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Isolation and Characterization of Spontaneous Avirulent Variants of *Histoplasma capsulatum*

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A selection procedure was developed which allowed us to isolate spontaneous isogenic avirulent clones from virulent strains of *Histoplasma capsulatum*. The avirulent yeasts had a unique phenotype: they did not aggregate like the parental strains but grew as dispersed budded and unbudded single cells in liquid medium. On solid medium, the avirulent variant strains grew as smooth-textured colonies, whereas the virulent parental strains grew as rough convoluted colonies. Virulence testing in mice demonstrated that the smooth variants gave 50% lethal dose values similar to those of the avirulent Downs strain. Growth curves for the paired rough and smooth strains were similar. Furthermore, they had the same protein profiles when crude cell fractions were separated on one-dimensional polyacrylamide gels or when whole-cell extracts were separated by two-dimensional gel electrophoresis. Electrophoresis of culture supernatants, however, revealed a difference in a released low-molecular-weight peptide that may be related to virulence. In addition to their usefulness in comparative virulence studies, these avirulent strains should prove valuable for *H. capsulatum* genetic experiments because of the unique ability of these yeasts to grow without clumping.

At least 40 million people in the United States have been infected with *Histoplasma capsulatum*, a dimorphic fungus especially common in the soil of the Ohio-Mississippi river valley (1). Only a small percentage of those infected develop clinically apparent histoplasmosis, but the spectrum of disease can range from mild to severe, either exclusively pulmonary or disseminating to other tissues. Infection generally begins by inhalation of conidia or mycelial fragments, although these soon convert to budding yeast in the 37°C environment of the lung. Persistent infection and spread rely on survival within alveolar macrophages, which are unable to kill the phagocytosed yeasts and thus allow continued intracellular multiplication (see reference 7).

The interaction of virulent *H. capsulatum* with macrophages and mice has been studied in several laboratories, but the biochemical and molecular characteristics associated with virulence remain unknown. A serious obstacle to such studies has been the lack of appropriate avirulent strains for direct comparisons with existing virulent strains. The only strain variation described for *H. capsulatum* is the brown (B) and albino (A) classification of Berliner (3), who based this distinction on the morphological appearance of mycelial colonies in vitro. Primary isolates of *H. capsulatum*, when grown as mycelia, often contain both A and B types, although unselected subculture in vitro results in predominantly A type colonies. Tewari and Berkhout (15) reported that the B type yeasts were more virulent than the A type yeasts of the same strain. Superficially, this B/A type difference might appear similar to the virulent/avirulent phase variation common to many other pathogens. However, the actual B/A difference in virulence for mice and rabbits is relatively minor (4, 15), probably rendering this variation inadequate for a true virulent-avirulent strain comparison.

The only well-characterized avirulent *H. capsulatum* strain, Downs (6), is also inappropriate for such a comparison. Downs exists only as an A mycelial type, has been extensively subcultured in vitro, has lost its ability to sporulate, and has no known virulent counterpart. In fact, recent taxonomic analysis of *H. capsulatum* strains by mitochondrial DNA and rDNA restriction patterns has established a separate genetic class for Downs (16).

It was our goal to isolate avirulent strains isogenic to standard strains of *H. capsulatum* in order to identify factors contributing to virulence. Such virulent-avirulent strain pairs would permit a direct comparison of genes and gene products associated with virulence. In growth of other pathogens, such as *Yersinia enterocolitica* (9) and *Streptococcus mutans* (13), clumping is often associated with the virulence of a particular strain. *H. capsulatum* yeast cells typically grow in broth culture as large aggregates which are not easily or efficiently dispersed. We therefore developed a reliable selection procedure which allowed us to isolate and clone nonaggregating variants from two virulent strains of *H. capsulatum*. This paper describes the initial biological and biochemical comparisons between the virulent strains and their nonclumping avirulent derivatives.

