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Characterization of *Aspergillus sydowii* (Thom et Church), a fungal pathogen of Caribbean sea fan corals

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Abstract

In the Caribbean, the fungus *Aspergillus sydowii* is currently causing an epizootic among sea fan corals (*Gorgonia* spp.). To elucidate potential factors that may have facilitated the emergence of this disease, we characterized and compared temperature requirements, susceptibility to coral crude extracts, and metabolic profiles of pathogenic (marine) and non-pathogenic (terrestrial) strains of *A. sydowii*. Growth of all *A. sydowii* strains were observed at all temperatures tested (22–36 °C) with an optimum of approximately 30 °C. Sea fan crude extracts inhibited growth of *A. sydowii* but were less effective at higher temperatures. Thus, temperature is likely to have a strong influence on the dynamics of the *Gorgonia–Aspergillus* interaction by promoting the growth of the pathogen while reducing the efficacy of host resistance. Metabolically, marine *A. sydowii* strains pathogenic to sea fans were distinct from non-pathogenic terrestrial strains.

Introduction

Diseases can have a major impact on populations and communities in the tropical marine environment (Gladfelter, 1982; Lessios, 1988; Hughes, 1994; Clarke, 1996; Aronson & Precht, 1997). Many marine diseases are influenced by environmental factors, especially changes in temperature, and there is concern about how diseases of marine organisms will be affected if the current warming trend continues (Goreau et al., 1998; Harvell et al., 1999). For example, outbreaks of cholera have been associated with climatic fluctuations because of the link between the pathogen, *Vibrio cholerae*, and plankton (Colwell, 1996). Another example is the oyster parasite *Perkinsus marinus* which underwent a range extension from the Chesapeake Bay northward to the Gulf of Maine due to warming winters which decreased over-winter mortality (Ford, 1996). Thus, in order to predict the impact of a disease, it is important to examine host-pathogen interactions across the range of environmental conditions in which they are found.

In the Caribbean, there is an ongoing epizootic among sea fan corals (*Gorgonia ventalina* and *G. flabellum*) caused by the fungus, *Aspergillus sydowii* (Smith et al., 1996; Nagelkerken et al., 1996, 1997; Geiser et al., 1998). The symptoms of this disease, aspergillosis, include lesions, galling, and purpling of the tissue, which can lead to death of the colony (Smith et al., 1998). In 1995, aspergillosis was present throughout most of the Caribbean including the Florida Keys (Nagelkerken et al., 1997).

The fungus *Aspergillus sydowii* is a mesophilic soil saprobe which is also known as a food contaminant and occasionally as an opportunistic pathogen of humans (e.g. Olutiola & Cole, 1977; Rinaldi, 1983; Smith, 1989; Gharelb & Nour El Dein, 1990). As with many opportunistic diseases, its pathogenicity is dependent on the genetic composition and immune status of the host, and also on the extent and duration of

Table 1. Aspergillus sydowii strains used in this study

Abbrev.	Strain	Habitat	Origin
REF	USDA-NRLL224	Terrestrial	Silk
KW	Key West, Florida, U.S.A.	Marine	Sea fan
SA	Saba, Netherland Antilles	Marine	Sea fan
SS	San Salvador, Bahamas	Marine	Sea fan

the exposure (Rinaldi, 1983). For aspergillosis, the health of the host is particularly important. For instance, aspergillosis caused by *Aspergillus fumigatus* is often fatal to immune-compromised humans (Dixon & Walsh, 1992), but much less frequently so among healthy individuals (Pontón et al., 1991).

While a few species of *Aspergillus*, including *A. sydowii*, have been isolated from the ocean before (Roth et al., 1964; Sweeney et al., 1976; Kendrick et al., 1982; Abrell et al., 1996; Belofsky et al., 1998; Raghukumar & Raghukumar, 1998; Toske et al., 1998), they are not considered normal inhabitants of the marine environment. Nonetheless, there appears to be at least one striking difference between marine and terrestrial strains of *A. sydowii*. Geiser et al. (1998) found that strains isolated from terrestrial sources were not pathogenic to sea fans whereas those previously isolated from diseased sea fans were.

Sea fan corals possess antifungal secondary compounds which may play a role in disease resistance (Kim et al., 2000). However, the efficacy of host defenses can be compromised if the host is stressed (e.g. Ross et al., 1996; Arkoosh et al., 1998; Lenihan et al., 1999). For corals, temperature (both high and low) is an important stressor, which can lead to the breakdown of host-zooxanthellae symbiosis (i.e. bleaching; Brown, 1997). Given that the temperature requirements of most microbial agents are higher than those of their hosts, elevated water temperatures are predicted to shift the coral–fungus interaction to favor the pathogen.

