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Research Article

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Correlation between Pathogenicity and Temperature Sensitivity in Different Strains of *Histoplasma capsulatum*

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Abstract

We compared the mycelial to yeast transitions of the Downs strain of *Histoplasma capsulatum* (low level of virulence) with those of G184A and G222B, two more virulent strains having different levels of pathogenicity for mice. When the morphological transitions are initiated by a temperature shift from 25° to 37°C, all three strains undergo similar physiological changes, but these are less severe in G184A and G222B than in the Downs strain. The transitions from mycelial to yeast morphology in both of the more virulent strains are also one-third more rapid than in Downs. We also find that the differences in temperature sensitivity of the three strains can be correlated with the temperature required for complete uncoupling of oxidative phosphorylation. The differences in sensitivity to elevated temperatures extend to the growth of yeast cells of all three strains.

Considered together, our results suggest that sensitivity to elevated temperatures may be a key factor accounting for differences in virulence and that uncoupling of oxidative phosphorylation may be the primary event in the morphological transition in all three strains.

Introduction

Histoplasma capsulatum is a dimorphic fungal pathogen that grows as a multicellular mycelium in soil and as a unicellular yeast in the reticuloendothelial cells of infected humans and animals (1). The morphogenic process and the factors that affect it are of considerable interest, because growth of the yeast phase appears to be required for pathogenicity (2).

We have studied the transition from mycelial to yeast morphology in the Downs strain of *H. capsulatum*, a clinical isolate that has been passaged continuously in our laboratory for ~13 yr (3). In culture, the mycelial to yeast transition can be triggered by a shift in temperature from 25° to 37°C. Three distinct stages in the morphological transition have been delineated (4). Stage 1, immediately after the temperature shift, is characterized by uncoupling of oxidative phosphorylation and a rapid decline in ATP levels. Over the next 24 h, there is a progressive decrease in respiration rate, and in the rates of RNA and protein synthesis. After 24 to 40 h, the cells enter a dormant period (stage 2) that lasts 4–6 d. Stage 2 cells are characterized by very low rates of respiration, grossly decreased concentrations of mitochondrial electron transport components, and strong inhibition of RNA and protein synthesis. Exogenous cysteine or other sulfhydryl-containing compounds are required during stage 2 to rectivate mitochondrial respiration via shunt pathways that bypass inhibited portions of the electron transport system (5). Stage 3 is characterized by increasing concentrations of cytochrome components, resumption of normal respiration, induction of the yeast phase-specific cysteine oxidase, and completion of the transition to yeast morphology (4, 6).

The Downs strain of *H. capsulatum* is an attenuated variety (reference 7; LD_{50} for AKR mice 7 d after infection is 20–22 $\times 10^6$ yeast per ml). Initial experiments on several more virulent *H. capsulatum* strains (G184A, G186A, G217B, and G222B; LD_{50} values for AKR mice are 0.5–9.0 $\times 10^6$ yeast per ml) showed that the 25° to 37°C temperature shift resulted in only a transitory decline in ATP levels to ~50% of those of control cells at 25°C (7).

In the present work, we carried out more detailed studies of the morphological transition of two more virulent strains (G184A, LD_{50} for AKR mice 7 d after infection is 9×10^6 yeast per ml; and G222B, LD_{50} is 6×10^6 yeast per ml). These studies showed that the mycelial to yeast transitions induced by a temperature shift from 25° to 37°C are similar to that of the Downs strain, but the changes are less extreme. Respiration was only partially uncoupled from oxidative phosphorylation, the decline in ATP levels was transitory, and respiration rates decreased to a lesser extent than in the Downs strains. Concentrations of cytochrome components fell to 11–38% of initial values, but they remained detectable throughout the transition. There was no distinct dormant phase, and the transition of both of the more virulent strains to yeast morphology was one-third more rapid than in the Downs strain. The changes in respiration and ATP levels were more marked in G184A than in G222B. Considered together, these results are consistent with the idea that a heat-related insult triggers the morphological transition in all three strains, and that pathogenicity is correlated with their sensitivity to elevated temperature. Similarly, the prolonged dormant phase of the Downs strain (stage 2) may reflect a greater sensitivity to temperature shifts compared with other strains.

These hypotheses were tested further by examining the mycelial to yeast transition of the Downs strain at 34°C and the two more virulent isolates at 40° to 43°C. The physiological changes during the morphologic mycelial to yeast transition of Downs at 34°C were similar to those of the more virulent strains at 37°C; conversely, when the more virulent strains were shifted to temperatures of 40° to 43°C, their mycelial to yeast transitions were similar to that of Downs at 37°C. As before, G184A ap-

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peared to be more temperature sensitive than the more virulent G222B.

Finally, the sensitivity to elevated temperatures extended to the yeast phase of all three strains. At 35°C, growth of Downs, G184A, and G222B yeast cells was indistinguishable. At 37°C, Downs yeast cells showed a significant lag phase compared with G184A and G222B yeast cells, and the growth rate was also slower in Downs. At 39°C, the lag phase in Downs was longer, the growth rate less, and a lower growth yield was apparent, compared with G184A and G222B. At 41°C, Downs did not grow at all. G222B and G184A still showed residual growth at 41°C and the rate of growth and yields were higher in cultures of G222B than G184A. Based on these results, we hypothesize that temperature sensitivity is a key factor accounting for the differences in virulence of these three strains of *H. capsulatum* and that uncoupling of oxidative phosphorylation is the trigerring event in the mycelial to yeast transition of all three strains.

Methods

Strains. The Downs strain of *H. capsulatum* is a clinical isolate that has been passaged in our laboratory for ~13 yr (3). The G184A and G222B strains of *H. capsulatum* were originally obtained from the American Type Culture Collection (Rockville, MD).

Culture conditions. Cells were grown in 2% glucose, 1% yeast extract as described previously (5). Cultures were started with a constant inoculum of cells and were grown to midlog phase, which occurred after 48 to 72 h of incubation at 25°C for mycelia and 37°C for yeast. Growth of yeast cells in the liquid culture was monitored turbidimetrically by measuring absorbance at 650 nm in 18-mm culture tubes in a Bausch and Lomb spectronic 20 spectrophotometer. Absorbance values were correlated with cell number counted in a Levy-Hauser cell-counting chamber. In the experiments on transforming cells, mycelial cultures were diluted 1:5 in fresh medium, divided among several flasks, and incubated at the higher temperatures, which ranged from 34° to 43°C. The flasks were harvested at different times, and respiration rates were measured. Other portions of the cultures were filtered and dried at 42°C to determine dry weights.

Determination of virulence. The lethality of the three strains of *H. capsulatum* for AKR mice was determined by the number of deaths after tail vein injection of 0.2-ml suspensions of graded doses of viable yeast or early germinated mycelia (1×10^5 to 1×10^7 cells per ml) in phosphate-buffered saline. Early germination of mycelia was documented microscopically and the cultures were used when 90% of the fungi had germ tubes that were at least twice the diameter of the yeast cells. Cell suspensions for infection were quantitated using a cell-counting chamber and the numbers of viable cells were determined by colony counts on brain heart infusion agar (Difco Laboratories, Detroit, MI) that was supplemented with growth factor and cysteine by the procedure of Burt et al. (8). There were 10 mice in each dose group. The lowest inocula of *H. capsulatum* cells, which killed 50% of the mice within a week of infection (LD_{50}), were determined by probit analyses (9).

