analysis many multinucleate cells and buds were sited for both wild-type and transformed strains of *B. dermatitidis* (Fig. 4A and B) (data not shown). Since most buds analyzed are separated from the mother cell by a septum, it is not possible to exclude a model in which uninucleate buds generate multinucleate yeast by multiple rounds of nuclear division following formation of septa. In a minority of cases ( $\sim 10\%$  of 300 buds analyzed), individual septate buds possessed a single nucleus (Fig. 4C to F) (data not shown). However, in many instances unseptated buds possessed more than one nucleus, as seen in

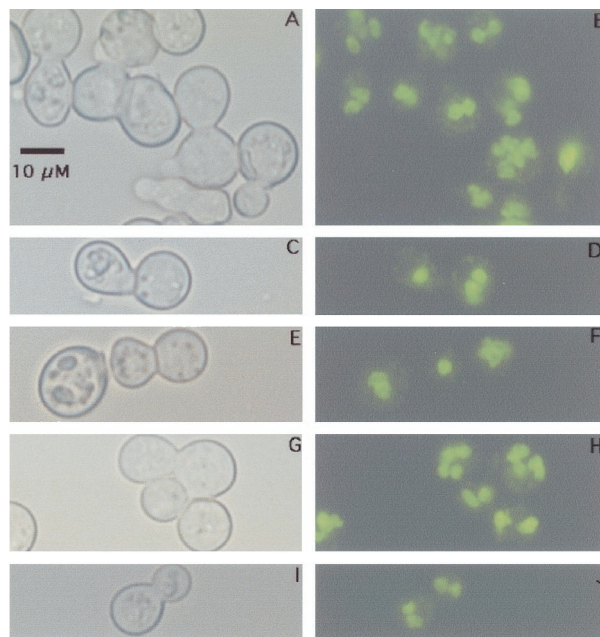


FIG. 4. Multinucleate *B. dermatitidis* yeast cells produce buds with one or more nuclei. Early-log-phase yeast cells of strain 26199 were harvested, fixed, RNase treated, and stained with Sytox Green. Panels are paired horizontally so that the same field is shown viewed with bright-field microscopy (A, C, E, G, and I) or fluorescence (B, D, F, H, and J, respectively). (A and B) Several multinucleate cells are shown. (C, D, E, and F) Budding pairs with septa and a single nucleus apparent in the bud. (G, H, I, and J) Budding pairs without septa and multiple nuclei in the bud. All panels are shown with the same magnification. A scale bar is shown in panel A.

Fig. 4G to J, suggesting that multinucleate buds may form by nuclear migration rather than solely by nuclear division. The results indicate that models for both uni- and multinucleate bud formation during *B. dermatitidis* yeast growth should be considered for explaining the production of homokaryons.

## DISCUSSION

***A. tumefaciens*-mediated transformation of *B. dermatitidis* and *H. capsulatum*.** Although *A. tumefaciens*-mediated transformation of *B. dermatitidis* resembles transformation by electroporation with regard to the insertional fate of DNA at apparently random positions in the genome and the frequent formation of direct-repeat concatemers, it differs in important ways. First, the transformation frequency is higher with *A. tumefaciens* (18) (Tables 1 and 2) (data not shown). This is important for strains for which electroporation failed to generate transformants but that exhibited low-frequency transformation via *A. tumefaciens* (Tables 1 and 2) (data not shown). The T-DNA transformation system has also allowed the introduction of constructs, such as the *lacZ* expression cassette, which was not possible via electroporation (30) (Tables 1 and 2) (data not shown). As seen for several filamentous fungi (12), *B. dermatitidis* spores could also be used as a target for *A. tumefaciens*-based transformation (Table 1).

For both *B. dermatitidis* and *H. capsulatum*, the transformation frequency via *A. tumefaciens* is 5- to 10-fold higher by use

TABLE 5. Expected proportion of homokaryotic and heterokaryotic progeny produced by random sorting at each generation and cumulative effect of this sorting over generations