MATERIALS AND METHODS

Strains, media, and growth conditions. Strains G186A (ATCC 26029), G186B (ATCC 26030), G184A (ATCC 26027), G184B (ATCC 26028), G217A (ATCC 26031), G217B (ATCC 26032), G222A (ATCC 26033), and G222B (ATCC 26034) were obtained from the American Type Culture Collection (Rockville, Md.). The yeast form of these strains was stored as colonies on sealed GYE (2% glucose, 1% yeast extract) plates at 4°C. Subcultures from these stock plates were maintained as yeast on GYE or HMM (see below) plates at 37°C in a humidified atmosphere of 95% air–5% CO_2. HMM is a defined synthetic medium consisting of 1 liter of nutrient mixture F-12 (Gibco, Grand Island, N.Y.) supplemented with 18.2 g of glucose, 1 g of sodium glutamate, 84 mg of cystine, and 5.96 g of HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (P. L. Worsham and W. E. Goldman, manuscript in preparation). Yeast cells for each experiment were harvested from log-phase HMM broth cultures grown in Erlenmeyer flasks filled to one-fifth of their capacity. All cultures were grown at 37°C at 150 rpm in an orbital shaker (Queue Systems, Parkers-

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burg, W.V.) modified to maintain an atmosphere of 95% air–5% CO_2.

For growth studies, 500-ml broth cultures were inoculated with 10^6 yeasts per ml, as determined by hemacytometer counts. The culture flasks were placed in a 37°C shaker at 150 rpm. One milliliter was removed from the culture at each time point and diluted in 1 M NaOH to disperse clumps, and yeasts were counted with a hemacytometer. The experiments were continued for 5 to 6 days.

Enrichment of nonclumping yeast strains. Log-phase yeast cells in broth culture (achieved after 48 to 72 h) were allowed to settle for 10 min. The cells which remained in suspension were removed and subcultured in fresh medium. This was repeated seven to nine times. After the final enrichment cycle, the yeasts which did not settle were subcultured onto GYE agar plates. The resulting colonies were observed under a dissecting microscope. Colonies with a smooth appearance were picked with sterile toothpicks and cloned. Soft (0.4%) agar plates, which enhanced the difference in colony morphology (see Fig. 1), were used as a final verification of the smooth colony phenotype.

Virulence testing. Log-phase *H. capsulatum* yeasts were harvested from broth cultures, washed twice with fresh medium, and then dispersed to single cells and small clumps (two to six yeasts) by aspiration through a 23-gauge needle. The reported calculations of yeast cell numbers are based on individual yeast hemacytometer counts corrected for viability (typically >95%). Percent viability was determined by comparing hemacytometer-estimated CFU (clumps plus single cells) with the actual number of colonies from dilution plating on HMM agar. Graded doses in 0.2 ml were inoculated intravenously (tail vein) into 8- to 10-week-old female CDF_1 mice, with groups of five mice for each dose. Mice were monitored daily for determination of the 50% lethal dose (LD_{50}).

In experiments to select for in vivo revertants, spleens were harvested from infected mice, minced, and plated on HMM with gentamicin. UV mutagenesis was done by timed exposure to UV light resulting in 99.5% yeast killing, followed by repeated subculture of the surviving yeasts in the dark for 1 week (Worsham and Goldman, manuscript in preparation).

Cell fractionation. Log-phase yeasts for one-dimensional polyacrylamide gel electrophoresis (PAGE) were grown as 5-ml broth cultures. The cells were collected by centrifugation at 1,500 × *g* for 10 min and then washed once in phosphate-buffered saline. Yeasts were suspended in 1 ml of phosphate-buffered saline with 25 mM phenylmethylsulfonyl fluoride; all subsequent steps were done at 4°C. One-third volume of acid-cleaned glass beads (0.45 to 0.50 mm; VWR Scientific) were added, and the cells were broken by vortexing for 5 min. After the beads were allowed to settle, the lysate was collected and then centrifuged at 4,000 × *g* for 3 min. The supernatant from this step was then centrifuged at 14,000 × *g* for 15 min. Both the supernatant and the pellet from this centrifugation were analyzed as non-cell wall fractions. A third fraction enriched from cell walls was obtained by suspending the 4,000 × *g* pellet and centrifuging it at 1,500 × *g* for 10 min through a 50% glycerol cushion. Phase-contrast microscopy revealed that intact yeast cells were retarded by the glycerol layer, but cell wall fragments were pelleted.