The LD_{50} value of the Downs strain for mice is 20×10^6 yeast per ml; values for G184A and G222B are 9×10^6 and 6×10^6 yeast cells per ml, respectively. LD_{50} values of early germinated mycelia were essentially the same as those of yeast.

Reagents. Oligomycin and carbonyl cyanide *m*-chlorophenylhydrazone (Cl-CCP)[1] were obtained from Sigma Chemical Co. (St. Louis, MO) and were dissolved in absolute ethanol just before use. Ethanol, at the concentrations used in these experiments, had no effect on oxygen uptake.

Isolation of mitochondria. Yeast, mycelia, and cells at intermediate stages of the morphological transition were isolated by filtration through No. 1 filter paper (Whatman Laboratory Products Inc., Whatman Paper Div., Clifton, NJ), washed with distilled cold water, and resuspended in 0.33 M sucrose, 1 mM ethylene glycol bis-*N,N'*-tetraacetic acid, pH 7.0, and 0.3% bovine serum albumin. Glass beads, one-third volume, were added, and the cells were broken by three 15-s bursts in a Braun cell homogenizer (model MSK; Fisher Scientific Co., St. Louis, MO) and cooled intermittently with liquid CO_2. The homogenate was centrifuged twice at 800 *g* (10 min) and the mitochondria were pelleted at 17,000 *g* (30 min). The mitochondrial pellets were resuspended in mitochondrial respiration buffer consisting of 0.3 M sucrose, 8 mM NaH_2PO_4, 5 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), and 8 mM Tris-HCl, pH 7.2.

Measurements of oxygen consumption. Cells were suspended in cell respiration buffer containing 1% mannose, 1 mM $CaCl_2$, and 1 mM dimethylglutaric acid (pH 7.2). Oxygen concentration was measured polarographically using a KIC oxygraph equipped with a Clark-type oxygen electrode (Gilson Instruments, Middleton, WI). Cell respiration rates were expressed as microliters of O_2 per hour per milligram dry weight of cells.

Coupling of oxidative phosphorylation. Coupling of oxidative phosphorylation was assayed by the ability of an uncoupler (Cl-CCP) to stimulate respiration in the presence of oligomycin, an inhibitor of ATP synthetase (7). Cell respiration assays were carried out in the presence of salicylhydroxamic acid (SHAM) to inhibit the alternate oxidase and force electron flux through the cytochrome system (10).

Spectrophotometric measurements. Spectrophotometric measurements were carried out with an Aminco-DW2 dual and wavelength spectrophotometer (5). The cuvette used for low temperature spectra (77°K) had a path length of 1 mm, and the cuvette used for room temperature spectra had a path length of 1 cm. Mitochondria were suspended in 0.3 M sucrose, 8 mM NaH_2PO_4, 8 mM Tris, pH 7.2, and 1 mM EDTA.

Measurement of ATP levels. Cells were collected by rapid filtration and immediately frozen in liquid N_2. ATP was extracted with perchlorate and assayed by the glucose-6-phosphate dehydrogenase/hexokinase method (11). Each assay used 10–15 mg dry weight of cells. ATP concentrations were expressed as micromoles ATP per gram dry weight.

Protein measurements. Proteins were measured by the Lowry method (12).

Results

Physiological changes during the mycelial to yeast transition induced by a temperature shift from 25° to 37°C

In previous work, we characterized physiological changes during the mycelial to yeast transition of the Downs strain of *H. capsulatum* induced by a temperature shift from 25° to 37°C (4, 5, 7). The same temperature shift is sufficient to induce the morphological transition of the more virulent G184A and G222B strains of *H. capsulatum*. We began the present study by comparing physiological changes in these virulent strains with those in Downs after temperature shift from 25° to 37°C. We found the same pattern of physiological changes, but the responses were muted in the more virulent strains.

Uncoupling of respiration. Fig. 1 compares the effect of a temperature shift from 25° to 37°C on respiration in the mycelia of the Downs strain and more virulent strains, G184A and G222B. Coupling between respiration and oxidative phosphorylation in whole cells was assayed by the ability of the uncoupler Cl-CCP to stimulate respiration in the presence of oligomycin, an inhibitor of ATP synthetase. Stimulation of respiration by Cl-CCP indicates that respiration was coupled to ATP synthesis, whereas lack of stimulation indicates uncoupled respiration (13, 14). The respiration measurements were carried out in the pres-

1. *Abbreviations used in this paper:* Cl-CCP, carbonyl cyanide *m*-chlorophenylhydrazone; SHAM, salicylhydroxamic acid.

