

## The *URA5* Gene Is Necessary for *Histoplasma capsulatum* Growth during Infection of Mouse and Human Cells

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**The *Histoplasma capsulatum* *URA5* gene, which has recently been cloned and disrupted by allelic replacement, encodes orotidine-5'-monophosphate pyrophosphorylase. Inactivation of *URA5* by either targeted or UV mutagenesis results in disruption of the pyrimidine biosynthetic pathway and uracil auxotrophy. We examined the effect of uracil auxotrophy due to a *ura5* mutation on *H. capsulatum* virulence in both cell culture and whole-animal models. Uracil auxotrophs of two *H. capsulatum* restriction fragment length polymorphism classes were found to be avirulent in cultured murine and human cells, as well as in mice. Moreover, virulence could be restored either by supplying a functional *URA5* gene in trans or by supplying exogenous uracil during infection in vitro. These experiments demonstrate that the pyrimidine biosynthetic pathway is essential for *H. capsulatum* growth and virulence.**

The dimorphic fungus *Histoplasma capsulatum* is the causative agent of the most common systemic mycosis in the United States, histoplasmosis (9, 13, 46). Endemic to the Mississippi and Ohio River valleys, infection with *H. capsulatum* most often manifests itself as a mild respiratory infection with flu-like symptoms. However, *H. capsulatum* infection can lead to severe systemic infection and even death in immunocompromised hosts, such as AIDS or cancer patients (4, 15, 17, 18, 47). *H. capsulatum* is thermally dimorphic, existing as a saprophytic mold in the soil or at room temperature in the laboratory and as a yeast in the host or at 37°C in the laboratory. Upon inhalation of the mold form, the organism enters pulmonary macrophages, where it converts to the yeast form, replicating inside the phagolysosome and eventually lysing the cell (19, 31, 36). In immunocompetent individuals infection is limited by the cell-mediated immune response; however, the response is fungistatic rather than fungicidal (3, 6). It is thought that persistent infection may be reactivated when the patient becomes immunocompromised, resulting in a severe, potentially fatal systemic infection (7). Therefore, the identification of gene products essential for survival and growth of *H. capsulatum* in the host is important for the development of antifungal treatments. Although there have been reports of virulence differences between strains (24, 29), between variants of strains (5, 44, 50, 54), or after chemical treatment (28, 30), the previous lack of molecular genetic tools for this pathogen has precluded the construction of isogenic strains and definitive identification of genes necessary for virulence.

It has been demonstrated for several bacterial and fungal pathogens, including *Bacillus anthracis* (16), *Listeria monocytogenes* (27), *Yersinia pestis* (43), *Salmonella typhimurium* (1, 2, 14), *Cryptococcus neoformans* (37, 38, 45), and *Candida albicans* (11, 20, 23, 26, 40, 41), that organisms with mutations involving components of the purine or pyrimidine biosynthetic

pathway are less virulent in cultured cells or whole animals. The *H. capsulatum* *URA5* gene encodes orotidine-5'-monophosphate pyrophosphorylase (OMPpase), a component of the pyrimidine biosynthetic pathway, and *ura5* mutants require exogenous uracil for growth in culture (54). In this study, we examined the ability of two *H. capsulatum* strains that lack a functional *URA5* gene due to UV mutagenesis (G217B *ura5*-23) or targeted gene disruption (G184AS  $\Delta$ *ura5::hph*) to infect cultured cells and mice. For these experiments, we developed a nonradioactive cell proliferation assay to examine the virulence of *H. capsulatum* in the mouse macrophage-like cell line RAW264.7, as well as a cytotoxicity assay to examine *H. capsulatum* virulence in the human monocyte line U937, which does not proliferate following phorbol myristate acetate (PMA) treatment to induce monocyte-to-macrophage differentiation. We found that the *URA5* gene is essential for virulence in both murine and human cell lines, as well as in a mouse infection model, and that either resupplying *URA5* by transformation or supplying exogenous uracil during infection of cultured cells restores virulence. This study is the first demonstration of Koch's molecular postulates for a gene essential for virulence for this fungus. Moreover, it provides the basis for future examination of *H. capsulatum* genes essential for growth of the organism in the host cell by exploiting the absolute requirement for uracil during infection.