| Nuclear content <sup>a</sup> | No. of combinations <sup>b</sup> | Expected fraction at each generation |               |                    | Cumulative result                                   |   |
|------------------------------|----------------------------------|--------------------------------------|---------------|--------------------|---|---|
|                              |                                  | Parental homokaryons                 | Heterokaryons | Mutant homokaryons | Maximum fraction of mutant homokaryons <sup>c</sup> | Generations to mutant fixation <sup>d</sup> |
| ○○○●                         | 6                                | 3/6                                  | 3/6           | 0                  | 0.250   | 18.8  |
| ○○●●                         | 6                                | 1/6                                  | 4/6           | 1/6                |   |   |
| ○○○○○●                       | 20                               | 10/20                                | 10/20         | 0                  | 0.167   | 30.2  |
| ○○○○●●                       | 20                               | 4/20                                 | 16/20         | 0                  |   |   |
| ○○●●●●                       | 20                               | 0                                    | 16/20         | 4/20               |   |   |
| ○○○○○○○●                     | 70                               | 35/70                                | 35/70         | 0                  | 0.125   | 41.7  |
| ○○○○○○●●                     | 70                               | 15/70                                | 55/70         | 0                  |   |   |
| ○○○○●●●●                     | 70                               | 1/70                                 | 68/70         | 1/70               |   |   |
| ○○●●●●●●                     | 70                               | 0                                    | 68/70         | 15/70              |   |   |

<sup>a</sup> Nuclei per yeast prior to budding. Open circles, parental nuclei; solid circles, mutant nuclei. Only a select number of possible arrangements of parental and mutant nuclei are shown.

<sup>b</sup> Total number of combinations for sorting the number of nuclei per bud ( $B$ ) from the total number of nuclei per yeast ( $N$ ), by using the formula  $N!/B!(N-B)!$ . For these calculations the number of nuclei per bud is set at one-half the total number of nuclei.

<sup>c</sup> From the formula of Chen et al. (10):  $q_0 - 3q_0p_0(1 - 1/N)^t = q_t$ , where  $q_0$  and  $p_0$  are the fractions of mutant and parental nuclei in the yeast at generation 0,  $q_t$  is the fraction of the population that are homokaryotic mutants at generation  $t$ , and  $N$  is the total nuclear content before budding. The maximum fraction is the value of  $q_t$  when  $t = \infty$ , at which time  $q_t$  equals  $q_0$ . Values are given only for the situation of a single mutant (transformed) nucleus per cell.

<sup>d</sup> Calculated as the generations to give 99% of the expected maximum fraction of mutant homokaryons in the population by using the formula above. The number of generations to fixation is set at the value of  $t$  when  $q_t$  equals 99% of  $q_0$ .

of uracil prototroph selection compared to selection for resistance to hygromycin (Table 4). This finding could result from the high level of hygromycin necessary to suppress the growth of spontaneous hygromycin-resistant colonies, and a similar difference in transformation efficiency has been previously reported for *H. capsulatum* transformed via electroporation (40).

As found with electroporation, there can be variation in the transformation efficiency between strains and experiments (Tables 1 to 4). The cause for this variation was not determined, but it could result from differences in cell wall components or DNA metabolism, linked to differences in growth conditions and cell cycle. Preliminary experiments indicated that older plate cultures of *B. dermatitidis* were less efficiently transformed with *A. tumefaciens* (unpublished data).

A second important difference between *A. tumefaciens*- and electroporation-based transformation is the fate of transforming DNA. Published data for *B. dermatitidis* indicate that approximately two-thirds of transformants via electroporation had more than one site of DNA integration (18), and this fate also occurs in *H. capsulatum* (unpublished data). For both *B. dermatitidis* and *H. capsulatum*, T-DNA insertions occur at single sites in the genome in more than 80% of the cases tested (Fig. 2). This fate is essential for the easy cloning and analysis of insertion alleles. Since neither *B. dermatitidis* nor *H. capsulatum* has a facile genetic system for linkage mapping of a mutant phenotype to a DNA insertion site, the presence of multiple insertion sites would severely complicate analysis of mutants. With a single site of insertion, that site can be confirmed as the source of a mutant phenotype by complementing the defect with a wild-type allele. The wild-type allele would be

cloned using the sequence information from the allele containing the T-DNA insertion. With more than one site of insertion, each would have to be cloned, analyzed, and complemented to determine which one is responsible for the mutant phenotype. Concatemers with multiple copies of the T-DNA at the site of integration (Fig. 2) should not interfere with cloning of the flanking DNA by use of a protocol such as Adapter PCR (34), which depends only on the plasmid sequence being available for primer binding.

**Generation of homokaryotic transformants in multinucleate *B. dermatitidis* yeast.** As used in plant mutagenic screens and as expected in general, insertional mutagenesis should often disrupt gene function and cause a recessive phenotype. However, with one nucleus harboring an insertion mutation in a yeast cell containing two or more wild-type nuclei, the yeast would often retain the wild-type phenotype. Therefore, it would be necessary to convert heterokaryotic yeast to the homokaryotic condition to uncover a recessive phenotype.