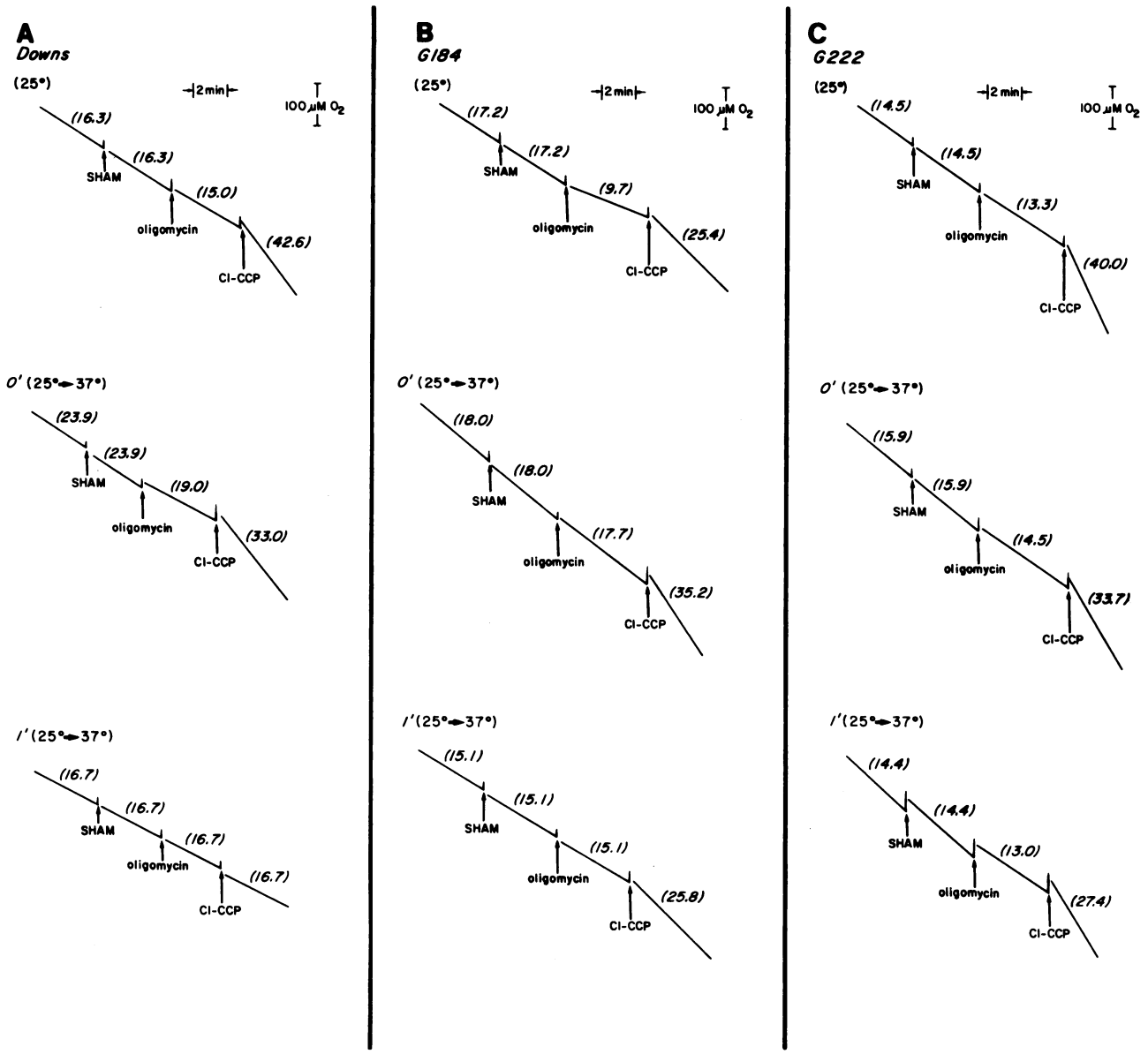

Figure 1. Oxygen electrode recordings of respiration of mycelial cells at 25°C, immediately after (0) and 1 min after shift up to 37°C. To initiate the experiments, 0.5 ml of cultures in 10-ml tubes were switched from one water bath at 25°C to another at 37°C. 3-ml portions were then added to the cuvette in the oxygraph at the designated time points. (*A*) Downs; (*B*) G184A; and (*C*) G222B. The values in parentheses are rates of oxygen consumption (μl of O_2 per hour per milligram dry weight). Additions were oligomycin (5 μg/ml) and Cl-CCP (0.5 mM). Salicylhydroxamic acid (0.2 mM) was added to inhibit the alternate oxidase. The experiment was repeated six times with independent cultures and essentially identical results were obtained.

ence of SHAM, an inhibitor of alternate oxidase activity (10), to force all the electron flow through the cytochrome system. Fig. 1 *A* shows respiration in the Downs strain at 25°C, and at zero time and 1 min after a temperature shift from 25° to 37°C. Respiration is tightly coupled at 25°C (about a 2.8-fold stimulation of respiration by Cl-CCP) and is rapidly uncoupled after the temperature shift up (no stimulation by Cl-CCP within 1 min after the shift up in temperature).

Similar experiments for G184A and G222B show that respiration in these strains remains coupled to oxidative phosphorylation after the temperature shift from 25° to 37°C (Fig. 1, *B* and *C*). However, the degree of stimulation by Cl-CCP is decreased in both strains, from 2.6 before the temperature shift to 1.7-fold after the temperature shift in G184A, and from 3.0 before the temperature shift to 2.1-fold after the temperature shift in G222B. This decreased stimulation by Cl-CCP suggests that partial uncoupling may have occurred. The respiration experiments for the Downs, G184A, and G222B strains were repeated on six independent cell cultures of each strain with essentially the same results.

ATP levels. The less severe effect of temperature shift on coupling of oxidative phosphorylation in the more virulent strains was paralleled by less severe effects on ATP levels. Fig. 2 shows that intracellular ATP levels drop rapidly to zero in the Downs strain after the temperature shift, whereas intracellular ATP levels in G184A and G222B fall to 29 and 43% of the initial levels, respectively, and then recover to almost preshift levels in 60 min.

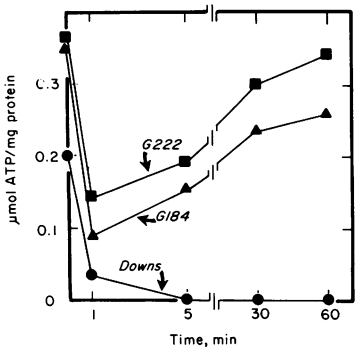

Figure 2. Effect of temperature shifts from 25° to 37°C on intracellular ATP levels. To initiate the experiment, 10 ml of cultures in 50-ml Erlenmeyer flasks were switched from one water bath at 25°C to another at 37°C. The experiment was carried out with three independent cultures and essentially identical results were obtained.

Respiration rates. Fig. 3 shows respiration rates in the Downs strains, as a function of time after a temperature shift from 25° to 37°C. In these experiments, we were able to discern a very transient increase in respiration immediately after the shift to 37°C followed by the progressive decline, characteristic of stage 1 of the transition. This decline continued for ~24 h, at which time cell respiration rates were essentially zero. The dormant period (stage 2) lasted ~5 d, after which the cells entered stage 3, which was characterized by a resumption of respiration and growth of yeast phase cells. Yeast forms began to appear in the Downs culture at ~8–10 d when respiration rates were increasing, and the cultures were predominantly yeast by 12–13 d.

As in the case of ATP levels, the changes in respiration rates during the transition of G184A and G222B were less pronounced than in the Downs strain. Although the same three stages can be distinguished, the rate at which respiration decreased during stage 1 was less precipitous in the more virulent strains, and spontaneous respiration in G184A and G222B only declined to 46 and 56% of the initial levels, respectively. In addition, the morphologic transitions were more rapid in these stains than in Downs. Yeast forms appeared in the cultures by 6–7 d after the temperature shift, concomitant with the increase in respiration rates, and by 8–9 d the cultures were predominantly yeast.

Electron transport components. We previously showed that the decline in respiration in the Downs strain reflects decreased concentrations of electron transport components assayed spectrophotometrically. Similar changes occurred in G184A and G222B, but again, the changes were moderated compared to the Downs strain.

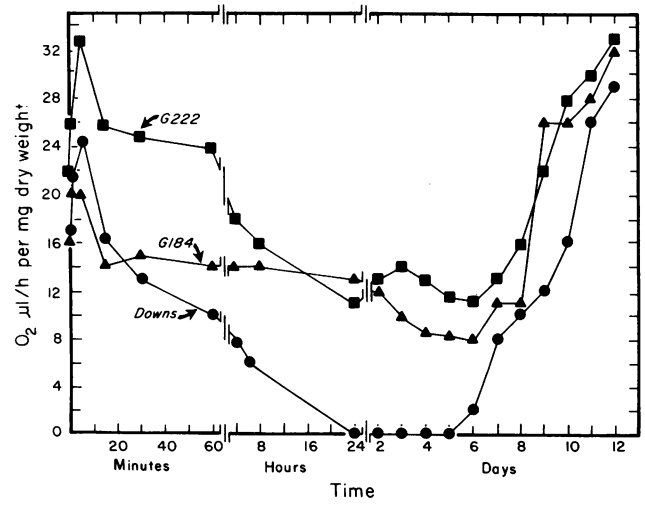

Figure 3. Respiration rates during the mycelial to yeast transition. The experiment was initiated by shifting mycelia (10-ml cultures in 50-ml Erlenmeyer flasks) from 25° to 37°C. Respiration was measured both in respiration buffer and fresh medium, with essentially identical results.