### MATERIALS AND METHODS

**Fungal strains.** *H. capsulatum* G217B, G184AS, and G184AS  $\Delta$ *ura5::hph* have been described previously (52, 53). G217B (ATCC 26032) is a clinical isolate of restriction fragment length polymorphism (RFLP) class 2; G184AS is derived from the clinical isolate G184A (ATCC 26027) (RFLP class 3), and G184AS  $\Delta$ *ura5::hph* was constructed by using allelic replacement with a hygromycin resistance marker which insertionaly inactivated the *URA5* gene (53). For this study, we isolated G217B *ura5*-23, a uracil-auxotrophic mutant of G217B, by UV mutagenesis and 5-fluoroorotic acid selection as described previously (54). We transformed both G217B *ura5*-23 and G184AS  $\Delta$ *ura5::hph* with plasmid pWU45, a pBR328 derivative containing the *Podospira anserina* *URA5* gene. This plasmid is identical to the previously described telomeric shuttle plasmid pWU44 (51), except for moving of the telomeric repeats from the *Bam*HI site to the *Bcl*II site of the base plasmid pBR328, restoring a functional tetracycline resistance gene. We used the *P. anserina* *URA5* gene for complementation due to the ease of detecting transforming DNA by Southern hybridization since it does not detectably hybridize to *H. capsulatum* genomic DNA (50, 51). This gene encodes the

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same OMPase enzyme activity as the *H. capsulatum* *URA5* gene and has not shown any differences from the native gene in transformation efficiency or the fate of transforming DNA (50, 51). Although pWU45 is a telomeric plasmid, for this study we used transformants (one from G217B *ura5-23* and two from G184AS *Dura5::hph*) in which the transforming marker was chromosomally integrated and mitotically stable without selection.

**Mammalian cells.** The mammalian cell lines used in this study were RAW 264.7 (ATCC TIB-71), a murine macrophage-like cell line, and U937 (ATCC CRL-1593.2), a human monocyte line, both acquired from the American Type Culture Collection.

**Growth media.** HMM, which has been described previously (52), was used for growth of *H. capsulatum* strains. HMM was supplemented with 0.1 mg of uracil/ml for the growth of uracil auxotrophs. RPMI medium (Gibco-BRL or Cellgro) supplemented with 10% heat-inactivated fetal calf serum (Gibco-BRL) (complete medium) was used for the growth of RAW264.7 and U937 cells. RPMI medium was supplemented with 0.1 mg of uracil/ml during infection with *H. capsulatum* when indicated. Penicillin and streptomycin (10 µg/ml each) were added to both HMM and RPMI medium. All cells were grown at 37°C with 5% CO<sub>2</sub>.

**Virulence assays.** The virulence of *H. capsulatum* in RAW264.7 cells was measured as a percentage of host cell viability by using a nonradioactive cell proliferation kit (Boehringer Mannheim) based upon incorporation of the thymidine analogue bromodeoxyuridine (BrdU). RAW264.7 cells were plated at a density of  $2 \times 10^4$  cells per well in 96-well plates (Costar) and allowed to adhere overnight. *H. capsulatum* strains were grown to approximately mid-log phase (2 days), and yeast cells were enumerated with a hemocytometer following 1/10 dilution in serum-free RPMI medium and vortexing to disperse aggregated cells. If the aggregates could not be completely dispersed, each small group of cells, usually containing two to six yeast cells, was counted as one infectious unit. Yeast cells were diluted in complete RPMI medium (with or without 0.1 mg of uracil/ml) and added to triplicate wells for a multiplicity of infection (MOI) of 0.5 yeast/mammalian cell. The plates were placed on a nutator (for gentle agitation) at 37°C, and infection was allowed to proceed for 4 h. Following removal of the extracellular yeast cells by washing with serum-free RPMI medium, complete RPMI medium (with or without 0.1 mg of uracil/ml) was added to each well and the plates were incubated for 4 days on a nutator at 37°C. The proliferation assay was carried out according to the manufacturer's protocol. Briefly, extracellular yeast cells were removed by washing with serum-free RPMI medium, and surviving RAW264.7 cells were incubated with BrdU for 2 h. The RAW264.7 cells were then fixed and incubated with an anti-BrdU antibody conjugated to alkaline phosphatase, which was later quantitated by using a colorimetric substrate and reading the *A*<sub>370</sub>. The *A*<sub>370</sub> of wells containing uninfected cells was set at 100% viability, and the *A*<sub>370</sub> of wells containing medium only was set at 0%. Control experiments indicated that there was no detectable incorporation of BrdU by *H. capsulatum* during a 2-h incubation designed to mimic these experimental conditions (data not shown).