For two-dimensional PAGE comparison, yeasts were harvested and washed as described above and then suspended in 750 μl of ice-cold breakage buffer (10 mM Tris hydrochloride, 10 mM phenylmethylsulfonyl fluoride, pH 7.5). One-third volume of acid-washed glass beads were added to the cell suspension, and the yeasts were broken by vortexing at high speed for 1 min. Excessive heating was avoided by cooling the suspension on ice, and the vortexing-cooling cycle was repeated three times. The cell lysate was transferred to clean tubes, and the glass beads were washed with 200 to 400 μl of breakage buffer. After the lysate and washes were pooled, DNase I (grade DPFF; Worthington Diagnostic Systems, Freehold, N.J.) and RNase A (type X-A; Sigma Chemical Co., St. Louis, Mo.) were added at final concentrations of 50 and 25 μg/ml, respectively. Lysates were centrifuged at 2,000 × *g* for 10 min to remove any glass beads, unbroken cells, and large debris. The pellet was discarded, and the proteins in the supernatant were precipitated with cold trichloroacetic acid for 10 min. Acetone-washed pellets were dried in a Speed Vac concentrator (Savant Instruments, Hicksville, N.Y.).

Culture supernatants. Supernatants from log-phase broth cultures were concentrated 25-fold at 4°C in an Amicon (Danvers, Mass.) ultrafiltration cell fitted with an Amicon YM2 membrane. The concentrated supernatant was diafiltered with 1 liter of 10 mM ammonium acetate (pH 7.2) and lyophilized. Alternatively, smaller samples were concentrated and diafiltered with Amicon Centricon-10 microconcentrators. Samples were stored desiccated at −20°C.

Supernatants were radiolabeled with Iodobeads (Pierce Chemical Co., Rockford, Ill.), following the instructions of the manufacturer. Each reaction contained 250 μl of concentrated culture supernatant, 1 mCi of ^{125}I (Amersham Corp., Arlington Heights, Ill.), and two beads.

PAGE. One-dimensional PAGE was performed by the method of Laemmli (8), with molecular weight standards purchased from Bio-Rad Laboratories (Richmond, Calif.). To determine protein concentrations, we used the method of Ehresmann et al. (5) or the Bio-Rad protein assay kit. Two-dimensional PAGE was performed by the method of O'Farrell (12). Mixtures of pH 3 to 10 and pH 5 to 7 ampholytes (Seralytes; Fisher Scientific) were used to form various isoelectric-focusing pH gradients. After the gels were fixed overnight in acetic acid-methanol-water (2:5:13), they were stained with ammoniacal silver nitrate by the method of Oakley et al. (11).

RESULTS

Phenotypic characterization. The selection of nonclumping strains from standard virulent *H. capsulatum* strains is described in Materials and Methods. In six independent selections, all nonaggregating variants of strains G186A and G184A had the same colony morphology when cultured as yeast; compared with the highly convoluted colonies of the parental (rough) strains, the variant colonies were smooth in texture (Fig. 1). This characteristic phenotype was enhanced when the colonies were grown on soft (0.4%) agar (Fig. 1). Mycelial colonies, however, did not exhibit any distinction between the rough and smooth strains (Fig. 1). In broth cultures of the yeast form, the smooth strain grew mostly as single or budding yeasts, with small clumps that were easily dispersed to single cells (Fig. 1).

The smooth phenotypic variants were stable and nonrevertable, as evaluated by several methods. Normal subculture and repeated platings generated no phenotypic revertants, even when the smooth variants were plated after UV mutagenesis. In addition, we also attempted to select rough revertants by a reverse strategy of the original selection technique, long-term broth culture with repeated collec-

tion of the more quickly settling clumps. Plating from cultures enriched in this fashion onto both soft and regular agar did not yield revertants.

We have been able to select and clone a series of smooth variants from two virulent strains of *H. capsulatum*, G186A and G184A. Calculations of the frequency of this event were not possible because of severe clumping of the parental strains, the lengthy subculturing period required for selection, and the generally poor plating efficiency of yeast cells. Attempts to generate smooth isolates from the B types of these strains have not yet been successful, nor has it been possible to obtain corresponding isolates from the virulent strains G217A, G217B, G222A, and G222B. None of these strains displayed the striking rough colony morphology observed with G184A and G186A, making it more difficult to distinguish the smooth phenotype.