The results of spore progeny testing following hygromycin selection demonstrated that a single round of colony growth under selection generates homokaryotic lines. Surprisingly, the results from the visual selection of *lacZ*<sup>+</sup> primary transformants indicated that homokaryotic progeny are generated even in the absence of antibiotic selection. This result was unexpected, as the drug-selective advantage of homokaryotic versus heterokaryotic transformants was believed to be the mechanism for obtaining homokaryons with antibiotic selection (15). An alternative model is that during budding only one nucleus segregates into a bud. If this were the case, only homokaryotic progeny would be produced from heterokaryotic



cells. These would be either of the transformed or of the parental type, but under hygromycin selection, only the transformed ones would survive. Although published studies have reported occasional yeast cells with a single nucleus (11, 13), there has been no systematic analysis of the number of nuclei transferred to a bud in *B. dermatitidis*. Our own nuclear staining analysis of yeast cells from early-log-phase cultures indicated a minority of cells (~10%) with a single nucleus. During this analysis, budding cells were found in which more than one nucleus was present in the bud before septum formation. This finding suggests that multinucleate daughter cells do not all form with a single nucleus but could form via migration of more than one nucleus. Together, the data indicate that there is relaxed control of the number of nuclei per bud, and models based solely on uninucleate buds are insufficient to explain the experimental results.

Another possibility is that, by random sorting of nuclei during generations of budding, there is a cumulative increase of homokaryotic progeny, which eventually outnumber heterokaryons in the population. A similar phenomenon, unselected segregation of organelles, occurs following somatic fusion of plant protoplasts (31) and pertains to nuclear sorting in *Aspergillus* species (27).

#### Mathematical modeling of homokaryosis in *B. dermatitidis*.

On the basis of random sampling of nuclei during budding, one can calculate the probability for each generation that a progeny cell will be either homo- or heterokaryotic. The proportion of each will depend on the total number of nuclei before budding and on the fraction of parental and transformed nuclei. To predict outcomes of colony formation based on random sorting, calculations were made with four, six, or eight total nuclei per yeast cell, of which one or more are mutant or transformed (the number selected for each bud was set at half

the total number of nuclei [Table 5]). These form a subset of the possible combinations that could apply to *B. dermatitidis* budding yeast and cover a range consistent with nuclear numbers determined microscopically (Fig. 4; also data not shown). In Table 5, the number of combinations ( $C$ ) of selecting nuclei ( $B$ ) for a bud from a pool of total nuclei ( $N$ ) is calculated by the equation  $C = N!/B!(N - B)!$ . As the number of nuclei increases, so does the number of possible combinations. In addition, the expected fraction of progeny with different genotypes at each generation depends on the number of parental and mutant nuclei in the prebud yeast.

The cumulative effect of losing a fraction of each set of offspring to the homokaryotic classes over many generations is that eventually the homokaryons greatly outnumber the heterokaryons. The heterokaryon population grows not at the expected rate of doubling at each generation (i.e., total cells =  $2^t$ , where  $t$  is the number of generations) but at a reduced rate (e.g.,  $1.33^t$ , if one-third of the cells formed are homokaryotic). In contrast, as homokaryotic cells are produced, they will double in subsequent generations. The population and genotype dynamics for the case of 4 nuclei per yeast cell with two nuclei per bud are shown in Fig. 5. From cells with one of four nuclei transformed, the expected progeny would be an equal proportion of parental homokaryons and heterokaryons. During subsequent generations, heterokaryons would give rise to progeny with expected proportions of two-thirds heterokaryons and one-sixth each parental and mutant homokaryons. The cumulative effect would decrease the proportion of heterokaryons in the population so that in 20 generations, 99.3% of the progeny would be homokaryotic, equally divided between parental and mutant types (Fig. 5). Twenty generations (~ $10^6$  yeast cells) fits with estimates for a visible *B. dermatitidis* colony (data not shown).