Fig. 4 shows 77°K difference spectra (succinate-reduced minus oxidized- and dithionite-reduced minus oxidized) of mitochondria from the mycelial phases of the Downs (Fig. 4 *A*), G184A (Fig. 4 *B*), and G222B (Fig. 4 *C*) strains of *H. capsulatum.* The spectra for all three strains show alpha peaks corresponding to cytochrome *c* (550 nm), cytochrome *b* (558 nm), and cytochrome aa_3 (605 nm). The spectra for G222B and Downs show an additional peak at 555 nm, which in the Downs strain was previously assigned to cytochrome c_1. This component is probably obscured by other peaks in the spectra for G184A. In all three strains, addition of dithionite causes additional reduction of *b*-type cytochromes, as noted previously in the Downs strain (5). The spectra also show beta peaks at 510–525 nm and Soret peaks for cytochrome *b* (423 nm) and aa_3 (443 nm). The Soret peak for cytochrome *c,* which is expected to appear at ~418 nm, is presumably obscured by the Soret peak for cytochrome *b.* The trough at ~465 nm is due to flavoproteins.

The remaining spectra in Fig. 4 show the effect of temperature shift on electron transport components in the three strains. Table I summarizes the content of electron transport components of mycelia and at different times after the shift up in temperature. In Downs (Fig. 4 *A*), the concentrations of electron transport components decline markedly over the first 24 h after the temperature shift. After 1 to 2 d, cytochromes *c* and aa_3 are virtually undetectable, and cytochrome *b* has decreased to approximately one-seventh of the initial level. The residual cytochrome *b* is not reduced by succinate, which suggests that it is inactive in respiration. The minimum concentration of electron transport components occurs at ~5 d after the temperature shift. After that, the concentrations of cytochrome components and flavoproteins increase to normal levels as yeasts begin to appear in the cultures, and the transition is completed by 12–13 d.

Fig. 4, *B* and *C,* show that G184A and G222B undergo similar but less extreme changes. After 1 to 2 d, concentrations of cytochromes aa_3, *b,* and *c* in G184A and G222B fall to 11–38% of initial levels, but they remain detectable throughout the transitions. The minimum level of cytochrome components in both stains is reached after 1 to 5 d. Cytochrome concentrations then increase as the morphological transitions proceed. Yeast cells first appear in the cultures on day 6, and the cultures are predominantly yeast by day 8, as cytochrome concentrations approach those of normal yeast.

Physiological changes during the mycelial to yeast transition induced by a temperature shift from 25° to 34° to 43°C

The preceeding results show that the patterns of physiological changes during the mycelial to yeast transitions in Downs, G184A, and G222B induced by temperature shift from 25° to 37°C are qualitatively similar, but they are more extreme in Downs than in G184A and G222B. One possible explanation is that Downs is simply more sensitive to elevated temperatures than G184A and G222B. To test this hypothesis, we studied the morphological transitions induced by shifting Downs from 25° to 34°C and mycelia of G184A and G222B from 25° to 40° or 43°C.

Uncoupling of respiration. Fig. 5 shows oxygen electrode recordings of respiration of Downs, G184A, and G222B after var-