The goal of this study is to characterize the temperature requirements of *Aspergillus sydowii*, and to examine how temperature affects the efficacy of the antifungal chemistry of *Gorgonia ventalina*. These experiments were carried out using three geographically disparate strains of *A. sydowii* known to be pathogenic to sea fan corals, and a non-pathogenic strain isolated from a terrestrial source (cf. Geiser et al., 1998). By using the pathogenic variants, it was possible to assess whether differences in temperature requirements and interaction with host chemical defenses were related to pathogenicity. Metabolic profiles based on 95 carbon source utilization patterns were also determined in order to elucidate physiological differences between pathogenic and non-pathogenic strains.

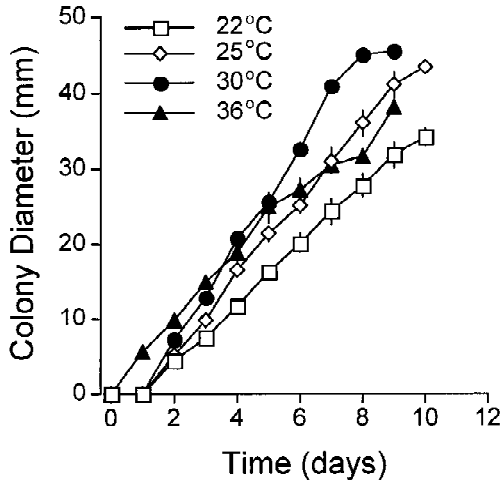

Figure 1. Changes in diameter of *Aspergillus sydowii* colony (mean ± SE) over 10 days averaged across the four strains.

Materials and methods

Effects of temperature on growth rate

The effect of temperature on growth rates was determined by measuring the daily increase in the diameter of fungal colonies grown on solid medium (e.g. Holmquist et al., 1983; Cuppers et al., 1997). These assays were carried out using three geographic strains of *Aspergillus sydowii* isolated from diseased sea fans and on one terrestrial strain isolated from silk (Table 1). Geiser et al. (1998) found that the terrestrial strain was not pathogenic to sea fans. Plates with PYG medium (0.1% peptone, 0.1% yeast extract, 0.3% glucose, 3% Instant Ocean [Aquarium Systems, Mentor, Ohio, U.S.A.]) were inoculated with a drop of spore solution made up with 550 000–700 000 spores/ml. Plates were maintained at 22, 25, 30 and 36 °C in growth chambers (n=10 replicates×4 temperatures×4 strains). Lag time was determined as the number of days until there was visible growth (i.e. until the diameter was 2 mm). Once there was visible growth, colony size (the average of two perpendicular measurements of colony diameter) was measured daily for up to 10 days. Daily observations on colour and shape of the colony were also taken. Mean growth rates were compared using a

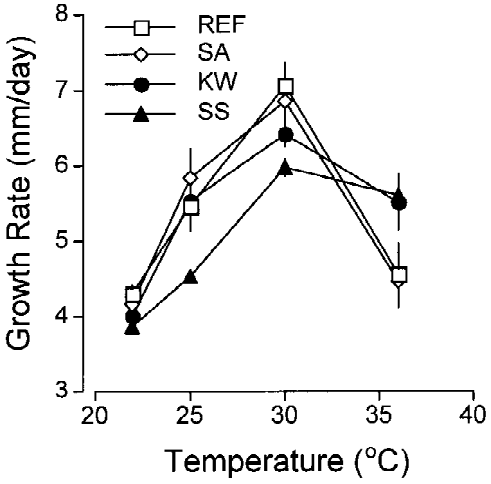

Figure 2. Role of temperature on growth of *Aspergillus sydowii* strains (n=10 per strain per temperature). Error bars represent ±1 standard error. Strain abbreviations as in Table 1.

