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Aspergillus Hyphae in Infected Tissue: Evidence of Physiologic Adaptation and Effect on Culture Recovery

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Microbiologic cultures of fungi are routinely incubated at ambient temperatures in room air, and the rate of recovery of *Aspergillus* species from clinical specimens is poor. Failure of current culture methods to mimic the physiologic temperature and low-oxygen environment found in hypha-laden infected tissue may underlie this poor recovery. Experiments were performed to compare the recovery of *Aspergillus* spp. incubated at 35°C in 6% O_2–10% CO_2 with that at 25°C in room air. The samples tested included *Aspergillus*-infected tissue specimens from a dog model and human autopsies, experimental anaerobically stressed *Aspergillus* inocula, and 10,062 consecutive clinical specimens. Culture at 35°C in 6% O_2–10% CO_2 significantly enhanced the recovery of *Aspergillus* spp. from the infected autopsy tissue samples. Incubation at 35°C alone resulted in approximately 10-fold-improved culture recovery from the experimentally stressed hyphae, and the 6% O_2–10% CO_2 atmosphere independently favored growth under temperature-matched conditions. Finally, incubation at 35°C (in room air) improved the overall recovery of *Aspergillus* spp. from clinical specimens by 31%. Culture at 35°C in a microaerobic atmosphere significantly enhances the recovery of *Aspergillus* spp. from various sources. *Aspergillus* hyphae growing in infected tissue appear to be adapted to the physiologic temperature and hypoxic milieu.

Invasive aspergillosis (IA) is the most common opportunistic invasive mold infection in the United States. This infection is associated with high mortality in heavily immunosuppressed patients, such as those with leukemia or marrow transplants (12). In addition to the mediocre activity of antifungals, the poor prognosis of IA is related to suboptimal and late diagnoses (3, 9, 12, 26, 27). Although various diagnostic tests have been developed for IA, such as *Aspergillus* galactomannan and PCR assays, (15, 18), culture isolation of the *Aspergillus* organism along with clinical and radiologic correlation remains the current standard (1).

Several studies have shown that the use of routine clinical mycology methods results in a low rate of *Aspergillus* recovery from clinical material, yet the reasons for this poor recovery of aspergilli remain obscure (2, 17, 25, 26). In the current fungal culture method, most media incubate at room temperature (25°C) in ambient air (11, 13, 16, 23). Such ambient incubation was introduced several decades ago when IA occurred infrequently. Although ambient incubation conditions are known to support the growth of a wide variety of yeasts and molds from environmental sources, it is unclear whether these conditions are optimal for *Aspergillus* recovery from infected tissue.

The histopathology of IA typically shows numerous *Aspergillus* hyphae intermingled with tissue necrosis, inflammatory infiltration, thrombosis, and vascular invasion by the fungus (4). Conidia, while present in the environment and seen during aerobic culture of the organism, are absent in tissue infected by IA (4). We hypothesize that invading *Aspergillus* hyphae adapt to the physiologic temperature and hypoxic milieu of the host tissue. When this adapted organism is suddenly transferred to an artificial culture medium and incubated under ambient air and temperature, however, it is unable to grow under the new conditions. In this study, using *Aspergillus*-infected tissue, experimentally prepared anaerobically stressed *Aspergillus* inocula, and clinical specimens, we demonstrated that incubation under conditions intended to simulate the physiologic environment of tissue (35°C in a 6% O_2–10% CO_2 atmosphere) indeed resulted in improved growth and recovery of *Aspergillus*.

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MATERIALS AND METHODS

Fungal strains. *Aspergillus fumigatus* strains consisted of American Type Culture Collection strain 90906 and seven *A. fumigatus* clinical isolates. In addition, we used one clinical isolate each of *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*, and *Aspergillus versicolor*.