Virulence and growth characteristics. Conventional virulence testing by intravenous inoculation of mice revealed that all the smooth variants were as avirulent as the Downs strain. LD_{50} were determined in 8-week-old female CDF_1 mice for both the rough and smooth strains from G186A (Fig. 2). The LD_{50} at 16 days postinoculation was 7.7×10^5 for G186A and 1.3×10^7 for the corresponding smooth strain. Similar results were obtained with four independently derived smooth clones, including variants of G184A. The avirulent Downs strain yeasts generally gave LD_{50}s of approximately 2×10^7 (10).

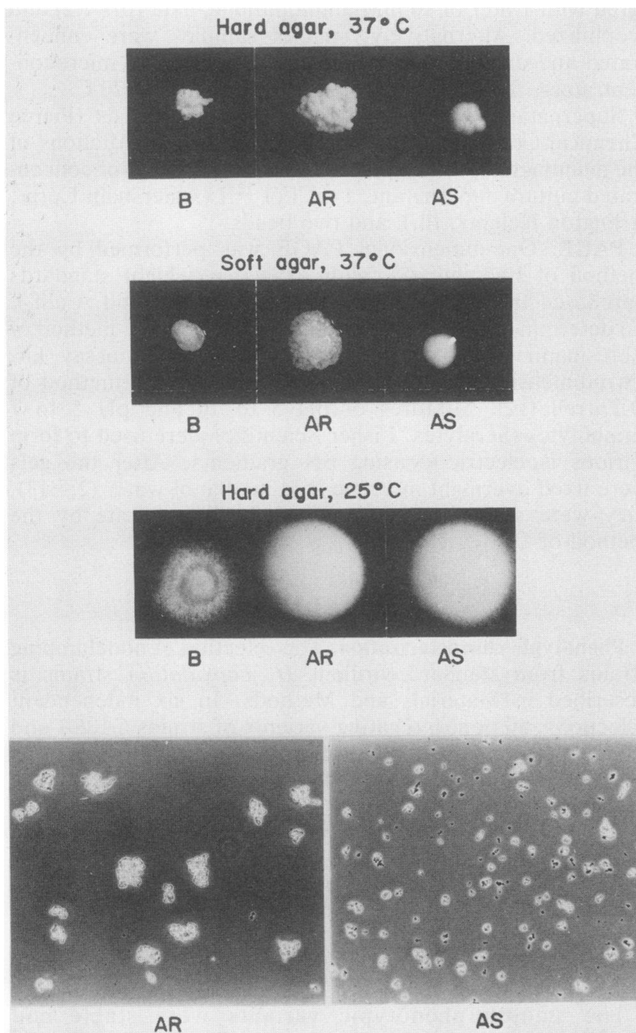

FIG. 1. Morphology of *H. capsulatum* strains and variants. Strains: B, G186B; AR, G186A rough; AS, avirulent smooth variant of G186A. Yeast colonies (37°C) of the rough and smooth strains were easily distinguishable, unlike mycelial colonies (25°C). Bottom panel shows microscopic appearance of yeast phase grown in liquid medium at 37°C (×168).

