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EXPERIMENTAL ARTICLES

Ecophysiological Differences between Saprotrophic and Clinical Strains of the Microscopic Fungus *Aspergillus sydowii* (Bainier & Sartory) Thom & Church

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Abstract—A number of ecophysiological differences were shown for saprotrophic and clinical strains of the potentially pathogenic microscopic fungus *Aspergillus sydowii*. The colony growth rates were determined for four saprotrophic and five clinical fungus strains on Czapek medium within the ranges of temperature (5, 10, 15, 20, 30, 35, 37, 40, 42°C) and humidity (0.8, 0.85, 0.9, 0.95, 099 a_w), as well as on media with other sources of organic matter (Sabouraud medium, Hutchinson medium with cellulose, and water agar). The capacity for growth of *A. sydowii* strains on a broad spectrum of organic substrates was determined with the EKOLOG method for multisubstrate testing. The clinical and saprotrophic strains of *A. sydowii* differed in the colony growth rates under the same temperature and humidity combinations, as well as in the capacity for growth on different organic substrates. At decreased water activity (0.90–0.85 a_w), the temperature interval for growth of the saprotrophic strains was narrower (30 ± 2°C) than for the clinical strains (25–30°C). Comparison of growth on different media revealed the highest growth rates of the clinical strains on Sabouraud protein-containing medium. The method of multisubstrate testing showed that the saprotrophic strains grew on sugars better than the clinical ones.

Keywords: Aspergillus, environmental and clinical strains, growth, ecological factors

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In recent years, special attention in the studies of the ecology of microscopic fungi has been given to the group of potentially pathogenic (opportunistic) microfungi, which are widespread in the environment as saprotrophes, but in a number of cases may reveal pathogenic properties, predominantly in immunodeficient individuals [1]. A number of issues pertinent to opportunistic fungi have not been clarified yet. For example, do opportunistic fungi possess special properties or may most microfungi be potentially pathogenic for humans? Do natural strains of opportunistic fungi and those isolated from humans differ in their properties?

Of the filamentous microscopic fungi common in the environment, the fungi of the genus *Aspergillus* are primarily known as the causative agents of opportunistic mycoses [2, 3]. For certain species of *Aspergillus* (predominantly *A. fumigatus*), the studies are conducted to compare the molecular properties of the clinical and saprotrophic strains, usually with a view to searching for the factors determining virulence. These works are mainly epidemiological and are designed to compare the strains isolated from patients and nosocomial environments, i.e., to look into the possibility of nosocomial aspergillosis [4] for assessing the risk of infection by the environmental fungi inhabiting hospital wards and premises, air, and water [5]. Despite the fact that there exist certain data on the similarity between the molecular properties of the clinical and saprotrophic strains [6], the results are not unambiguous in most cases [7].

At the same time, it is not clear whether or not the physiological and ecological properties of the strains of opportunistic mycelial fungi isolated from the clinical material and from the environment are different. In other words, it is unclear whether certain adaptive processes of the fungus occur when it develops in a macroorganism and whether clinical strains are able to survive in natural habitats.

The opportunistic microscopic fungus *A. sydowii* (Bainier & Sartory) Thom & Church is a saprotrophe but is able to cause invasive aspergilloses and skin mycoses, is isolated from the bronchial discharge, etc. [1, 8, 9]. In our previous research, we showed that while the molecular properties (the ITS and D1/D2 regions) of the saprotrophic and clinical strains of this species did not differ, they exhibited differences in the spore formation rate under different ecological conditions [10].

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Table 1. *A. sydowii* strains used in the work

No.	Strain no.	Short name	Source of isolation
1	KBP-FC014	C14	Clinical isolate from the bronchoalveolar lavage fluid, Hematological Research Center, Russian Academy of Medical Sciences, Moscow
2	KBP-FC095	C95	Clinical isolate from the bronchoalveolar lavage fluid, Moscow Municipal Scientific and Practical Center for Tuberculosis Control
3	KBP-FC096	C96	Clinical isolate from the bronchoalveolar lavage fluid, Moscow Municipal Scientific and Practical Center for Tuberculosis Control
4	KBP-FC125*	C125	Clinical isolate from the bronchoalveolar lavage fluid, Moscow Municipal Scientific and Practical Center for Tuberculosis Control
5	NRRL 254 (type strain)	C93	Clinical isolate, Waycross, Georgia, United States
6	VKM F-441	E84	Soddy podzolic soil, Moscow region
7	VKM F-968	E85	Seawater, the Black Sea, Russia
8	VKM F-2488	E86	Ice, Antarctica
9	VKM F-3293*	E87	Ftorolone (polymer), Moscow

Note: In the numbers of strains obtained from the collections, the prefixes denote the name of collection: VKM, All-Russian Collection of Microorganisms; KBP, Collection of the Soil Biology Department, Faculty of Soil Science, Moscow State University; NRRL, National Agricultural Research Center, Agricultural Research Service of Culture Collections, Peoria, Illinois, United States; MMRPCTC, Moscow Municipal Scientific and Practical Center for Tuberculosis Control.