To prepare U937 cells for infection, 10 mM PMA was added to cells at a concentration of  $1.5 \times 10^3$  cells/ml. This treatment has been shown to induce monocyte-to-macrophage differentiation in these cells (39). Treated cells are adherent to plastic and no longer proliferate. Adherent cells were collected after 24 h, counted, plated in 96-well plates at a concentration of  $6 \times 10^4$  cells/well, and allowed to adhere for 48 h. *H. capsulatum* strains were grown to mid-log phase (2 days), and yeast cells were prepared for infection as described above. Infections were performed in triplicate wells at an MOI of 0.5 for 4 h, extracellular yeast cells were removed by washing with serum-free RPMI medium, and complete RPMI medium (with or without 0.1 mg of uracil/ml) was added to each well. The plates were incubated for 7 days on a nutator at 37°C, and fresh complete RPMI medium was added to each well on day 4. On day 7, viable U937 cells were stained with a solution of 0.2% crystal violet in 2% ethanol. After wells were washed six times with serum-free RPMI medium, 100 µl of the stain was added to each well for 10 min, and then excess stain was removed by washing the plate with distilled H<sub>2</sub>O. Stained cells were solubilized with 1% sodium dodecyl sulfate for 30 min before *A*<sub>590</sub> was read. The *A*<sub>590</sub> of wells containing uninfected cells was set at 100% viability, and the *A*<sub>590</sub> of wells containing medium only was set at 0%.

For infection of both cell lines in the presence of exogenous uracil, uracil was included in the control uninfected wells used to establish 100% viability. We have not observed any deleterious effect of uracil on mammalian cells. However, uracil was added to control cells in these experiments to allow us to evaluate the effect of uracil solely on the infecting *H. capsulatum* yeast.

**Infection of mice with *H. capsulatum*.** Male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Groups of mice ( $n = 6$ ) were infected intranasally with  $2.5 \times 10^5$  *H. capsulatum* yeast cells in a 50-µl volume.

**Organ culture for *H. capsulatum*.** Lungs and spleens were homogenized in balanced salt solution and serially diluted and dispensed (100 µl) onto plates containing brain heart infusion agar (2% agar [wt/vol]) supplemented with 5% (vol/vol) defibrinated sheep erythrocytes, 1% glucose, 0.1 mg of uracil/ml, and 0.01% (wt/vol) cysteine hydrochloride. Plates were incubated at 30°C, and CFU were enumerated after 7 to 10 days. Data are expressed as means  $\pm$  standard errors of the means per organ.

## RESULTS

**Effect of uracil auxotrophy on *H. capsulatum* virulence in a murine cell line.** We examined the effect of uracil auxotrophy on the virulence of *H. capsulatum* strains of two different RFLP classes in the murine macrophage-like cell line RAW 264.7. G217B, which belongs to RFLP class 2, has previously been shown to be virulent in murine macrophages, both primary isolates (8, 42, 48, 49) and the cell line P388D1 (10); however, its virulence in RAW264.7 cells has not previously been tested. Although to our knowledge the virulence of G184AS, RFLP class 3, in RAW264.7 cells has not been tested, previous studies have shown that G184AS is avirulent in the murine cell line P388D1 (10). To examine virulence in RAW 264.7 cells, we adapted a nonradioactive cell proliferation assay to measure the number of RAW264.7 cells viable after infection with *H. capsulatum*, presuming that virulence of *H. capsulatum* strains is inversely proportional to the viability of the RAW264.7 host cell. As shown in Fig. 1A, both G217B and G184AS were virulent, that is, infection resulted in low RAW 264.7 cell viability, at an MOI of 0.5, with G217B exhibiting slightly greater virulence (10% viability) than G184AS (20% viability). However, the uracil auxotrophs G217B *ura5-23* and G184AS *Dura5::hph* were found to be avirulent in RAW264.7 cells (Fig. 1A). Infection with these strains resulted in high RAW264.7 cell viability, often at values of greater than 100%. These high values were not a result of yeast cell incorporation of BrdU and may be due to the activation of RAW264.7 cell proliferation resulting from the presence of avirulent yeast. To determine if a lack of available uracil was responsible for the reduced virulence of the *URA5* mutants, we supplied uracil in the medium during infection. The addition of 0.1 mg of uracil/ml restored virulence to both G217B *ura5-23* and G184AS *Dura5::hph*, each resulting in approximately 6% RAW264.7 cell viability (Fig. 1B). Resupply of a functional *URA5* gene on a plasmid, pWU45, in addition to restoring uracil prototrophy to the yeast in vitro, restored virulence to a G217B *ura5-23* transformant (~9% viability) and two independent G184AS *Dura5::hph* transformants (~10 to 11% viability) (Fig. 1A). These results indicate that avirulence of the *ura5* mutants was due to uracil auxotrophy and could be complemented by addition of exogenous uracil or by supply of a functional *URA5* gene in this infection model.