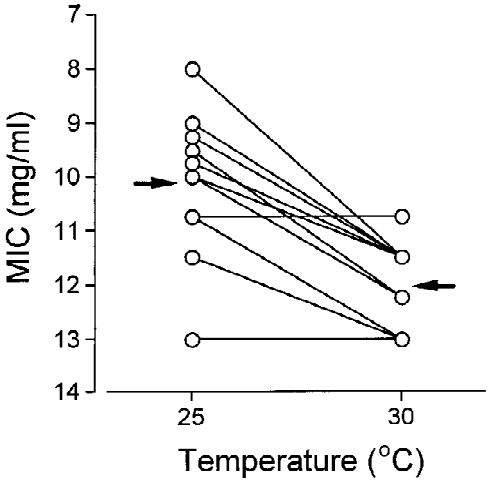

Figure 3. Antifungal activity (as indicated by minimum inhibitory concentration assays) of *Gorgonia ventalina* crude extracts against *Aspergillus sydowii* FK at two temperatures. Each pair connected by a line indicates the same crude extract tested at the two temperatures. Mean MICs are indicated by arrows.

2-way ANOVA. These data satisfied the assumptions of normality (Kolmogorov Normality test, p=0.4028) and of equal variances (Levine test, p=0.391).

Antifungal assays

Kim et al. (2000) showed that *Gorgonia ventalina* posses crude extracts that inhibit germination of aspergilli spores. To examine the effect of temperature on the efficacy of crude extracts, we carried out antifungal assays at two temperatures: 25 °C and 30 °C.

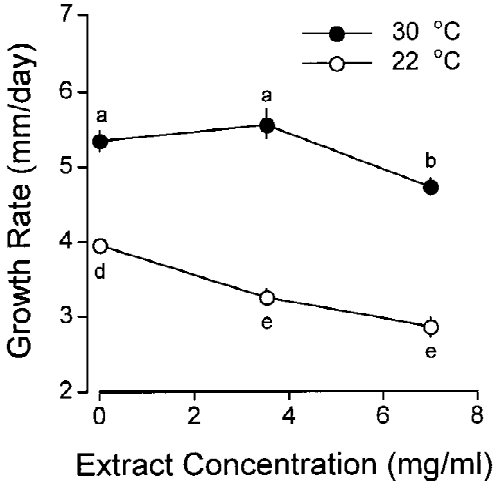

Figure 4. Effects of temperature and sea fan crude extract concentration on growth of Aspergillus sydowii (n=5–6 replicates per concentration per temperature). Error bars represent ±1 standard error. Letters indicate statistically different groups (Scheffé post-hoc tests, $p < 0.05$).

For this assay, we collected 12 sea fans (*G. ventalina*) from Tennessee Reef (Florida, U.S.A.) which were then cut into four similarly-sized fragments. All fragments were extracted individually in dichloromethane (DCM) for 24 h, dried under N_2, and weighed to determine crude extract content. The antifungal activity of the crude extracts was determined using a minimum inhibitory concentration (MIC) assay described in Kim et al. (2000). Briefly, using 96 microwell plates, we determined the lowest extract concentration which inhibited the germination of spores. Thus, a low MIC value indicates high antifungal activity. In each well containing a known concentration of spores (San Salvador strain), sea fan extracts and Alamar Blue (Accumed, Westlake, Ohio, U.S.A.) were added and mixed vigorously using a pipette. Alamar Blue is a non-toxic dye used for colorimetric detection of the metabolic activity (i.e. germination of spores) of filamentous fungi (Espinel-Ingroff et al., 1997). Positive (acetone) and negative (50 mg/ml of hygromycin [Calbiochem, California, U.S.A.]) controls were included on each plate. Because the data were categorical, the effects of temperatures were compared using a Wilcoxon Signed Rank Test.

We also examined the effect of crude extracts on growth rates of fungal colonies. In this assay, the crude extract was added directly to the PYG medium immediately before pouring the plates. The final concentrations of extract in the medium were adjusted to 3.5 and 7.0 mg/ml, which are within the activ-

ity range of the crude extracts (Kim et al., 2000). Acetone and DCM were added to the controls to account for their presence in the extract solution. All plates were placed in a laminar flow hood with the lids slightly ajar to allow the DCM and acetone to evaporate off. To the center of each plate, 2 μl of spore solution (600 000 spores/ml) of *A. sydowii* (isolated from Key West, FL, U.S.A.) were added. In total, the two treatments (extract concentration and controls) were replicated 5 times each for incubation at 22 and 30 °C (i.e. n=2 treatments×2 temperatures×5 replicates=20 plates). Growth rate was calculated from the daily increase in diameter of fungal colonies and was analyzed using a 2-way ANOVA after testing for normality (Kolmogorov Test, p=0.238) and homogeneity of variances (Levine Test, p=0.831).