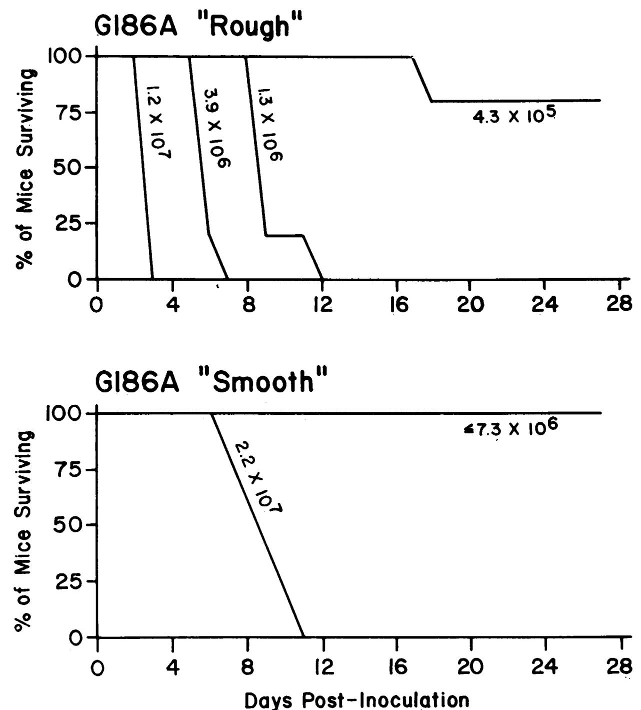

FIG. 2. Dose response of CDF_1 mice to intravenous inoculation of *H. capsulatum* strains. Inoculum sizes (yeasts per mouse) are indicated on each curve. Calculations of the LD_{50} at day 16 are 7.7 $\times 10^5$ G186A rough and 1.3×10^7 for G186A smooth.

Culture of minced spleen tissue from mice that died from high inocula of smooth yeasts yielded no *H. capsulatum* colonies. To increase the changes of isolating a virulent revertant, smooth strain yeasts were mutagenized with UV light, grown in broth culture for 1 week, and inoculated into mice at high doses. No colonies emerged from this mutagenized population when spleen tissue was cultured. Therefore, similar to our results in vitro, we were unable to select in vivo for rough revertants (or any other surviving organisms) from cloned smooth strains.

The lack of virulence in the smooth strains was found not to be due to an obvious defect in growth ability. In vitro growth rate experiments were performed on the paired rough and smooth strains. Growth in several different media with different carbon sources (glucose or mannose) was similar for both the virulent and avirulent strains. Representative HMM broth culture growth curves are displayed in Fig. 3. Linear regression analysis of the data from log-phase growth allowed us to calculate a doubling time of 7.35 h for the rough strain (regression coefficient, 0.981); the generation time for the corresponding smooth strain was 7.24 h (regression coefficient, 0.994). The similarity in doubling times also implies that differences in growth rates were not responsible for the initial selection of the smooth variants.

Biochemical characterization. This series of directly derived avirulent strains allowed us to make paired compari-

sons between virulent and avirulent *H. capsulatum* clones. Initially, we focused on characterization of proteins to assess the overall relatedness between these strains and possibly identify virulence-associated factors. To reveal major strain-specific protein differences, we compared the protein profiles of partially purified cell fractions as well as culture supernatants. Paired samples were electrophoresed on one-dimensional gels with various polyacrylamide concentrations (8, 10, 12, 15, and 18%) and cross-linking ratios (2, 8). Silver staining allowed the detection of minor protein species and permitted color-based discrimination between closely migrating proteins.

When different cell-associated protein fractions (cell wall-enriched and non-cell wall) were compared (Fig. 4), we observed no consistent quantitative or qualitative differences between the rough and smooth strains of G186A. However, the same protein fractions from the closely related strain G186B revealed many major and minor differences. Two-dimensional PAGE of total cell-associated proteins confirmed and extended these observations: no consistent protein differences could be detected within a pI range of 3.5 to 8.5 and a molecular weight range of 15,000 to 200,000 (data not shown).

Since analysis of cell-associated proteins could not discriminate between the rough and smooth strains, we compared proteins released during log-phase broth culture. Separation of peptides on 15 or 18% polyacrylamide gels revealed a major strain-specific difference (Fig. 5). The rough strain released a peptide of approximately 10,000 M_r, while the smooth strain released a peptide of about 8,000 M_r in similar amounts. In addition, the two peptides had distinctly different colors when stained with ammoniacal silver nitrate. When culture supernatants were iodinated and electrophoresed, autoradiography revealed the same bands (verifying their identity as peptides) as well as other less abundant proteins (Fig. 6). The autoradiographs indicated that each strain produced both the 10,000- and 8,000-M_r peptides; however, the rough strain released greater amounts of the larger peptide and the smooth strain released more of the smaller peptide. The differential release of these low-molecular-weight peptides was the only major protein variation we could detect between the paired virulent and avirulent strains of *H. capsulatum*.