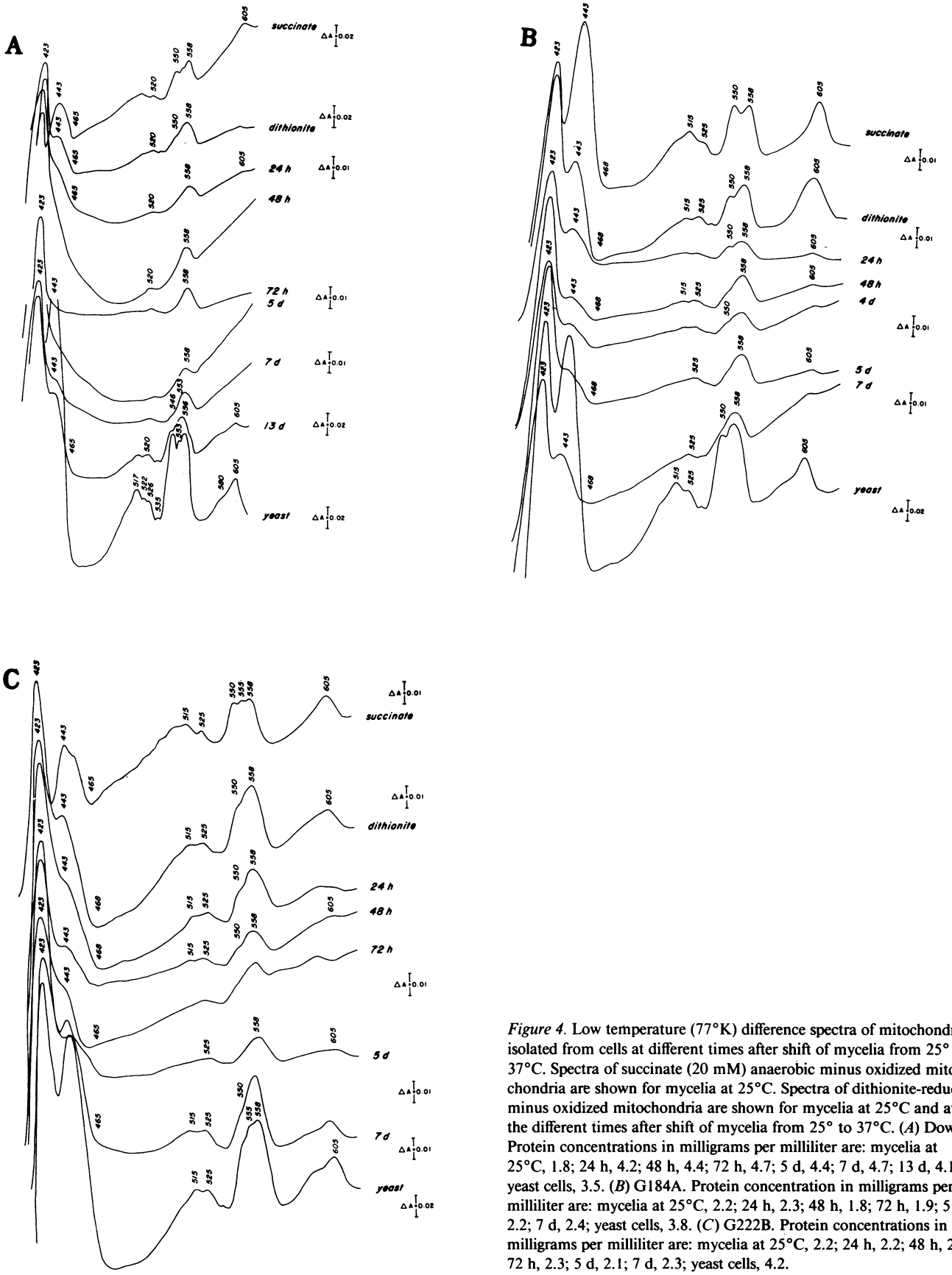

Figure 4. Low temperature (77°K) difference spectra of mitochondria isolated from cells at different times after shift of mycelia from 25° to 37°C. Spectra of succinate (20 mM) anaerobic minus oxidized mitochondria are shown for mycelia at 25°C. Spectra of dithionite-reduced minus oxidized mitochondria are shown for mycelia at 25°C and at the different times after shift of mycelia from 25° to 37°C. (*A*) Downs. Protein concentrations in milligrams per milliliter are: mycelia at 25°C, 1.8; 24 h, 4.2; 48 h, 4.4; 72 h, 4.7; 5 d, 4.4; 7 d, 4.7; 13 d, 4.1; yeast cells, 3.5. (*B*) G184A. Protein concentration in milligrams per milliliter are: mycelia at 25°C, 2.2; 24 h, 2.3; 48 h, 1.8; 72 h, 1.9; 5 d, 2.2; 7 d, 2.4; yeast cells, 3.8. (*C*) G222B. Protein concentrations in milligrams per milliliter are: mycelia at 25°C, 2.2; 24 h, 2.2; 48 h, 2.2; 72 h, 2.3; 5 d, 2.1; 7 d, 2.3; yeast cells, 4.2.

*Table I. Concentrations of Cytochromes in Mitochondria Isolated from Transforming Cells after a Temperature Shift from 25°–37°C**

	Downs			G184A			G222B		
Days	cyt aa_3	cyt *b*	cyt *c*	cyt aa_3	cyt *b*	cyt *c*	cyt aa_3	cyt *b*	cyt *c*
0	0.16	0.28	0.28	0.28	0.27	0.23	0.16	0.21	0.15
1	ND‡	0.11	0.04	0.04	0.09	0.08	0.08	0.16	0.12
2	ND	0.06	ND	0.04	0.12	0.10	0.06	0.10	0.07
3	ND	0.06	ND	0.03	0.10	0.08	0.04	0.08	0.05
5	ND	0.04	ND	0.04	0.13	0.11	0.05	0.09	0.05
7	ND	0.07	ND	0.06	0.16	0.13	0.15	0.23	0.15
9	ND	0.06	ND						
12	ND	0.12	0.08						
13	0.06	0.18	0.12						
Yeast	0.24	0.19	0.15	0.24	0.20	0.21	0.23	0.20	0.18

cyt, cytochrome. * Cytochrome concentrations were calculated from low temperature difference spectra of dithionite-reduced minus oxidized mitochondria. The values are expressed as nanomoles per milligram mitochondrial protein. ‡ ND, nondetectable.

ious temperature shifts. Fig. 5 *A* shows that shifting the Downs strain from 25° to 34°C, which is still sufficient to induce the morphological transition, and does not result in complete uncoupling of oxidative phosphorylation, judged by stimulation of respiration by addition of the uncoupler Cl-CCP. However, respiration does appear to be partially uncoupled, as the stimulation by Cl-CCP decreased from 2.6-fold before the temperature shift to 1.8-fold 1 min after the temperature shift. At the same time, Fig. 5, *B* and *C*, show that respiration in G184A and G222B is completely uncoupled after shifts to the higher temperatures of 41° and 43°C, respectively. This experiment was repeated at least six times on separate cultures with essentially the same results.

ATP levels. Fig. 6 shows the effects of different temperature shifts on ATP levels in the three strains. In the case of Downs, the shift to 34°C results in a less severe decline in ATP levels than the shift to 37°C, which is consistent with the finding that respiration is not completely uncoupled by the shift to 34°C (Fig. 6 *A*). This change in ATP levels more closely resembles those in the more virulent strains shifted to 37°C (Fig. 6, dashed lines).

In the case of G184A and G222B, the shift to a higher temperature (40°C) results in a faster and deeper decline in ATP levels than the shift to 37°C (Fig. 6 *B*). The data show that ATP levels fall to 10% of the initial values in G184A and 30% in G222B. The ATP levels remain depressed for at least 60 min. These changes in ATP levels more closely resemble those in the Downs strain shifted to 37°C. The finding that ATP levels do not fall to zero in the virulent strains is consistent with the finding that they must be shifted to somewhat higher temperatures (41° and 43°C for G184A and G222B, respectively) to completely uncouple oxidative phosphorylation.

Respiration rates. The effects of different temperature shifts on respiration rates parallel their effects on oxidative phosphorylation and ATP levels. As shown in Fig. 7 *A*, the rate of decline in respiration rates of the Downs strain is less rapid at 34°C than at 37°C, and respiration rates fall to only 40% of initial levels. Respiration rates begin to increase 9–10 d after the temperature shift, about when yeasts appear in the cultures.

When G184A and G222B were shifted to a higher temperature (40°C), the patterns of change in respiration rates more closely resemble that in Downs strain shifted to 37°C (Fig. 7 *B*). It is particularly striking that when shifted to higher temperatures, both virulent strains show an extended dormant phase in which spontaneous respiration rates fall to zero. The decrease in respiration in response to the temperature elevation to 40°C appears to be more rapid in G184A than in G222B.

Electron transport components. Table II shows that the changes in concentrations of mitochondrial electron transport components in Downs shifted to 34°C are moderated compared with those when the strain is shifted to 37°C. The level of cytochrome aa_3 decreased to 66% of the initial level; cytochrome *b* to ~42% of the initial level; and cytochrome *c* fell to 46%. At the same time, the changes in the more virulent strains shifted to 40°C are similar to those in the Downs strain shifted to 37°C. In particular, cytochromes *c* and aa_3 are not detectable spectrophotometrically during stage 2 of the transition and cytochrome *b* falls to ~10% of the initial levels.