**Effect of uracil auxotrophy on *H. capsulatum* virulence in mice.** Since uracil-auxotrophic *H. capsulatum* strains were avirulent in cultured murine macrophage-like cells, we next examined whether these results were indicative of avirulence in whole-animal infections. Twelve mice were infected intranasally with the *H. capsulatum* strains indicated in Fig. 2. After 7 and 14 days, six mice were sacrificed, and the lungs and spleens were examined for the presence of *H. capsulatum* by plate culture. The level of G217B remained fairly constant (~4 log<sub>10</sub> CFU) in both lung and spleen cultures at weeks 1 and 2 postinfection. The results at 1 week postinfection show that G184AS, which was previously found to be avirulent in an intravenous mouse infection model (21), yielded only 0.3 log<sub>10</sub> fewer CFU in the lung than G217B (Fig. 2A). However, G184AS yielded 1.1 log<sub>10</sub> fewer CFU than G217B in the spleen at week 1 (Fig. 2C). The number of CFU present the second week after infection with G184AS indicates that the yeast was being cleared from the lung (~2.5 log<sub>10</sub> fewer CFU) (Fig. 2B), though CFU levels in the spleen were similar to those at week 1 (Fig. 2D). Although G184AS was clearly less virulent than G217B, levels of yeast recoverable at 1 week postinfection were high enough to detect differences between infection with the wild-type and uracil-auxotrophic strains. Consistent with

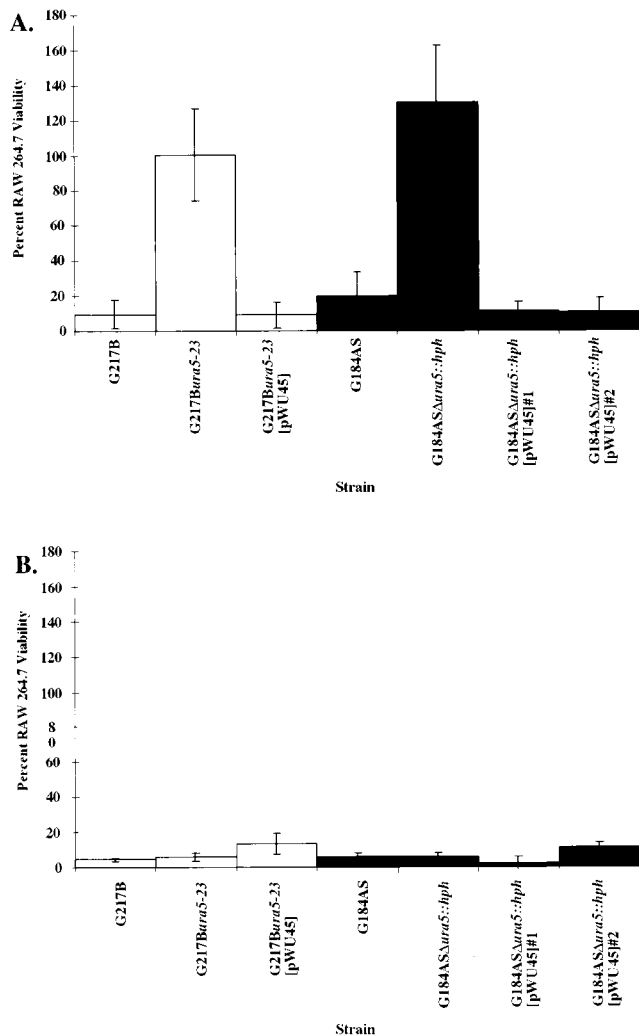


FIG. 1. Effect of uracil auxotrophy on the virulence of *H. capsulatum* in RAW264.7 cells. Virulence is expressed as a function of RAW264.7 cell viability after infection. Virulence was assessed by measuring the incorporation of the thymidine analogue BrdU, with the amount of BrdU taken up by uninfected RAW264.7 cells set at 100% viability. Each bar represents RAW264.7 viability after infection with the indicated *H. capsulatum* strain. The averages of triplicate wells from a representative experiment are shown, with standard deviations indicated by error bars. Similar results were observed in three independent experiments. (A) RAW264.7 cell viability resulting from infection with *H. capsulatum* without the addition of uracil to the culture medium. (B) RAW264.7 cell viability resulting from infection with *H. capsulatum* with the addition of uracil to the culture medium, including the uninfected wells used to establish 100% viability.