Culture media. Culture media included Sabouraud-dextrose-agar with Emmons modification (SDAE) plates, Mycosel tube medium containing chloramphenicol and cycloheximide (MCC), Sabouraud-dextrose-agar tubes (SDT), and brain-heart-infusion tubes (BHI) with gentamicin and chloramphenicol. Media were obtained commercially from Becton Dickinson Microbiology Systems.

Infected lung tissue. Human lung tissue specimens were obtained as part of a routine autopsy fungal culture from two patients who died from IA. Samples were transported at room temperature in a sterile container with no additives. The specimens were processed within 1 to 2 h of autopsy. The tissue was homogenized in 1 to 2 ml of sterile saline with a tissue grinder. Equal volumes were planted on SDAE plates and incubated under standard conditions at 25°C in ambient air or under experimental conditions at 35°C in 6% O_2–10% CO_2. Lung tissue was also derived from a dog model of IA (U.S. patent 64448720). Briefly, a flexible bronchoscope was used to introduce agarose-beaded *A. fumigatus* conidia into the middle lobe of the right lung of immunosuppressed beagle dogs. Lung specimens were obtained from untreated control dogs (~10 g, days

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4 to 10) and transported in sterile containers at 25°C within 1 h of necropsy. The tissue was disrupted by the procedure above. Equal volumes (six 50-μl spots) were planted on SDAE plates or other media as described in Results and incubated at 25°C in ambient air or at 35°C in 6% O_2–10% CO_2.

Preparation of anaerobically stressed experimental inocula. The *Aspergillus* strains were grown for 7 to 14 days on potato-dextrose agar, and conidia were harvested in sterile 0.9% NaCl without dimethyl sulfoxide, glycerol, or Tween 80. Large particles were settled for 10 min, and the supernatant was harvested, aliquoted, and frozen at −70°C. To prepare experimental inocula, 0.1 ml of thawed aliquot ($\sim 4 \times 10^6$ conidia) was added to RPMI 1640 medium with 0.166 M MOPS (morpholinepropanesulfonic acid) to pH 7.2 (R6504; Sigma, St. Louis, Mo.). The liquid cultures were incubated at 35°C in a "Campy" atmosphere (BBL271045 Campy-Pac; Becton Dickinson Microbiology Systems) in 25-mm tissue culture flasks; this approximates 6% O_2–10% CO_2. We observed that Campy gas allows germination and hyphal growth without sporulation. After 48 h, the flasks were transferred to an anaerobic environment (BBL271040, Ana-Pac; Becton Dickinson Microbiology Systems) at 35°C for an additional 48 to 72 h, simulating an anaerobic lesion. Cultures with macroscopic or microscopic sporulation were discarded. Hyphae were harvested in 1 ml of sterile saline and immediately ground with 20 strokes with a 50-ml tissue homogenizer (3500SA; Kendall), which resulted in 1 to 5 nucleated cells per fragment. A total of 5 ml of sterile saline was added, and large particles were settled for 2 to 3 min. The top half of the suspension was then transferred to an empty tube and mixed; subsequently, serial 10-fold dilutions were made in sterile saline. Each dilution provided paired inocula for plates studied under test and control incubation conditions.

Clinical specimens. From November 2001 to October 2003, 10,062 clinical specimens were submitted for fungal culture; 90% were sputum, bronchial wash, or lavage specimens. Samples were inoculated on five media and were divided into groups for room air incubation at 25 or 35°C. Growth results were grouped according to 25 versus 35°C growth for each medium and analyzed for recovery of *Aspergillus* species. The 25°C media were SDT, MCC, and SDAE, whereas the 35°C media were BHI and SDAE. Review of records was conducted under M. D. Anderson protocol LAB02-200.

Statistics. Categorical data were analyzed by the chi-square test, Fisher's exact test, or the exact, two-sided value for the McNemar test with StatXact 4 software (Cytel Software Corp.) (21). Scalar values were compared using Student's *t* test for paired results.