Rate of growth of yeast phase cells as a function of temperature

Downs, G184A, and G222B showed parallel differences in LD_{50}s for mice when tested as either early mycelial transformants or yeast phase cells. If pathogenicity is related to temperature sensitivity, then yeast phase cells of the three strains should show the same relative sensitivity to high temperatures as cells passing through the mycelial to yeast transition. To test this prediction, we obtained growth curves of yeast cells of the three strains at different temperatures (Fig. 8). At 35°C (Fig. 8 *A*), the curves for all three strains were indistinguishable and the doubling times were 13.5 h. At 37°C (Fig. 8 *B*), the curves for G222B and G184A remained indistinguishable, with doubling times of 13 h. However, Downs yeast showed a longer lag phase and a decreased growth rate (doubling time of 16.5 h) compared with the other strains. At 39°C (Fig. 8 *C*), the differences between Downs and the more virulent strains were even more pronounced. There was a longer lag period and a further decrease in growth rate (doubling time of Downs was 24 h compared with 13.5 h for G184A and G222B). The growth yield of Downs at the stationary phase was also significantly less than for G184A and G222B. At 41°C (Fig. 8 *D*), Downs did not grow at all. The other two strains continued to grow, but the rate of growth for

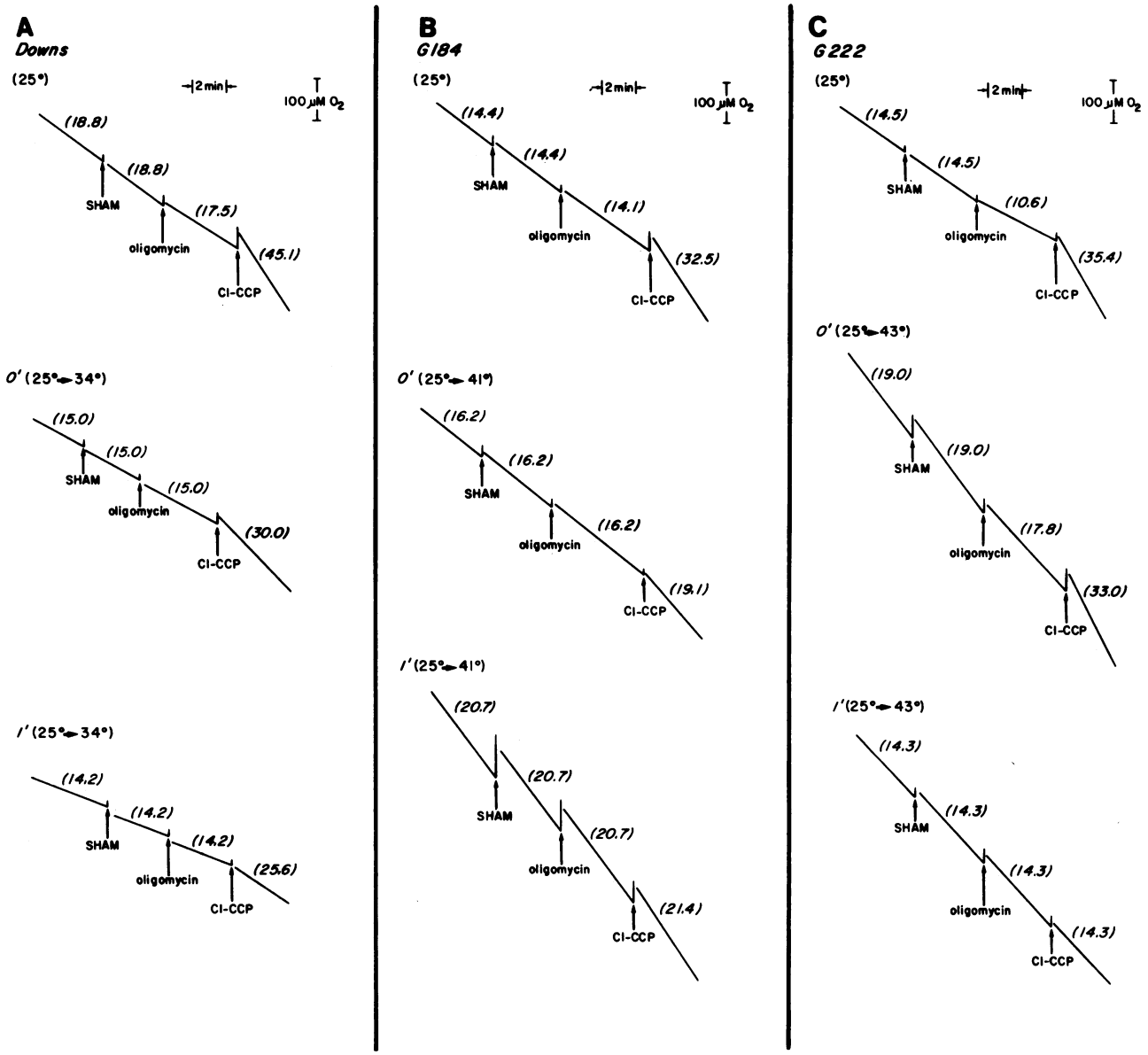

Figure 5. Oxygen electrode recordings of respiration of mycelial cells at 25°C, immediately after (0) and 1 min after shift up in temperature. The procedures were the same as for Fig. 1. (*A*) Downs; (*B*) G184A; and (*C*) G222B. The values in parentheses are rates of oxygen consumption (microliters of O_2 per hour per milligram dry weight). Additions were oligomycin (5 μg/ml) and Cl-CCP (0.5 mM). Salicylhydroxamic acid (0.2 mM) was added to inhibit the alternate oxidase. The experiment was repeated six times with independent cultures and essentially identical results were obtained.

G184A (doubling time of 33 h) was substantially slower than for G222B (doubling time of 30 h). G222B also grew to a higher cell number than G184A. These experiments were repeated three times on separate cultures with essentially the same results.

Discussion

The present work shows that the patterns of physiological changes during the mycelial to yeast transitions of more virulent strains of *H. capsulatum* are fundamentally similar to those previously reported for the less virulent Downs strain. In all cases, the triggering event appears to be a heat-related insult induced by the temperature shift up. The temperature shift leads to declines in ATP levels, respiration rates, and concentrations of electron transport components (stage 1). The cells then enter a stage of lower metabolic activity (stage 2), and finally recover and transform to yeast morphology (stage 3), presumably reflecting adaptation to growth at higher temperatures.

Our results show further that a major difference between the more virulent strains and the Downs strain is that the former are more resistant to temperature shifts. When the temperature is shifted from 25° to 37°C, as is ordinarily done to induce the morphological transitions, changes in ATP levels, respiration rates, and electron transport components are more severe in the Downs strain than in the virulent strains. The morphological transition of the Downs strain can be made to resemble those of the more virulent strains simply by shifting it to a lower temperature (34°C). At the same time, the morphological transitions of the more virulent strains can be made to resemble that of the Downs strain by shifting them to higher temperatures (40°–43°C). We note also that G184A is more sensitive to elevated

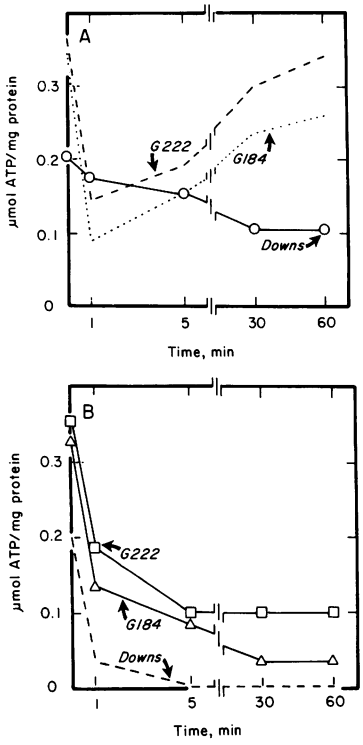

Figure 6. Effect of temperature shifts on intracellular ATP levels. The procedures were the same as for Fig. 2. (*A*) The Downs culture was shifted from 25° to 34°C. ATP levels for G184A and G222B shifted from 25° to 37° (Fig. 2) are indicated by dashed lines for comparison. (*B*) The G222B and G184A culture were shifted from 25° to 40°C. ATP levels for the Downs strain shifted from 25° to 37°C (Fig. 2) are indicated by dashed lines for comparison.

temperature than G222B. This difference is as predicted from the relative LD_{50}s of G184A and G222B.