Metabolic profiles

We determined the metabolic profiles of both pathogenic and non-pathogenic strains of *Aspergillus sydowii*. In addition, several other non-pathogenic, terrestrial strains were examined for comparison: *Aspergillus flavus* [ATCC-PI917], *A. versicolor* [USDA-NRLL226], and *A. fumigatus* [ATCC-PI1061]. Metabolic profile data were collected using the commercially available Biolog GN microwell plates (Biolog Inc., Hayward, CA; see Bochner, 1989). Briefly, each strain was tested for metabolic activity in the presence of 95 different carbon sources (plus water as a control) which are incorporated into colorimetric (i.e. tetrazolium) assays. Results were read on an automated plate reader and absorbance readings 40% higher than the control wells were scored as positive. Thus, each strain was represented by 95 binary characters which were compared in a cluster analysis (unweighted pair group averaging procedure).

Results

Effects of temperature

The radial growth rate of *Aspergillus sydowii* was generally linear over the duration of the experiment (Fig. 1). With the exception of fungi grown at 36 °C, all others showed a 1 day lag period before visible growth occurred. Subsequently, growth rate was constant, although after 8 days, fungi growing at 30 °C appear to slow. As shown in Figure 2, temperature had a strong effect on growth rates (F=30.82, p=0.0001) which was maximal at 30 °C. Overall growth rates did not vary among strains (F=0.944, p=0.429); however, we noted a significant interaction between temperature and strain (F=2.7979, p=0.0127) which was due to the increased sensitivity of the REF and SA strains to high temperatures.

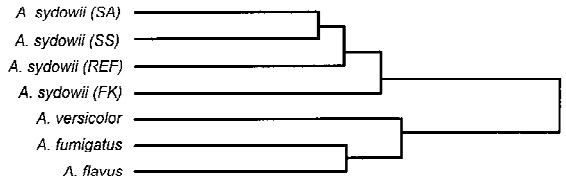

Figure 5. Cluster analysis of *Aspergillus* spp. based on carbon source utilization patterns. Strain abbreviations as in Table 1.

Effects of crude extracts

Crude extracts from *Gorgonia ventalina* (n=12) inhibited the germination of *Aspergillus sydowii* spores at concentrations ranging from 8 to 13 mg/ml (Fig. 3). The efficacy of the extracts was temperature dependent (Wilcoxon Signed Rank Test, p=0.0093) with the extracts being significantly less active at 30 °C (mean±SE=12.0±1.40) than at 25 °C (10.1±1.19).

Extract–temperature interaction

Temperature and extract concentration were important determinants of fungal growth (Fig. 4). Overall, radial growth rates of fungal colonies were higher at 30 °C than at 22 °C (F=315.1, p=0.0001). Extract concentration also affected growth rates (F=25.39, p=0.0001) but at different levels depending on temperature (i.e. interaction F=5.758, p=0.0085). The inhibitory effect of crude extracts (i.e. decrease in growth rate) was detected at both 3.5 and 7.0 mg/ml when *Aspergillus sydowii* was incubated at 22 °C. In contrast, decrease in growth rates was only detected at 7.0 mg/ml when the fungus was grown at 30 °C. At a crude extract concentration of 7.0 mg/ml, growth rate of cultures at 22 °C was decreased by 27%, compared to a decrease of 12% among cultures at 30 °C. Regardless of temperature, cultures grown on extracts at 7 mg/ml produced more aerial hyphae – those which grow vertically above the plane of the colony – than cultures grown on extracts at the lower concentrations.

Metabolic profiles

Cluster analysis of the metabolic data indicated that all strains of *Aspergillus sydowii* were more similar to one another than to other *Aspergillus* species (Fig. 5).

Among *A. sydowii*, pathogenic strains were not clearly delineated from the non-pathogenic, terrestrial strain (REF, USDA NRRL 242). Indeed, the terrestrial strain was more closely related to the Saba (SA) and San Salvador (SS) strains than was the Florida Keys (FK) strain. However, there was substantial variation in metabolic profiles within *A. sydowii* strains as suggested by the comparable levels of variation observed across species.