RESULTS

Infected tissue. Infected tissue samples obtained from two dogs and two human autopsies were cultured under the modified microaerobic conditions (35°C in 6% O_2–10% CO_2) and ambient conditions. Each specimen was ground, and paired inoculation spots on SDAE plates were made for the different conditions and incubated for 1 week. For the 20 inoculation spots from dogs (10 spots per dog, 5 spots per condition), no growth was observed on the ambient plates, whereas 6 spots (from both dogs) grew *Aspergillus* spp. under the modified microaerobic conditions (0 of 10 versus 6 of 10; $P = 0.005$; Fisher's exact test). The human specimens were inoculated into 15 spots; only 4 spots (all from one specimen) showed growth under the ambient conditions in comparison with the growth of all of the spots under the modified microaerobic conditions (4 of 15 versus 15 of 15; $P = 0.0001$). These results suggest that incubation at 35°C in 6% O_2–10% CO_2 favors the growth of *Aspergillus* obtained from infected tissue. These conditions are similar to the physiologic temperature and probable hypoxic and hypercapnic tissue microenvironment typical of thrombotic lesions in IA. Thus, *Aspergillus* hyphae in infected tissue are likely adapted to the pathophysiologic milieu of the host.

Experimental *Aspergillus* inoculum. To further investigate the effect of the incubation temperature and atmosphere, experimentally stressed *Aspergillus* inocula were prepared to simulate the hyphal state of the organism in infected tissue. Serial 10-fold dilutions of the experimental inocula derived from five common *Aspergillus* spp. (*A. fumigatus*, *A. flavus*, *A. terreus*, *A. niger*, and *A. versicolor*) were tested. The most dramatic effect on growth was seen at the limiting dilution, which typically corresponds to 10 to 100 stressed hyphal fragments per ml in the inoculum. As shown in Fig. 1, incubation at 35°C in 6% O_2–10% CO_2 consistently resulted in quick, robust growth of all of the *Aspergillus* spp. In contrast, the plates incubated at 25°C in room air showed no growth of any of the species, even after prolonged incubation, which suggests loss of hypha-inoculum viability. In separate experiments of paired *A. fumigatus* anaerobically stressed inocula, all 23 inoculated spots that were incubated at 35°C in 6% O_2–10% CO_2 showed growth, whereas none of the spots incubated under ambient conditions grew after 7 or more days ($P < 0.0001$; Fisher's exact test). Therefore, *Aspergillus* hyphae that were experimentally preconditioned via anaerobic exposure preferentially grew at 35°C in 6% O_2–10% CO_2.

The effects of the atmosphere and temperature were assessed individually in separate experiments. Twelve paired inocula were cultured at a fixed temperature of 25°C: one group of 12 inocula were cultured in room air, and the other group of 12 inocula were cultured in 6% O_2–10% CO_2. The room air group showed a mean colony diameter of 1.38 cm with a standard deviation of 1.15 cm, while the microaerobic group had a mean colony diameter of 3.06 cm with a standard deviation of 0.56 cm ($t = 7.35$; $P < 0.001$). This demonstrates better growth of *Aspergillus* hyphae with microaerobic incubation. The effect of the incubation temperature was more dramatic. As shown in Table 1 (two leftmost columns), room air plates incubated at 35°C exhibited consistently better culture recovery of the organism than plates incubated at 25°C in inocula from 10 to 10^4 hyphae/ml. Table 3 shows improved recovery of stressed hyphae at 35°C when compared to both 25 and 30°C conditions ($P = 0.005$ and 0.046, Fisher's test). Results under 25 versus 30°C conditions were not significant ($P = 0.6$).

The culture recovery of stressed hyphae was also compared with that of unstressed hyphae and conidia (Table 1). For the unstressed hyphae and conidia, identical recovery rates were obtained at both 25 and 35°C (in room air), and full recovery (16 of 16 inoculated spots) was seen at 10^3/ml. In contrast, for the stressed hyphae, full recovery was seen at 10^4/ml in the 35°C incubation group and 10^5/ml in the 25°C group. Thus, compared with unstressed hyphae and conidia, stressed hyphae had an approximately 10-fold reduction in viability when cultured at 35°C and a further 10-fold reduction (total, 100-fold reduction) when cultured at 25°C.