results with RAW264.7 cells, the uracil auxotrophs G217B *ura5-23* and G184AS  $\Delta$ *ura5::hph* exhibited greatly diminished virulence. Only cultures from lungs 1 week after infection with G217B *ura5-23* produced any CFU (Fig. 2A), but complete clearance occurred by week 2 (Fig. 2B). The reduced virulence of the uracil auxotrophs was also shown to be due to the lack of a functional *URA5* gene in this infection model. Infection of mice with G217B *ura5-23* and G184AS  $\Delta$ *ura5::hph* pWU45 transformants showed complete or substantial restoration of virulence, reflected by the CFU cultured from both the spleen and lung. The G217B *ura5-23* transformant had a level of CFU slightly higher than the wild type in the lung ( $5.4 \log_{10}$  CFU) (Fig. 2A) and spleen ( $4.1 \log_{10}$  CFU) (Fig. 2C) at week 1, with some reduction in CFU at week 2 (Fig. 2B and D). Although the number of CFU present in the lung at week 1 resulting

from infection with G184AS  $\Delta$ *ura5::hph* [pWU45]#2 was greater than that of the parental strain, it was  $\sim 1.6 \log_{10}$  unit lower than that of G184AS (Fig. 2A). However, infection with G184AS  $\Delta$ *ura5::hph* [pWU45]#1 did show a more complete restoration of virulence, with  $4 \log_{10}$  CFU recovered from the lung and  $2.4 \log_{10}$  CFU recovered from the spleen at week 1 (Fig. 2A and C). Similar to G184AS, transformant yeasts were cleared from the lung and spleen by week 2 (Fig. 2B and D).

***H. capsulatum* infection of a human cell line.** A long-term goal of our studies is to uncover those factors important for *H. capsulatum* infection and persistence in humans. Although murine infections are a useful model, allowing examination of both cell culture and whole-animal infections, it is important to examine the effect of *H. capsulatum* uracil auxotrophy on the infection of human cells and in particular human mononuclear phagocytes. Previous studies involving *H. capsulatum* infection of human monocytes have relied upon explanted cells from human volunteers (32–35). For ease of acquisition and consistency between experiments, we examined the effect of *H. capsulatum* uracil auxotrophy on the infection of the human monocyte line U937, which is both differentiable and activable. U937 cells were differentiated with the phorbol ester PMA, and adherent cells were infected with the *H. capsulatum* strains. Because the cells are terminally differentiated, and therefore do not replicate, we could not use the nonradioactive cell proliferation assay as a measure of host cell viability. Instead, viable cells were stained with crystal violet after infection. A longer infection period (7 days) than used for RAW264.7 cells (4 days) was necessary since lysis of U937 cells was not observed until at least day 6. Similar to the infection of RAW 264.7 cells, both G217B and G184AS were found to be virulent at an MOI of 0.5 (Fig. 3A). However, unlike the infection of RAW264.7 cells, G184AS was found to be slightly more virulent, yielding only 14% U937 cell viability, than G217B, which yielded 42% U937 cell viability. Consistent with the previous experiments utilizing murine cell culture and whole-animal infections, the uracil auxotrophs G217B *ura5-23* and G184AS  $\Delta$ *ura5::hph* were found to be avirulent in U937 cells (Fig. 3A). Infection with either auxotroph resulted in 85% U937 cell viability. However, supplying uracil (0.1 mg/ml) exogenously restored virulence of G217B *ura5-23* and G184AS  $\Delta$ *ura5::hph* to wild-type levels (46 and 20% U937 cell viability, respectively) (Fig. 3B). Moreover, resupplying a functional *URA5* gene in trans also restored the virulence of the uracil-auxotrophic strains G217B *ura5-23* and G184AS  $\Delta$ *ura5::hph* (31% and  $\sim 18$  to 20% U937 cell viability, respectively) (Fig. 3A).