*The strain was not included in the studies of the influence of temperature and humidity.

MATERIALS AND METHODS

We used nine *A. sydowii* strains in our work. They were isolated from patients, from the environment, or obtained from collections (Table 1). The initial identification of the strains was carried out according to the relevant determinative manuals and laboratory guides [11, 12] using 7–10-day old cultures on Czapek medium and then confirmed by the molecular properties represented in the GenBank database [10].

The colony radial growth rate $K_r = dr/dt$ (mm/h) was determined as a measure of growth. Czapek medium with the addition of glycerol was used to create the predetermined conditions of the water activity [13]. The colony diameter was measured in two perpendicular directions every 12 h for 10 days. The colony growth rates were determined at 5, 10, 20, 25, 30, 35, 37, 40, and 42°C and at humidity of 0.99, 0.95, 0.90, 0.85, and 0.80 a_w (which corresponds to moisture pressure of −1.4, −7.0, −14.4, −22.3, and −30.6 MPa).

The colony growth rates of *A. sydowii* strains were also determined at 25°C on the standard solid media with different sources of organic matter in 10 replicates: Sabouraud medium with peptone, Hutchinson medium with cellulose, and water agar.

The growth of the clinical and saprotrophic fungal strains was also determined on a broader spectrum of organic substrates by multisubstrate testing (MST) in the EKOLOG system [14]. The system employs 96-well plates for immunological tests, with the wells containing the dry substrates and mineral components, which are dissolved on addition of the suspension studied. In multisubstrate testing, fungal spore suspensions were used as inocula. The optimum spore suspension concentration required for the culture introduced into the plate determined to be 1×10^6 spores/mL.

The spore suspension was obtained by washout from the test tubes containing the strains of the microscopic fungus. Sterile water (10 mL) was added to the test tube with the 7–10-day old fungal culture, and the test tube was then agitated for 5 min on a Bio-Vortex V1 shaker. The suspension optical density was then calculated. The suspension concentrations were determined in Goryaev chamber. Streptomycin (7.5 mg per plate) was used as an antibiotic. The spore suspension obtained and the antibiotic solution were mixed and applied to the plates with a multichannel dispenser, 0.2 mL of suspension per well. The experiments were made in four replicates.

We modified the standard MST procedure, which is usually performed when working with a soil suspension. Firstly, a somewhat changed spectrum of organic substrates was introduced into the plates. Since our aim was to investigate the functional diversity of the clinical and saprotrophic strains of *A. sydowii*, a number of organic substrates which were of little use for this specific task were excluded from the experiment (namely, Tween and putrescine).