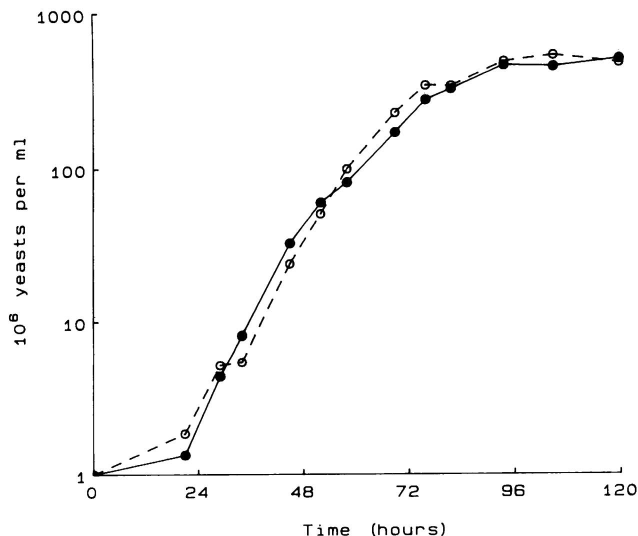

FIG. 3. Representative growth curves for G186A (●) and its smooth variant (○). Yeasts were grown in HMM at 37°C with constant orbital shaking.

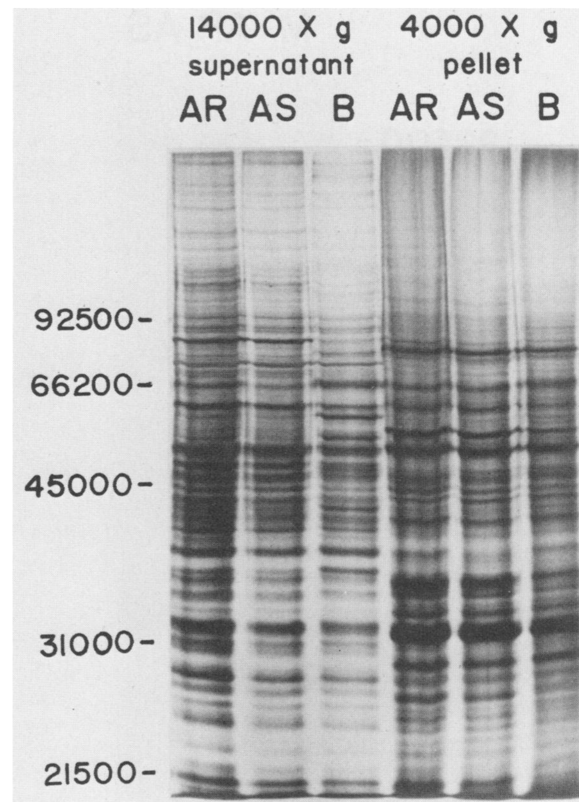

FIG. 4. Representative sodium dodecyl sulfate-PAGE of crude cell fractions from *H. capsulatum*. Strains: B, G186B; AR, G186A rough; AS, smooth variant of G186A. Gel, 10% acrylamide stained with ammoniacal silver nitrate; 25 μg of protein per lane. Migration and molecular weights of protein standards are indicated on the left.

DISCUSSION

A simple selection strategy, based on repeated subculturing of nonclumping yeast cells, generated a series of stable avirulent clones from standard virulent *H. capsulatum* strains. The stability of these smooth variants was evaluated both in vitro and in vivo. After many rounds of subculturing, both on agar plates and in liquid medium, no rough revertants were isolated. A reversal of the original selection procedure did not yield any rough revertants, even when UV mutagenesis preceded the selection. When mice were inoculated with UV-mutagenized or nonmutagenized smooth strain yeasts, no rough revertants could be cultured from harvested spleens. The stability of these variants suggests a number of possible genetic explanations for this phenotypic change. Even a small deletion affecting the expression of a virulence-associated (and aggregation-associated) gene would be nonrevertable. It is also possible that a factor(s) required for virulence is encoded on an extrachromosomal element. The loss of a plasmid, a transposable element, or a virus in the rough to smooth variation would be irreversible.