## DISCUSSION

The ability of a pathogen to survive within the host cell involves the activity of many gene products, including those that enable the organism to acquire nutrients within the harsh intracellular environment. By studying the effect of auxotrophic mutations on the survival of *H. capsulatum* in host cells, we hope to gain insight into the factors necessary for pathogenicity of the fungus. In this study, we showed that uracil auxotrophy due to loss of a functional *URA5* gene product, OMPase, results in loss of virulence for at least two RFLP classes of *H. capsulatum*. Infection of murine (RAW264.7) or human (U937) cells with the UV-generated auxotrophic mutant G217B *ura5-23* or the targeted *URA5* mutant G184AS  $\Delta$ *ura5::hph* results in high viability of the host cell compared to infection with the corresponding prototrophic strain, indicating reduced virulence. Similar results were observed during mouse infections. Although G184AS exhibits lower levels of

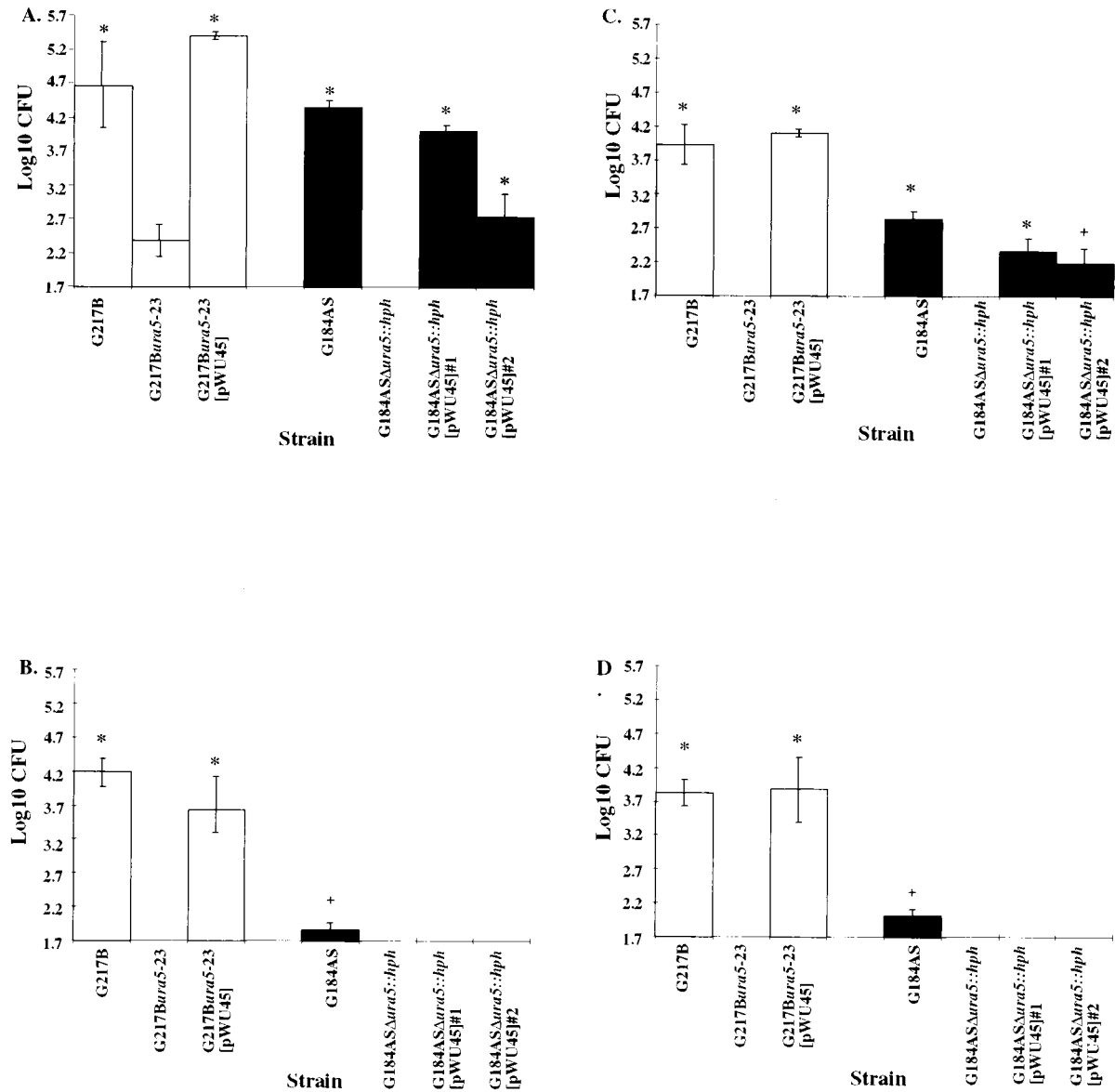


FIG. 2. CFU recovered from mice infected with *H. capsulatum*. Shown are the CFU obtained from homogenates of the lungs (A and B) and spleens (C and D) of six mice at 1 week (A and C) and 2 weeks (B and D) postinfection. The lower limit of detection for this experiment was 50 CFU, or 1.7 log<sub>10</sub> CFU. Error bars represent standard errors of the means. +,  $P < 0.05$  relative to the value for the corresponding uracil auxotroph, as determined by the Student *t* test; \*,  $P < 0.01$  relative to the value for the corresponding uracil auxotroph, as determined by the Student *t* test.

virulence in mice than does G217B, with lower numbers of CFU recovered from both the lung and spleen, the number of CFU observed after infection with G184AS is significantly different than that observed after infection with G184AS  $\Delta$ ura5::hph.