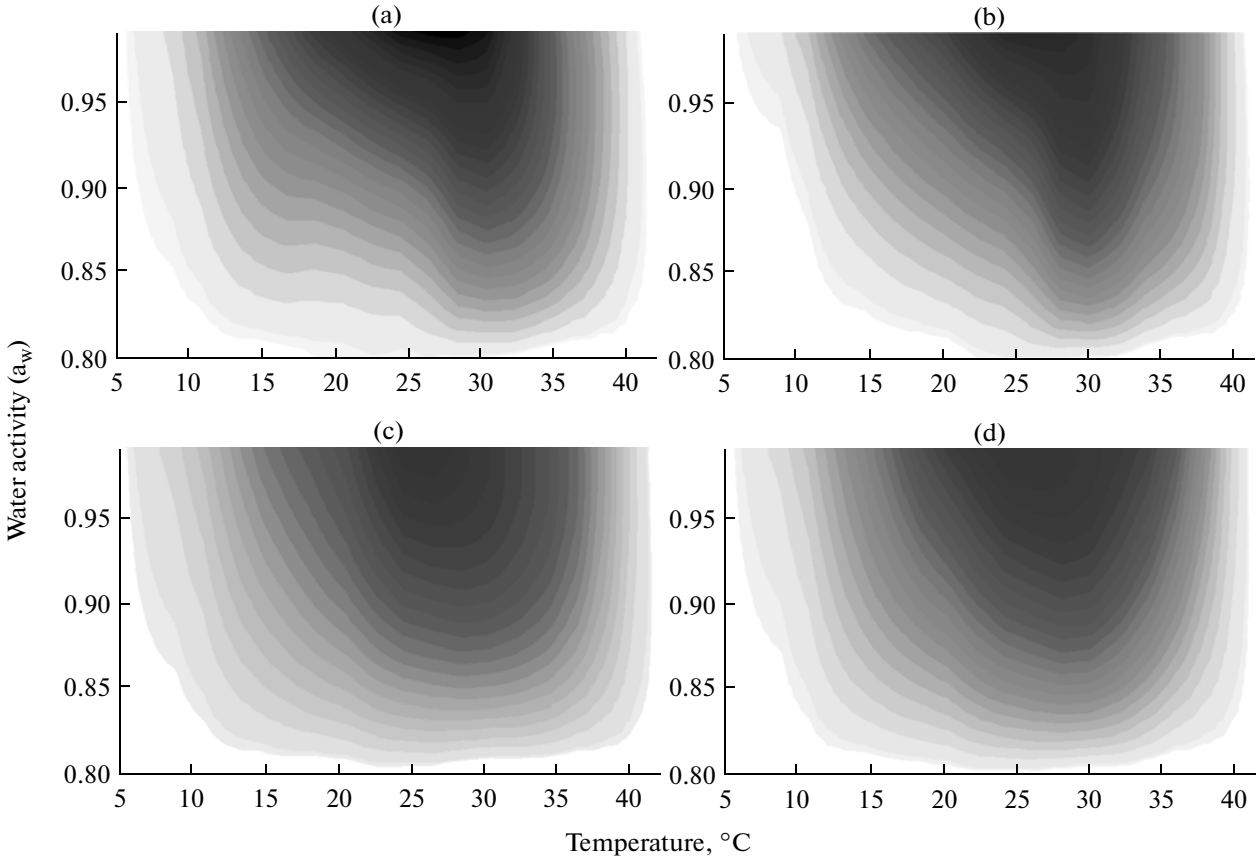

Fig. 1. Radial growth rates of *A. sydowii* strains in the temperature and water activity ranges studied: saprotrophic strain E84 (a); saprotrophic strain E85 (b); clinical strain C14 (c); and clinical strain C96 (d).

Secondly, the plates were incubated for 48–60 h at 25°C not in a resting state but on a shaker at 100 rpm (to avoid mycelial growth on the surface of the well wall).

Initially, MMT, dimethylthiazolyl tetrazolium bromide (saturated solution) was used as a stain. Subsequently, due to a number of discrepancies between the stain color intensity and the fungus growth rate, we rejected this stain. Therefore, thirdly, instead of determining a change in the stain color, we recorded the suspension optical density (at 405 nm) with the Sunrise multichannel photometer (Tecan) in the period of spore germination and germ tube formation but before formation of marked fungus pellets. Simultaneously, the development of *A. sydowii* was directly observed under a light microscope (×200 magnification) in order to assess the state of the cultures and the absence of contamination. Statistical data processing was carried out using the Microsoft Excel 2002 (Microsoft Office XP) and Statistica 6.0 (StatSoft, Inc., 1984–2001) software packages.

RESULTS AND DISCUSSION

It was established that a number of differences were revealed in the development of environmental and clinical strains at an interval of ecological conditions: at different temperature, humidity, and the presence of organic substances.

The growth of *A. sydowii* strains was possible in the 7–41°C range. The maximal growth rates of the strains were observed at 27–30°C. Growth at 37°C, the temperature of the human body, was observed for all *A. sydowii* strains (Fig. 1). The minimal humidity level which allowed for the growth of *A. sydowii* colonies was 0.83–0.85 a_w. The highest growth rates were observed at 0.97–0.99 a_w.

The clinical strains, compared to saprotrophic strains, usually had lower growth rates. At the same time, they were more capable of developing under less favorable conditions of low humidity. In the case of the saprotrophic strains, when the water activity decreased to 0.90–0.85 a_w, the range of temperatures favorable for growth was narrower (30 ± 2°C), while the clinical strains retained their capacity for active