The dramatic negative effect imposed by incubation at 25°C led us to hypothesize that stressed *Aspergillus* hyphae were energy starved and thus unable to adapt to the sudden transition to 25°C. Two experiments were carried out to test this hypothesis. In one experiment (Table 1), stressed hyphae were held at 4°C for up to 24 h before being cultured at 35°C. The resulting culture recovery was similar to that seen with straight 35°C incubation. This result suggests that the low metabolic activity at 4°C preserved the viability of the organism. In another experiment (Table 2), crossover incubation was performed at 25 and 35°C. While straight incubation at 35°C produced the best recovery, the incubation at 35°C for 24 h

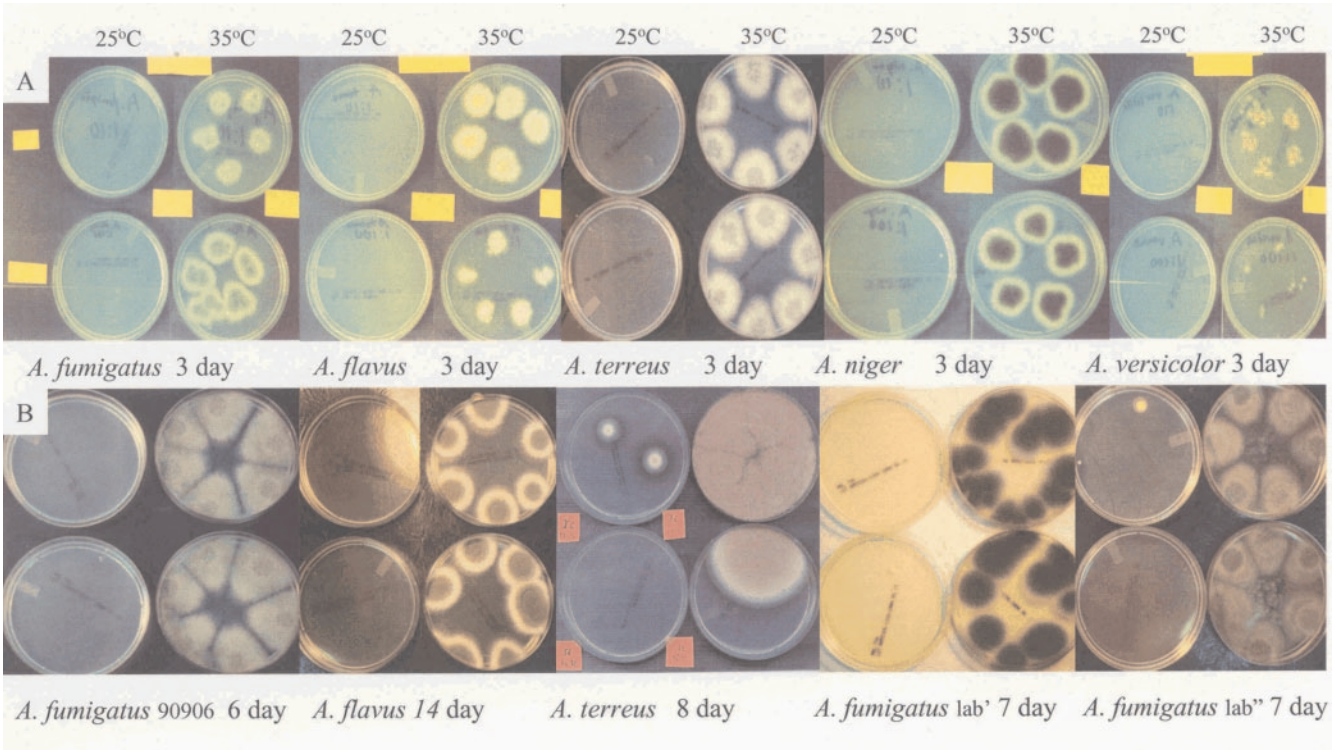

FIG. 1. Culture of experimentally stressed *Aspergillus* hyphae at 35°C in 6% O_2–10% CO_2 versus 25°C in room air for 3 days (A) and up to 14 days (B). Paired inocula for each dilution are shown side by side. The TIFF file images were produced with Corel Photo House software.)

plus further incubation at 25°C produced a better trend toward recovery than did the incubation at 25°C for 24 h plus further incubation at 35°C or straight incubation at 25°C. Therefore, while 25°C incubation was acceptable for unstressed hyphae and conidia, it was unsuitable for culture of hyphae that were preconditioned (stressed) via exposure at 35°C in an anaerobic atmosphere.

In addition to our standard SDAE plates, a variety of media and supplements were also tested, with medium differences having little impact on the growth of stressed hyphae (data not shown). The only rescue effect observed was with the incorporation of nitrate and pyruvate into SDAE (Table 2) not significant (NS).

Clinical samples. Analysis of the results of culture of clinical specimens was also revealing. Logistical considerations allowed only comparison of incubation in room air at 25 and 35°C; a microaerophilic atmosphere was not used. Of the 10,062 consecutive clinical specimens inoculated on 50,310 culture plates or tubes, 344 (3.4%) showed growth of an *Aspergillus* species from 837 *Aspergillus*-positive media. The media incubated at 25°C (SDT, MCC, and SDAE) had an *Aspergillus*-