Resupply of a functional *URA5* gene in trans restored virulence, thus fulfilling Koch's molecular postulates, with the caveat that a *P. anserina* *URA5* gene, encoding the same OMPase activity, was used for complementation. Virulence equal to that of the corresponding uracil-prototrophic strains was observed upon infection of either RAW264.7 or U937 cells with transformants of the uracil-auxotrophic strains carrying the plasmid pWU45, which contains a functional *URA5* gene. These data are consistent with the auxotrophic defect being the sole cause of the reduction in virulence in these infection models. Full or partial restoration of virulence by transforma-

tion with pWU45 was also observed in the infection of whole animals. Similar numbers of CFU were recovered from mice infected with the wild-type strain, G217B, and the transformed uracil-prototrophic strain, G217B *ura5-23* [pWU45], in both the lung and spleen. Although G184AS  $\Delta$ ura5::hph [pWU45]#1 and G184AS  $\Delta$ ura5::hph [pWU45]#2 did not show a level of virulence equal to that of the wild-type strain, G184AS, and in fact showed levels of virulence different from each other, the number of CFU observed after infection with these two strains was greater than that of the parental uracil-auxotrophic strain, G184AS  $\Delta$ ura5::hph, indicating that some level of virulence was restored. One explanation for the lack of full restoration of virulence could be that the *P. anserina* *URA5* gene used to complement uracil auxotrophy may exhibit diminished expression, or OMPase activity, in the mouse infection model for G184AS. Alternately, there may be a relatively