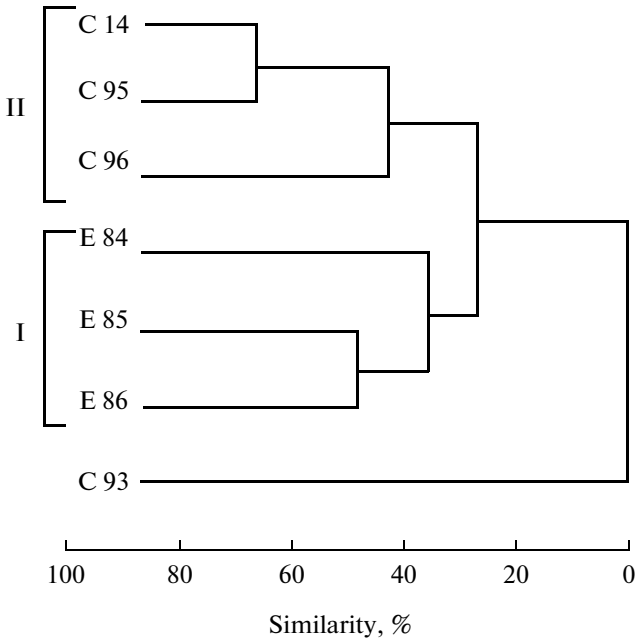

Fig. 2. Cluster analysis of *A. sydowii* strains based on comparison of the radial growth rates in different combinations of temperature and water activity. Group I, saprotrophic strains; group II, clinical strains.

growth in a wider range of temperatures (25–30°C) at such humidity. The examples of such changes for the environmental and clinical strains are shown in Fig. 1.

The Kr-based cluster analysis showed the environmental and clinical *A. sydowii* strains to be divided into clades according to their growth rates at different temperature and humidity conditions (Fig. 2).

The growth rates of the colonies of environmental and clinical strains on solid media with different sources of organic matter also revealed differences. Environmental strains grew better on Czapek medium with sucrose (Table 2). At the same time, on Hutchinson medium with cellulose as well as on water agar the growth of the colonies of environmental and clinical strains did not differ significantly. The growth rate was lowest on water agar. However, the clinical strains grew substantially better on the protein-containing Sabouraud medium than the group of saprotrophic strains. This was most evidently seen for the strains C14 (Kr = 0.126 ± 0.01 mm/h) and C93 (Kr = 0.09 ± 0.01 mm/h).

The analysis of growth using the method of multisubstrate testing on a wide spectrum of organic substances also revealed differences between the groups of environmental and clinical strains. They were recorded by optical density in the plate wells and confirmed microscopically by the absence of spore germination and the mycelium growth rate from the germinated spores. The results of the experiments revealed certain differences in the indices of physiological diversity of the environmental and clinical strains of *A. sydowii*. For example, the Shannon's diversity indices calculated for the consumption of the whole EKOLOG substrate spectrum by the fungal strains were H = 3.65 and H = 3.18 bits for the environmental and clinical strains, respectively. These differences stemmed from the fact that, on the whole, the environmental strains of *A. sydowii* grew somewhat better on sugars than the clinical strains (Fig. 3). No clear-cut differences between the groups of environmental and clinical strains grown on different amino acids were noted. Some clinical strains (e.g., C93, C125) grew better (the optical density was 3–4-fold higher) on creatine than the saprotrophic strains (E84, E85, E86, E87).

Based on the experimental findings obtained for the microscopic fungus *Aspergillus fumigatus*, which most often causes secondary mycoses, it is suggested that, with the great genetic diversity of the strains of this species in the environment, only some of them (1–2 strains) are able to infect humans. It remains to be understood in greater detail what properties of the microscopic fungus this capacity might be linked to [7]. So far, no clear differences in the molecular characteristics of the environmental and clinical strains studied have been established for various opportunistic *Aspergillus* species. It was therefore especially interesting to see whether the differences in other properties

Table 2. Radial growth rates (mm/h) of *A. sydowii* on solid media with different sources of organic matter under the standard conditions (25°C)

Medium	Saprotrophic strains	Clinical strains
Czapek medium with sucrose	0.082 ± 0.012	0.066 ± 0.006
Hutchinson medium with cellulose	0.068 ± 0.014	0.069 ± 0.019
Water agar	0.047 ± 0.014	0.043 ± 0.014
Sabouraud agar	0.044 ± 0.019	0.084 ± 0.014