TABLE 1. The effects of temperature and preconditioning on culture of experimental *Aspergillus* inocula[a]

Approximate no. of hyphal fragments[b]	Stressed hyphae at:				Unstressed hyphae at:		Unstressed conidia at:	
	35°C	25°C	35°C with preconditioning at 4°C		35°C	25°C	35°C	25°C
			4 h	24 h				
10^5	16/16	16/16	16/16	16/16	16/16	16/16	16/16	16/16
10^4	16/16	5/16	16/16	15/16	16/16	16/16	16/16	16/16
10^3	6/16	0/16	9/16	8/16	16/16	16/16	16/16	16/16
10^2	3/16	0/16	1/16	2/16	8/16	10/16	7/16	6/16
10^1	2/16	0/16	2/16	0/16	1/16	1/16	2/16	0/16

[a] Growth versus no growth is shown as the fraction of 16 inoculation spots on SDAE plates for each dilution and condition after 14 days of incubation (plates had eight spots of 12 μl each). The duration of preconditioning at 4°C was for 4 or 24 h, as indicated.

[b] The initial inoculum contained approximately 10^7 hyphal fragments or conidia per ml ("Stressed hyphae" columns). In separate experiments, conidial and aerobic hyphal fragment concentrations were adjusted to approximately 10^7/ml and were inoculated at the two incubation temperatures for comparison ("Unstressed hyphae," "Unstressed conidia" columns).

TABLE 2. The effects of temperature crossover and nitrate and pyruvate supplementation on culture of stressed *Aspergillus* hyphae[a]

Approximate no. of hyphal fragments[b]	Culture condition				
	35°C	35°C × 24 h, then 25°C	25°C × 24 h, then 35°C	25°C	Supplement at 25°C[c]
10^5	24/24	24/24	24/24	24/24	24/24
10^4	24/24	24/24	24/24	24/24	24/24
10^3	24/24	24/24	23/24	20/24	23/24
10^2	6/24	4/24	6/24	3/24	6/24
10^1	5/24	2/24	0/24	0/24	1/24
10^0	3/24	0/24	0/24	0/24	0/24

[a] Growth versus no growth is shown as a fraction of 24 inoculation spots on SDAE plates for each dilution and condition after 14 days of incubation.