The difference in temperature sensitivity of the three strains can be correlated with the temperature required for complete uncoupling of oxidative phosphorylation: 37°C in the Downs strain, 41°C in G184A, and 43°C in G222B. When the morphological transitions occur at lower temperatures, 34°C for Downs and 37°C for G184A and G222B, respiration is partially uncoupled, which is judged by the degree to which respiration is stimulated by Cl-CCP. Considered together, these findings suggest that partial or complete uncoupling of oxidative phosphorylation is an early event and very possibly the primary event in the morphological transition in all three strains. Although uncoupling of oxidative phosphorylation is by itself sufficient to account for the decline in ATP levels in stage 1 of the transition, our results leave open the possibility that other heat-related insults contribute to this decline.

The differences in temperature required for uncoupling of oxidative phosphorylation could reflect differences in lipid or

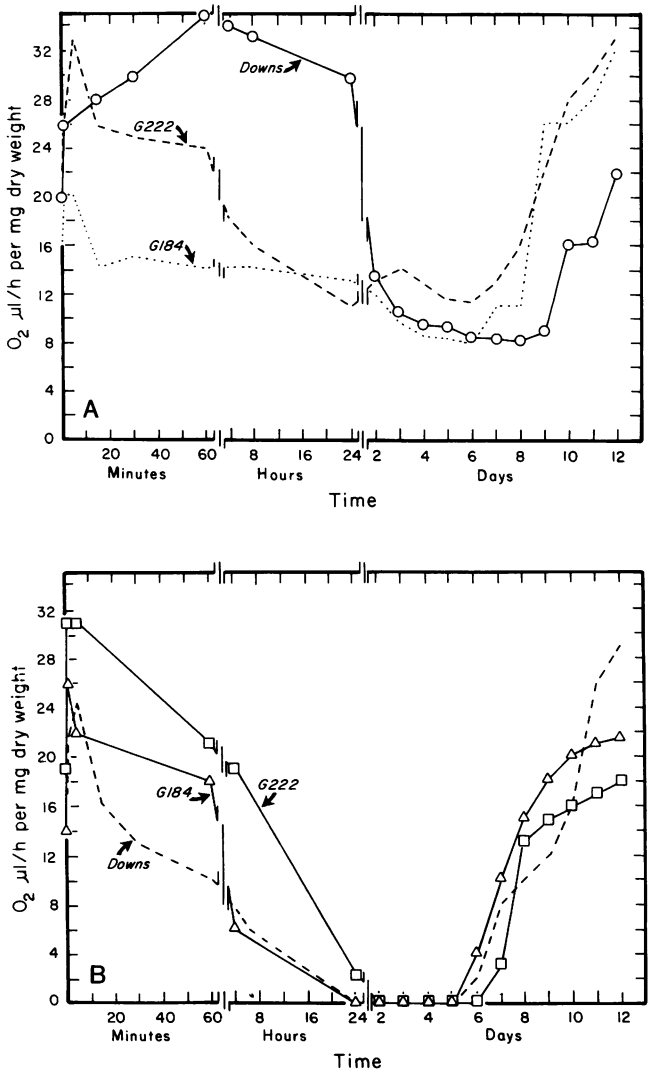

Figure 7. Respiration rates at various points during the mycelial to yeast phase transition. The experiment was initiated by shifting mycelia from 25° to 34° to 40°C. The procedures were the same as for Fig. 3. (*A*) The solid line shows respiration rates of Downs strain obtained at various times after temperature shift from 25° to 34°C. The dashed lines are the respiration rates of G184A and G222B shifted from 25° to 37°C. These are transcribed from Fig. 3. (*B*) The solid lines are respiration rates of G184A and G222B switched from 25° to 40°C. The dashed line shows respiration rates of the Downs strain after the switch from 25° to 37°C. These are transcribed from Fig. 3.

*Table II. Concentrations of Cytochromes in Mitochondria Isolated from Transforming Cells after Temperature Shifts from the 25°–34°C (Downs) or the 25°–40°C (G184A and G222B)**

	Downs			G184A			G222B		
Days	cyt aa_3	cyt *b*	cyt *c*	cyt aa_3	cyt *b*	cyt *c*	cyt aa_3	cyt *b*	cyt *c*
0	0.15	0.26	0.13	0.23	0.20	0.16	0.16	0.26	0.19
1	0.07	0.11	0.06	ND‡	0.06	0.04	ND	0.03	ND
2	0.10	0.16	0.10	ND	0.04	ND	ND	0.03	ND
3	0.10	0.15	0.11	ND	0.02	ND	ND	0.02	ND
12	0.22	0.20	0.14	0.07	0.15	0.10	0.10	0.19	0.12

cyt, cytochrome. * Cytochrome concentrations were calculated from low temperature difference spectra of dithionite-reduced minus oxidized mitochondria. The values are expressed as nanomoles per milligram mitochondrial protein. ‡ ND, nondetectable.