For cells with a greater number of nuclei, more-complex mathematical models apply (10, 25, 44). The model of Chen and coworkers can be used to calculate the number of generations necessary for "fixation" of the homokaryotic genotype. Fixation is defined as the number of generations required to reach 99% of the maximum attainable fraction of the population (10). The proportion of the population that are homokaryotic mutants at a given generation ( $q_t$ ) is equal to  $q_0 - 3p_0q_0(1 - 1/N)^t$ , where  $p_0$  and  $q_0$  are the initial proportions of parental and mutant nuclei, respectively, in the transformed cell,  $N$  is the number of nuclei in the cell prior to budding, and  $t$  is the number of generations. One outcome of this model is that when  $t$  equals  $\infty$ ,  $q_t$  equals  $q_0$ , and the maximum fraction of mutant homokaryons in the population is equal to the fraction of mutant nuclei in the initial transformed cell (Table 5). Once this predicted maximum is determined, the formula can be used to calculate the number of generations,  $t$ , to reach 99% of that maximum. Table 5 shows the results of calculations performed for a single mutant nucleus in cells with four, six, or eight total nuclei and demonstrates another property of the mathematical analysis, which is that the number of generations to fixation is roughly equal to 10 times the number of nuclei per bud (e.g., ~20 generations at 2 nuclei per bud).

The preceding analysis suggests that it should be possible to generate a colony that is mostly homokaryotic in 20 generations. But the example chosen, of yeast cells with four nuclei and buds with two, simplifies the more complex numbers found

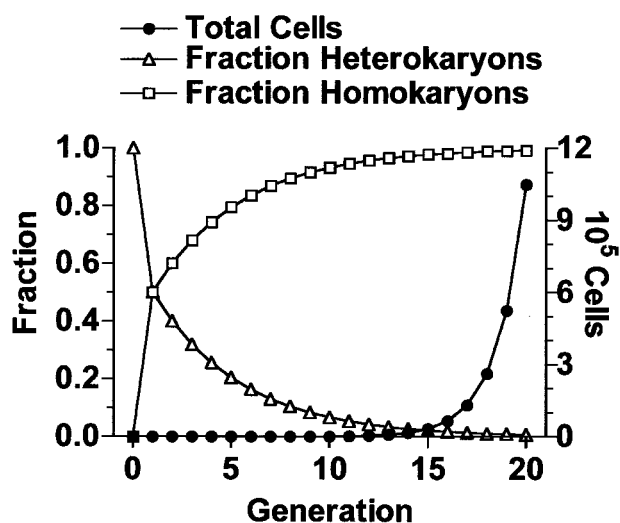


FIG. 5. Theoretical nuclear sorting to generate homokaryotic progeny. The calculations are based on the assumptions of four nuclei per cell at the time of budding, random selection of two nuclei for the bud, and equal nuclear duplication to regenerate four nuclei per cell before the next budding. The total number of cells doubles at each generation. The graph shows the consequence of moving cells in each doubling from the heterokaryotic to the homokaryotic group based on sorting probabilities.

for growing *B. dermatitidis* yeast. How does the presence of yeast cells with as many as eight nuclei affect the calculation? For yeast with more nuclei per cell, it will take more generations to obtain the same proportion of homokaryotic cells (Table 5). However, the number of generations may be lessened due to the fraction of cells that inherit a single nucleus. If 10% of the cells in each generation receive a single nucleus (1.8' kinetics), the cumulative effect is an 88% homokaryotic population in 20 generations, independent of other sorting effects. If, after nuclear division, sister nuclei preferentially migrate to a bud (perhaps determined by location in the cell), it would be functionally equivalent to a single nucleus migrating to a bud and would decrease the number of generations needed to fix the homokaryotic states. A combination of these effects could explain the experimental outcome of homokaryon production following transformation. At present, we cannot monitor individual nuclei during budding to answer specific questions on nuclear-sorting mechanisms. In future experiments we could fluorescently label nuclei to accomplish this goal (35).

Production of homokaryotic progeny is one of the keys to using *A. tumefaciens* T-DNA as an insertional mutagen to obtain recessive mutations in multinucleate *B. dermatitidis*. Together with transformation frequencies in the useful range and single sites of T-DNA insertion, these results fulfill the important criteria necessary for an efficient system of mutagenesis and a means for the subsequent cloning of the mutated genes in *B. dermatitidis* and *H. capsulatum*.

#### ACKNOWLEDGMENTS

This work was supported by grants from the U.S. Public Health Service and the American Lung Association and by a Burroughs Wellcome Scholar Award to B.S.K.

We are indebted to Jon Woods for *H. capsulatum* strains and plasmids and for advice concerning UV mutagenesis and uracil auxotroph isolation. We thank Chris Powell at the Wisconsin State Laboratory of Hygiene for *B. dermatitidis* and *H. capsulatum* strains. We thank Rujin Chen, Kanok Boonsrichai, and Bob Gordon for help with photography and graphics, Colin Kealey for technical assistance with plasmid construction, Patrick Masson for valuable discussions of the experimental results, and Gwynneth Coogan for assistance with mathematical analyses.

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