[b] The initial inoculum contained approximately 10^7 hyphal fragments per ml.

[c] Supplementation with 10 mM $NaNO_3$ and 10 mM potassium pyruvate.

positive rate of 1.5% (447 of 30,186), whereas the media incubated at 35°C (BHI and SDAE) had an *Aspergillus*-positive rate of 1.9% (390 of 20,124), a 31% improvement ($\chi^2 = 14.2$; $P = 0.0002$). However, we were interested in the media with presumed exposure to *Aspergillus* in the specimen; therefore, only the media associated with *Aspergillus*-positive specimens were analyzed further. Among 344 positive specimens with a total of 1,720 inoculated media, *Aspergillus* spp. were recovered from 447 of 1,032 (43.3%) media (SDT, MCC, and SDAE) incubated at 25 and 390 of 688 (56.7%) media (BHI and SDAE) incubated at 35°C, a 31% increase in the rate of recovery with 35°C incubation ($\chi^2 = 26.0$; $P < 0.0001$). When the relatively inhibitory Mycosel medium was excluded, 353 of 688 (51.3%) media (SDT and SDAE) incubated at 25°C showed *Aspergillus* recovery, which was still significantly lower than the recovery rate at 35°C (56.7%) ($\chi^2 = 4.00$; $P < 0.05$). When the 12 *Aspergillus*-positive autopsy samples were analyzed, 14 of the 24 (58.3%) media (SDT and SDAE) that were incubated at 25°C showed growth in contrast with 20 of the 24 (83.3%) media (BHI and SDAE) incubated at 35°C. This represented a 43% increase in the rate of recovery. Fourteen *Aspergillus* isolates were recovered at both temperatures, 6 were recovered only at 35°C, none were recovered only at 25°C, and 4 were negative at both temperatures ($P = 0.031$; exact, two-sided, one-degree-of-freedom McNemar test).

TABLE 3. Recovery of experimental *Aspergillus* inocula at 25, 30, and 35°C[a]

Approximate no. of hyphal fragments[b]	Stressed hyphae at:		
	35°C	30°C	25°C
10^5	32/32	32/32	32/32
10^4	26/32	22/32	17/32
10^3	10/40	3/40	1/40
10^2	1/40	0/40	0/40
10^1	0/40	0/40	0/40

[a] Growth versus no growth is shown as the fraction of 16 inoculation spots on SDAE plates for each dilution and condition after 9 days of incubation (plates had eight spots of 12 μl each). Dilutions with 10^3 and 10^2 fragments showed 11 of 80 spots for 35°C versus 3 of 80 at 30°C, or 1 of 80 at 25°C; $P = 0.046$ and 0.005, respectively, by Fisher's exact test.

[b] The initial inoculum contained approximately 10^7 hyphal fragments per ml.

DISCUSSION

Aspergillus species are nonfastidious mesophiles that grow in a wide variety of environments. They are obligate aerobic organisms and generally do not grow under anaerobic conditions, although enhanced germination of conidia may occur at lower O_2 levels (19, 22). The optimal growth temperatures are 37 to 45°C for *A. fumigatus*, 35 to 40°C for *A. terreus*, 25 to 37°C for *A. flavus*, and 17 to 42°C for *A. niger* (22). *Aspergillus* spp. are able to assimilate minerals from minimal medium and survive on simple carbon and nitrogen sources with no vitamin requirements (7, 8, 19, 20, 22). Thus, given their robust growth in the environment, the poor clinical yield from infected tissue seems paradoxical (25). The opportunistic nature of infections by these common saprobes argues against the evolution of a requirement for host-specific factors.