The only other example of a spontaneous phenotypic change related to *H. capsulatum* virulence is the B to A type mycelial colony variation (3). Tewari and Berkhout (15) and Daniels et al. (4) compared the virulence of B and A mycelial

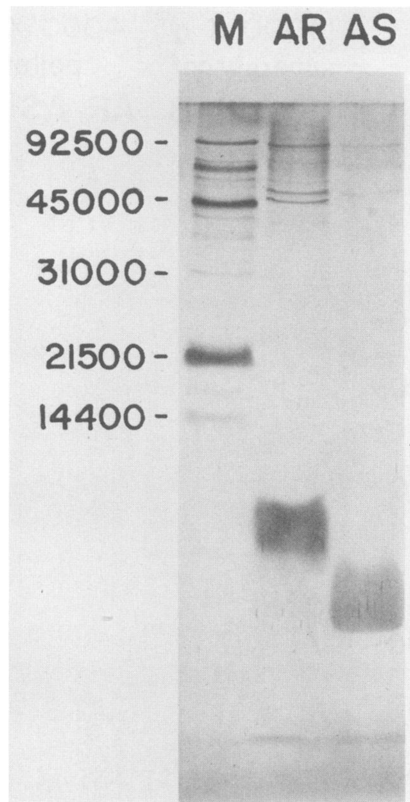

FIG. 5. Sodium dodecyl sulfate-PAGE of concentrated culture supernatants. Lanes: M, molecular weight standards; AR, G186A rough; AS, smooth variant of G186A. Gel, 18% acrylamide stained with ammoniacal silver nitrate; 30 μg of protein per lane. Molecular weights of protein standards are indicated.

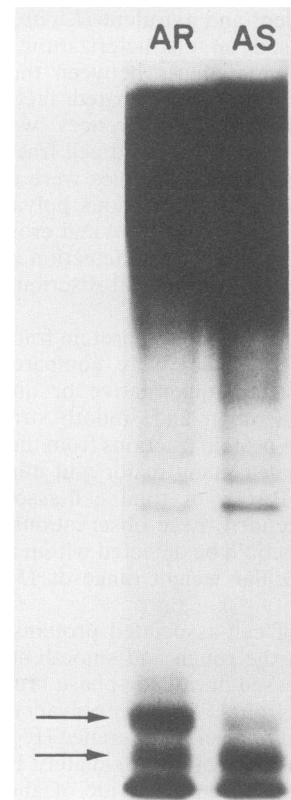

FIG. 6. Autoradiography of iodinated culture supernatant proteins separated on a 12% acrylamide gel. Stains: AR, G186A rough; AS, smooth variant of G186A. Each lane contained 10,000 dpm of ^{125}I. Arrows indicate bands corresponding to the 8,000- and 10,000-M_r peptides observed on silver-stained gels.

types from the same strains. In mice, the yeasts derived from B type mycelia were only slightly more virulent than the yeasts derived from A type mycelia (15). Virulence of the B and A types for rabbits was not significantly different (4). In contrast, the LD_{50} for our smooth strains were about 20-fold greater than the LD_{50} of their corresponding rough parental strains. Mouse virulence data for these smooth strains were similar to results for the Downs strain (a well-characterized but unrelated avirulent strain). The doubling times of the rough strains and their smooth derivatives were virtually the same; thus, differences in growth rate are probably not responsible for the loss of virulence.

The relatedness of the rough and smooth strains was initially examined by analyzing their protein profiles. Cell wall-associated proteins as well as non-cell wall proteins of the paired strains all appeared identical by one-dimensional and two-dimensional PAGE. The only difference detected was in the peptides released into the culture medium. A major peptide released by the rough strain yeasts appeared to be replaced by a faster-migrating species in the smooth strain culture supernatant. If these are related peptides, the change may reflect a deletion in the structural gene or an alteration in posttranslational processing. However, iodination experiments suggest that both strains are capable of making both peptide species. This implies that factors controlling the relative release of these peptides are different in the rough and smooth strains. Such factors may regulate expression of the two peptides, membrane or cell wall permeability, or peptide transport or processing.

Whatever the molecular explanation for the difference in expression or release of these peptides, the most striking message from the protein data is the remarkable similarity between the rough and smooth strains. Unlike the B/A mycelial difference (in which virulence is only marginally altered but proteins show major differences; see Fig. 4), the rough to smooth variation results in a profound loss of virulence with almost no change in protein profile. This increases the likelihood that the rough-smooth strain-specific differences we may detect are related to *H. capsulatum* virulence. It is possible that the sheer ability of *H. capsulatum* yeasts to grow in aggregates confers an in vivo survival advantage. The peptide released into the rough strain culture supernatant may be important for aggregation. Alternatively, aggregation per se may not be directly responsible for virulence; the inability to clump may simply reflect a molecular change in the cell surface which alters virulence for other reasons.