Discussion

Coral diseases have increased in number and virulence in the recent past (Goreau et al., 1998; Richardson, 1998; Harvell et al., 1999). Concomitant with increases in disease outbreaks have been increases in sedimentation, eutrophication, pollution, over-fishing and temperature (e.g. Grigg & Dollar, 1990; Williams & Bunkley-Williams, 1990). Although it has been suggested that poor water quality undermines the health of corals (e.g. Pastorok & Bilyard, 1985) and thus increases susceptibility to disease, there has been little direct evidence for this. One reason for this lack of evidence is that for only a few of the many pathologies described have the causative agents been identified and verified (Richardson, 1998). Thus, the discovery of the fungus *Aspergillus sydowii* as the pathogen of sea fans (*Gorgonia* spp.) has provided a tractable model system for examining how environmental factors affect the outcome of coral-pathogen interactions.

Because of the apparent seasonality of several coral diseases (e.g. Antonius, 1981; Rützler et al., 1983; Feingold, 1988; Kuta & Richardson, 1996), temperature has been thought to play an important role in disease emergence. There are at least two possible mechanisms by which temperature could affect host–pathogen interactions: by promoting the growth and activity of the pathogen and by reducing the efficacy of host defenses. Increased temperature has been shown to accelerate growth, and in some cases, the disease activity of coral pathogens. For instance, growth and activity of *Phormidium corallyticum*, the causative agent of black band disease (Carlton & Richardson, 1995), is temperature dependent with an optimum of 28–30 °C (Rützler et al., 1983). *Vibrio* AK-1, which induces bleaching in the coral *Oculina patagonica* (Kushmaro et al., 1996, 1997), also grows more rapidly at higher temperatures (Kushmaro et al., 1998) and binds more readily to the host cell surface (Toren et al., 1998). Increased temperature appears to have the additional effect of stressing corals and thereby making them more susceptible to disease. One manifest example of temperature-induced stress is bleaching, where there is a disassociation between the coral host and its symbiotic zooxanthellae resulting from prolonged exposure to high temperature (for review see Brown, 1997). In some cases, bleaching events have been followed by increased outbreaks of disease (Williams & Bunkely-Williams, 1990).

Similarly, temperature is likely have a strong influence on the dynamics of the *Gorgonia–Aspergillus* interaction. Long-term monitoring of *Gorgonia ventalina* populations in the Florida Keys indicates that disease prevalence is highest during the summer months when water temperature often reaches 30 °C (Kim & Harvell, unpublished data). The temperature dependence of *A. sydowii* growth rates reported here (Fig. 2) suggests that increased water temperature during the summer is likely to promote the emergence and pathogenicity of aspergillosis. In addition, there was a significant reduction in the potency of *Gorgonia ventalina* crude extracts against *A. sydowii* when assayed at 30 *versus* 25 °C (Figs 2 and 4). This reduction at the higher temperature may be due to inactivation of antifungal compounds, increased fungal resistance, or a combination of both. However, the fact that there was significant decrease in the growth rate of *Aspergillus sydowii* at 30 °C when crude extracts was at its highest concentration (7 mg/ml), indicates that the antifungal compounds are not inactivated at the higher temperature. Thus, the effect of high temperature appears to be to promote the growth of the fungus, allowing it to overcome the host's defenses.

A question raised by this work is whether the differences between the terrestrial, non-pathogenic strains of *Aspergillus sydowii* and the marine pathogenic strains are genetic or phenotypic. It is possible that there was rapid evolution of *A. sydowii* into a coral pathogen after it entered the marine environment. This is common among interspecific interactions and many of the best examples of rapid evolution are of introduced species (Thompson, 1998). To date, however, there is no evidence of genetic differentiation between terrestrial and marine strains of *A. sydowii* (Geiser et al., 1998). Similarly, we did not find any clear differences in temperature requirements (Fig. 2), susceptibility to host crude extracts (Fig. 3) or metabolic profiles (Fig. 5) between marine and terrestrial strains. However, that only marine strains of *A. sydowii* are pathogenic to sea fans (Geiser et al., 1998) indicates that there are likely to be fundamental (i.e. genetic)

differences between the marine and terrestrial strains. Uncovering those differences will be critical to understanding how *A. sydowii* emerged as a pathogen of sea fans.

There is a growing evidence that a rapidly changing climate will have a dramatic impact on the health of marine ecosystems (Epstein et al., 1999; Harvell et al., 1999) as was illustrated by the widespread bleaching and massive coral die-off following the 1997/98 El Niño event (Wilkinson et al., 1999). In light of the possibility that the death of some these corals resulted from subsequent infections (Harvell et al., 1999), a clear understanding of the factors mediating host-pathogen interactions will be essential for better predicting the impacts of a changing environment on corals and coral reefs, and better devising appropriate management protocols.

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