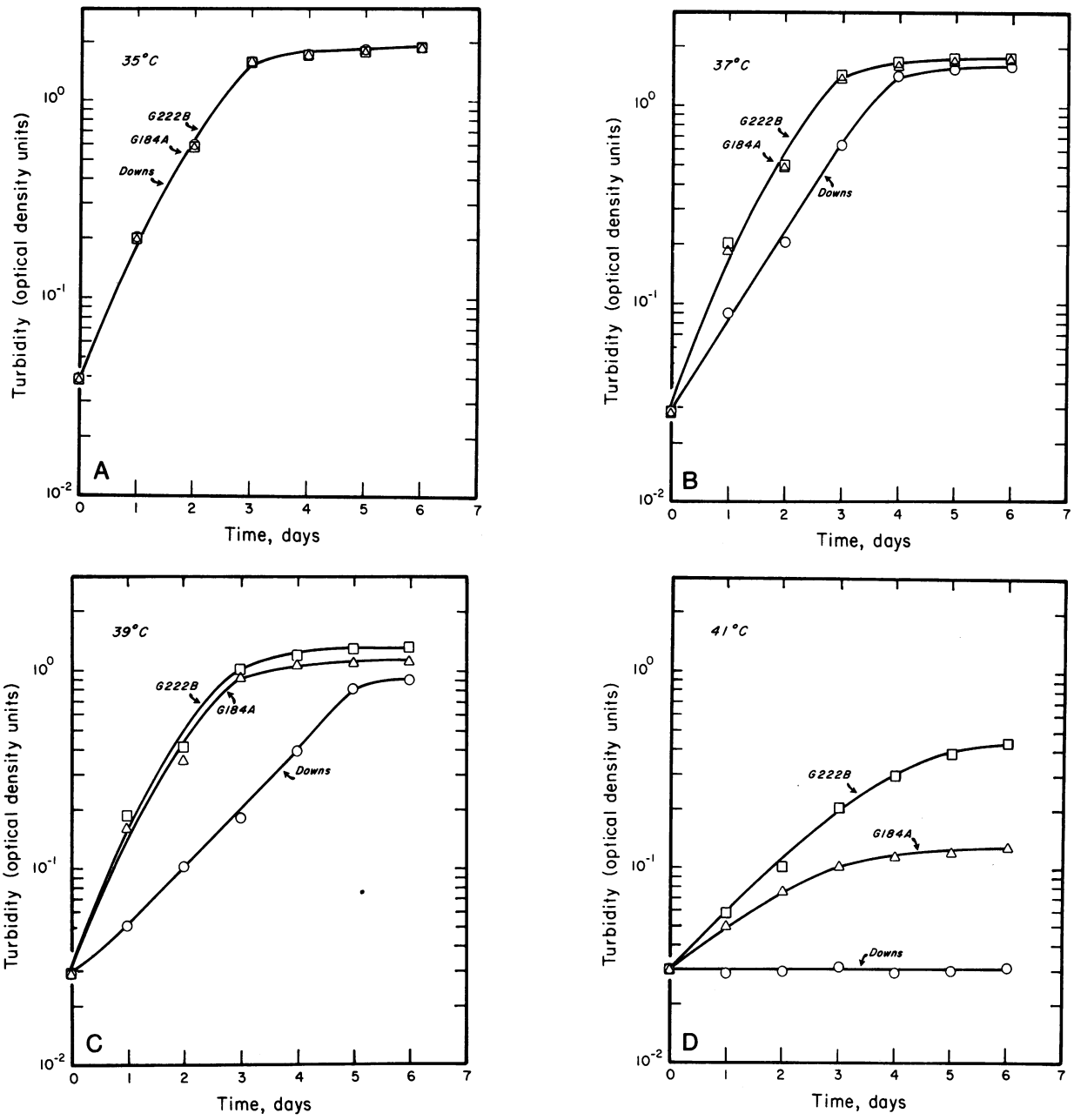

Figure 8. Growth curves of Downs, G184A, and G222B yeast cells at 35°C (*A*), 37°C (*B*), 39°C (*C*), and 41°C (*D*). Initial innocula of 1 × 10^6 cells/ml for each of the cultures were obtained by appropriate dilutions of growing cultures. Cell densities were quantitated by hemacytometer counts and optical density readings at 650 nm with a Bausch and Lomb-Spectronic 20. 0.4 OD U on the ordinate scale in the linear portion of the growth curve corresponds to 3.2 × 10^6 cells/ml. These experiments were repeated three times on different cultures with essentially the same results.

protein compositions of mitochondrial membranes in the strains, or more specific differences in the composition of the ATP synthetase complex. The strain differences among the strains might also be specified by a small number of nuclear and/or mitochondrial genes, since digest patterns of messenger transfer DNA and ribosomal DNA of each of the strains used in our study show that they belong to a different class (15). In the yeast form of the Downs strain, respiration is tightly coupled to oxidative phosphorylation at 37°C. Therefore, the mycelial to yeast transition must include some alteration in the mitochondrial membrane or ATP synthetase complex that permits respiration to remain coupled at 37°C.

When mycelia of Downs are incubated at 37°C the transition is prolonged, and cysteine or other suylfhydryl groups are required for survival (4). Previous studies with the Downs strain showed that cysteine is required during stage 2 for the operation of shunt pathways which permit electron transport to bypass inhibited portions of the cytochrome system. These shunt pathways appear to depend upon residual cytochrome oxidase, which appears to be present below levels that can be detected spectrophotometrically. In contrast, mycelia of G184A and G222B transform rapidly at 37°C and do not require any cysteine or other sulfhydryls in the medium for survival at this temperature. However, when the temperature is raised to 40°–43°C, these

strains become dependent on cysteine-activated shunt pathways during stage 2 (our unpublished observations). The results for the more virulent strains show that cysteine is required to complete the transition only under conditions in which electron transport components are severely depleted. Therefore, it is likely that the primary, if not the only function of cysteine in stage 2 of the transition, is to activate the respiratory shunt pathways.

We previously pointed out that there may be a relationship between temperature shifts that trigger the morphological transition and heat shock (7). Heat shock proteins are induced in all the strains in response to temperature shifts (16). These proteins may contribute to the survival of the organism after the initial temperature shift. It seems unlikely that heat shock proteins per se are directly involved in morphogenesis. However, it is possible that heat shock proteins and morphogenic proteins are under similar control and are induced in parallel early in the transition. An intriguing possibility is that proteins which function in morphogenesis have heat shock promoters.

Recently, Van Der Ploeg and co-workers (17) have reported that a heat shock response also occurs after the transfer of the parasitic protozoa *Trypanosoma brucei* and *Leishmania major* from the temperature of their poikilothermic insect vectors (25°C) to that of their homeothermic mammalian host (37°C). This may be another example of a differentiation process involved in pathogenicity that is triggered by a temperature shift and resultant heat shock response.

The mycelial phase of *H. capsulatum* is the normal morphology for growth in soil (1). It has been proposed that only the yeast phase is pathogenic (18). This hypothesis is supported by the histopathology of the disease, in which only the yeast phase is seen inside of macrophages in infected tissues. More recent experiments in our laboratory showing that inhibition of the mycelial to yeast transition by *p*-chloromercuriphenylsulfonic acid renders the virulent strains nonpathogenic for mice provide stronger evidence that only the yeast phase is pathogenic (our unpublished observations). Because conversion to the yeast phase appears to be required for progressive infection, it is likely that the rapidity and efficiency of the transformation and/or the subsequent growth of the yeast phase are important determinants of virulence. This conclusion is supported by the correlation we observed between sensitivity to elevated temperature of the mycelial and yeast phases and virulence for mice of all three of the strains we used in our experiments. Therefore, the hypothesis that a heat-related insult is a common element in the mycelial to yeast transition, and that the efficiency of the transformation and subsequent growth of yeast depend on the sensitivity to elevated temperatures of all three strains provides an explanation for the low level of virulence of the Downs strain of *H. capsulatum* and the difference in virulence between G184A and G222B. Note that our results leave open the possibility that temperature sensitivity and pathogenicity are not causally related to each other (our unpublished observations), but are both controlled by a third factor (e.g., membrane lipids).

The normal body temperature of mice is between 37° and 38.1°C (19), sufficient to exploit the differences in the transition and growth of yeast among the strains. We would predict that all three *H. capsulatum* strains would be less virulent at higher body temperatures. The resistance of birds, which have a body temperature > 40°C, to *H. capsulatum* infection supports this notion. In the same way, the Downs strain should be more virulent in a host with a body temperature below 37°C.

Our work also suggests a possible strategy to develop effective live vaccines for infections with dimorphic fungi. Infection with temperature-sensitive mutants that are similar to the Downs strain might provide the most effective protection against infection with the lowest chance of toxicity.

Acknowledgments

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