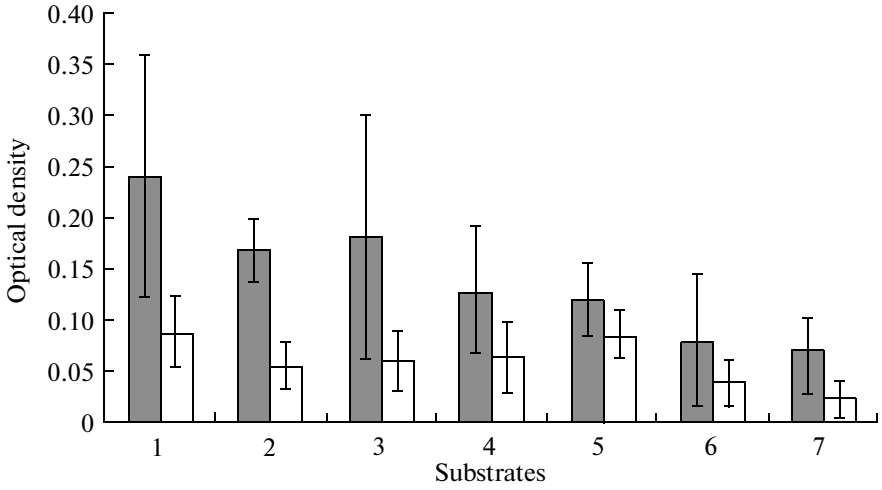

Fig. 3. Changes in the optical density in the EKOLOG system upon growth of clinical (white columns) and saprotrophic (gray columns) *A. sydowii* strains on sugars. Substrates: xylose (1), cellobiose (2), glucose (3), sucrose (4), maltose (5) ribose (6), and arabinose (7).

manifested themselves. For example, higher resistance to certain antibiotics was shown for a number of the clinical *Aspergillus* strains isolated from patients (*A. flavus, A. nidulans*) [15], but this may be only natural if the patients were subjected to preliminary medication. However, the level of production of gliotoxin, which is important for the fungal invasion into a macroorganism, did not differ in *A. fumigatus* clinical and environmental strains [16]. When the complex of the *Sporothrix schenckii* species, which are widespread in the tropical regions and cause sporotrichosis, was studied, the dimorphic species identified as *S. globosa* did not show differences between the clinical and environmental strains in growth at different temperatures and in the spectrum of the substrates consumed [17]. The study of nine environmental and 12 clinical *Trichoderma longibrachiatum* strains did not show any significant differences between the clinical and soil strains in resistance to antimycotics, consumption of individual amino acids, and capacity for growth at different temperatures or different pH [18].

Using the sample of nine strains of the opportunistic fungus *A. sydowii*, we showed the differences existing in the ecophysiological properties of the groups of its environmental and clinical strains. They manifest themselves in the colony growth rates at different combinations of abiotic conditions—temperature and humidity. Importantly, the clinical strains showed a capacity for high growth rates in a wider temperature and humidity range, rather than more active growth at higher temperatures, which is what is usually attempted to reveal [19]. As shown by us earlier, the clinical strains have a higher reproduction level under the same ecological conditions [10]. Note that greater differences in spore formation were observed at low humidity. In our opinion, the differences revealed may be regarded as the indication of an adaptation strategy aimed at surviving under stress conditions. Considering the fact that clinical strains are able to survive in soils [10], their capacity for development under more extreme conditions may be an important property for their preservation in the environment, with the present-day tendencies of climatic changes among other things.

On the other hand, a certain substrate specialization, i.e., differences in the growth rates on nutrient media with different sources of organic matter, was noted in the groups of the strains studied. The clinical strains of *A. sydowii* grew much better on Sabouraud proteinaceous medium than on media with sucrose or cellulose and, especially, on starvation agar, a nutrient-poor medium. On the contrary, better growth on the medium with sucrose was noted for saprotrophic organisms.

Similar tendencies were observed when we assessed the development of the clinical and environmental strains with the method of multisubstrate testing on a wide spectrum of organic substrates. In contrast to the standard method, we made assessments not by stain intensity but by changes in the optical density in the wells inoculated with the fungus, which was sometimes noted as a more revealing way of analyzing fungal growth in the work with a similar Fungilog procedure [20]. The environmental and clinical strains appeared to have clearer distinctions by the groups of compounds, rather than by consumption of the individual organic substrates. The expediency of such approaches, i.e., assessment of consumption of the groups of substrates (guilds) was shown earlier when analysis of the functional diversity of the fungal community in soils was made [21]. The differences revealed by our multisubstrate testing primarily concern the capacity for using sugars. The environmental *A. sydowii* strains studied usually grew better on the

sugars present in plants, in particular, on xylose, cellobiose, and arabinose.

Thus, we showed by the example of the mycelial microscopic fungus *A. sydowii* that ecophysiological differences may be revealed between the clinical and environmental strains of opportunistic fungi. Such data are important for both understanding the adaptation strategies, which clinical strains may develop, and assessing the possibility of their survival under the environmental conditions ever changing by anthropogenic influence.

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