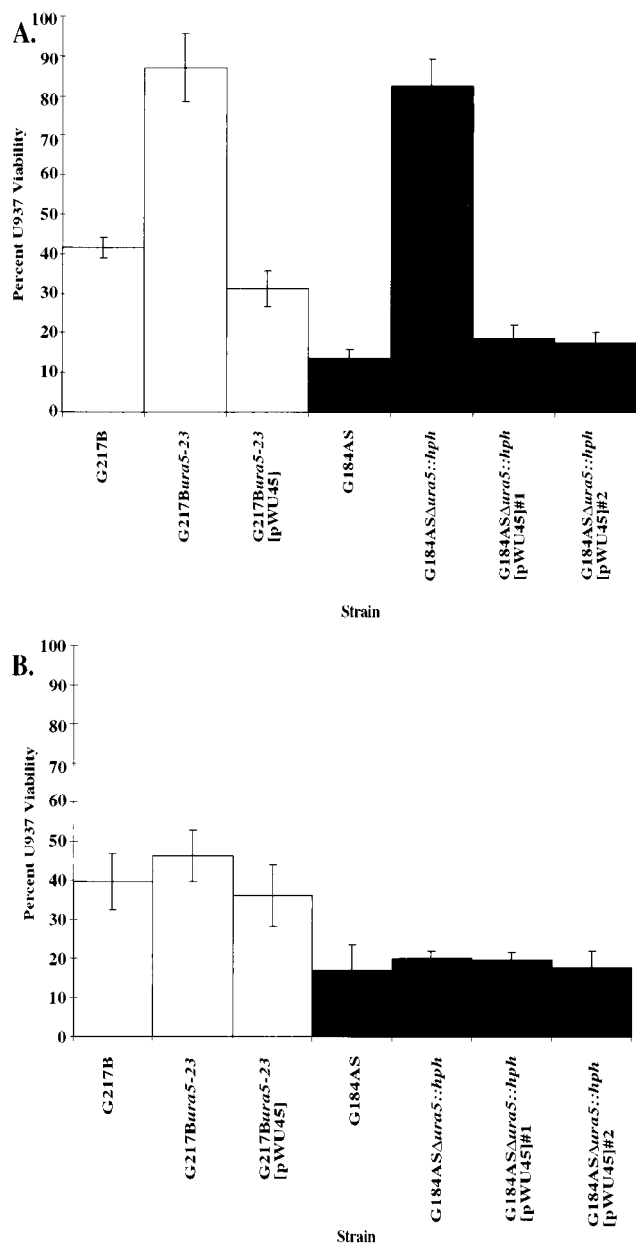


FIG. 3. Effect of uracil auxotrophy on the virulence of *H. capsulatum* in U937 cells. Virulence is expressed as a function of U937 cell viability after infection. Viability was assessed by staining with crystal violet. Dye uptake by uninfected cells was set at 100% viability. Each bar represents U937 cell viability after infection with the indicated *H. capsulatum* strain. The averages of triplicate wells from a representative experiment are shown, with standard deviations indicated by error bars. Similar results were observed in three independent experiments. (A) U937 cell viability resulting from infection with *H. capsulatum* without the addition of uracil to the culture medium. (B) U937 cell viability resulting from infection with *H. capsulatum* with the addition of uracil to the culture medium, including the uninfected wells used to establish 100% viability.

greater requirement for *P. anserina* *URA5* expression or OMPase activity specifically in this model system. It has been reported that transformation with a different plasmid containing this marker results in incomplete restoration of OMPase activity (55). However, there was no evidence for this phenomenon with G217B derivatives and no indication of suboptimal expression in G184AS derivatives during in vitro growth or infection of cultured cells. Another explanation for the incom-

plete restoration of virulence of the two G184AS  $\Delta$ ura5::hph transformants is that the integration site of the plasmid may have an effect on other factors involved in the infection process. However, both transformants exhibit lower levels of virulence than G184AS. Such incomplete complementation following gene disruption and restoration of the wild-type gene by transformation has been observed in pathogenic fungi previously (12, 22, 25). Obviously such phenomena merit careful consideration of the particular infection model and the microbial strain background used for testing effects on virulence.

The addition of exogenous uracil during the infection of cultured cells also restored virulence, indicating that the yeast cells are able to acquire and utilize uracil during infection of macrophages but that uracil is perhaps either not present in the intracellular environment or present in concentrations too low to support the growth of *H. capsulatum* under normal infection conditions. The low level of RAW264.7 and U937 cell viability resulting from infection in the presence of uracil was not due to some toxic effect of uracil itself, since control uninfected cells grown in the presence of uracil were viable after mock infection. In fact, the viability of these cells as measured by BrdU or crystal violet uptake was set at 100%, and the viability of infected cells was compared to that of these cells.

These results serve to reinforce the importance of pyrimidine biosynthesis for survival and virulence. Several purine- and pyrimidine-auxotrophic mutants of both bacterial and fungal pathogens have been shown to have reduced virulence. Adenine auxotrophs of *S. typhimurium* (1, 2, 14), *B. anthracis* (16), and *L. monocytogenes* (27) have proved avirulent in mice. Likewise, a *C. neoformans* strain auxotrophic for adenine exhibits reduced virulence in rabbits (37), while some *C. neoformans* strains auxotrophic for uracil (45) or the amino acid arginine (38) have shown decreased virulence in mice. *pur* mutants of *Y. pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica*, which require guanine for growth, are also avirulent in mice (43). Decreased pathogenicity has been observed for proline (20, 41), lysine (20, 41), serine (20, 26), adenine (20, 23, 40, 41), and uracil (11, 20) auxotrophs of *C. albicans*. These results, together with those presented in this paper, strongly suggest that biosynthetic pathways may serve as excellent targets for antifungal therapy. Moreover, genes encoding proteins involved in purine and pyrimidine biosynthetic pathways may serve as markers for expression during infection. We are currently investigating the use of the *URA5* gene as a reporter for testing promoter activity during macrophage infection.

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