In the present study, we demonstrated that at least part of the poor culture yield of *Aspergillus* spp. was due to suboptimal laboratory methodology. While ambient air and temperature were adequate for culture of unstressed *Aspergillus* hyphae and conidia, these conditions were inadequate for culture of stressed *Aspergillus* hyphae from infected tissue or experimental anaerobic inocula. Simply incubating these inocula at 35°C improved recovery by ~10 fold, however. Additionally, microaerobic incubation offered a further modest improvement. Incubating our clinical fungal cultures at 35°C in room air resulted in an improvement in the recovery of *Aspergillus* spp. from 5 to 43%. Thus, we recommend incubation at 35°C as a practical approach to the recovery of *Aspergillus* from clinical fungal culture, with the exception of hair, skin, and nail cultures. The effects of microaerobic atmosphere or nitrate-pyruvate are only minor by comparison. One disadvantage to the higher temperature format is that growth is rapid, and cultures may need to be examined more frequently to prevent overgrowth of multiple molds. An additional concern for 35°C incubation is the possibility of decreased recovery of other molds. Our preliminary data suggest that this is not the case for *Zygomycetes* organisms, *Scedosporium* species, or *Fusarium* species (data not shown). Further evaluations for dermatophytes are in progress. It should be emphasized that MCC medium is known to partially inhibit *Aspergillus* and should be excluded from the setup of sterile source specimens. Furthermore, poor growth in MCC as well as enhanced growth due to 35°C and other medium effects may contribute to the 31% increase in recovery. When MCC was removed, only a 5.4% improvement in overall recovery was seen; however, autopsy material remained improved by 43%. It should be noted that growth enhancement due to BHI, independent of temperature, also cannot be excluded based on our clinical data set. However, a large battery of enriched media failed to impact recovery. The clinical data show that variation in media choice as well as conditions of incubation have an important impact on *Aspergillus* recovery in the clinical setting.

In addition, our findings suggest that the physiologic state of the stressed *Aspergillus* hyphae was different from that of the unstressed hyphae and conidia. Although fragile, as evidenced by the requirement of a higher inoculum concentration to obtain full culture recovery (Table 1), the anaerobically stressed hyphae grew much better at 35 than at 25°C; however, these temperatures supported equally good growth of conidia. Furthermore, unstressed aerobic hyphae produced in 6% O_2 showed no difference in recovery between 25 and 35°C. As shown in Table 3, 35°C recovery of stressed hyphae was significantly superior to both 25 and 30°C. Thus, a 5 to 10°C gap seems formidable for stressed hyphae to overcome. Our serial dilution experiments supported the notion that these stressed hyphae are energy starved; when cultured at the lower temperature (25°C), they were unable to adapt metabolically to the transition from 35 to 25°C. A 24-h incubation at 35°C (in room air) followed by incubation at 25°C showed a trend toward enhanced recovery (NS), possibly by providing the needed adaptation for growth at 25°C (Table 2). Alternatively, supplementation of the culture medium with nitrate (but not ammonia) or pyruvate provided some rescue of stressed hyphae, although the effect was weak. Nitrate reductase from *Aspergillus* spp. has been well described (6) but is generally associated with assimilation (5). However, our observation that nitrate

rescued anaerobic hyphae suggests that nitrate can serve as a terminal electron acceptor in place of oxygen in respiration. A similar function occurs with members of the *Enterobacteriaceae* family (10) and has been suggested for *Fusarium* spp. (24). In addition, pyruvate is a central molecule in anaerobic fermentation. Although most *Aspergillus* spp. are not fermentative, a partial fermentation pathway has been suggested by the presence of pyruvate decarboxylase in *Aspergillus nidulans* (14). Our data hinted that this may be true for *A. fumigatus* as well. Thus, preserving energy charge (35°C incubation, 4°C holding, nitrate reduction, and pyruvate fermentation) enhanced the survival of anaerobically stressed *Aspergillus* inocula. These findings, in concert with our findings with autopsy tissue and clinical samples, support the inference that *Aspergillus* spp. growing in tissue are adapted to the local microenvironment.

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