The isolation of these smooth strains is also of significant practical importance. Studies requiring single-cell cloning or generation of mutants have generally been unfeasible because the yeasts were difficult or impossible to disperse from large clumps in broth culture. Therefore, the availability of this series of nonclumping smooth strains will not only allow direct comparisons of virulent and avirulent strains, but will

also permit accurate counts of viable yeasts on solid medium and aid in a wide range of genetic studies.

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LITERATURE CITED

1. **Ajello, L.** 1971. Distribution of *Histoplasma capsulatum* in the United States, p. 103–122. *In* L. Ajello, E. W. Chick, and M. L. Furcolow (ed.), Histoplasmosis: Proceedings of the Second National Conference. Charles C Thomas Publishers, Springfield, Ill.
2. **Anderson, B. L., R. W. Berry, and A. Telser.** 1983. A sodium dodecyl sulfate-polyacrylamide gel electrophoresis system that separates peptides and proteins in the molecular weight range of 2500 to 90,000. Anal. Biochem. **132:**365–375.
3. **Berliner, M. D.** 1971. Biological implications of morphological variants in *Histoplasma capsulatum* primary isolates, p. 21–29. *In* L. Ajello, E. W. Chick, and M. L. Furcolow (ed.), Histoplasmosis: Proceedings of the Second National Conference. Charles C Thomas Publishers, Springfield, Ill.
4. **Daniels, L. S., M. D. Berliner, and C. C. Campbell.** 1968. Varying virulence in rabbits infected with different filamentous types of *Histoplasma capsulatum*. J. Bacteriol. **96:**1535–1539.
5. **Ehresmann, B.** 1973. Spectrophotometric determination of protein concentration in cell extracts containing tRNA's and rRNA's. Anal. Biochem. **54:**454–463.
6. **Gass, M., and G. S. Kobayashi.** 1969. Histoplasmosis. An illustrative case with unusual vaginal and joint involvement. Arch. Dermatol. **100:**724–727.
7. **Goodwin, R. A., Jr., and R. M. Des Prez.** 1978. Histoplasmosis. Am. Rev. Respir. Dis. **117:**929–956.
8. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
9. **Laird, W. J., and D. C. Cavanaugh.** 1980. Correlation of autoagglutination and virulence of yersiniae. J. Clin. Microbiol. **11:**430–432.
10. **Medoff, G., M. Sacco, B. Maresca, D. Schlessinger, A. Painter, G. S. Kobayashi, and L. Carratu.** 1986. Irreversible block of the mycelial-to-yeast phase transition of *Histoplasma capsulatum*. Science **231:**476–479.
11. **Oakley, B. R., D. R. Kirsch, and N. R. Morris.** 1980. A simplified ultrasensitive silver stain for detecting proteins in polyacrylamide gels. Anal. Biochem. **105:**361–363.
12. **O'Farrell, P. H.** 1975. High-resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250:**4007–4021.
13. **Otake, S., M. Hirasawa, T. A. Brown, Y. Kawabata, H. Kiyono, S. M. Michalek, J. R. McGhee, and T. Shiota.** 1981. Virulence of *Streptococcus mutans*: characterization of a serotype g antigen-defective mutant and its revertants. Infect. Immun. **31:**151–159.
14. **Rippon, J. W.** 1974. Medical mycology: the pathogenic fungi and the pathogenic actinomycetes, p. 321–347. W.B. Saunders, Philadelphia.
15. **Tewari, R. P., and F. J. Berkhout.** 1972. Comparative pathogenicity of albino and brown types of *Histoplasma capsulatum* for mice. J. Infect. Dis. **125:**504–508.
16. **Vincent, R. D., R. Goewert, W. E. Goldman, G. S. Kobayashi, A. M. Lambowitz, and G. Medoff.** 1986. Classification of *Histoplasma capsulatum* isolates by restriction fragment polymorphisms. J. Bacteriol. **165:**813–818.