RESEARCH PAPER

Virulence 6:4, 395–403; May/June 2015; © 2015 Taylor & Francis Group, LLC

Adaptation to thermotolerance in *Rhizopus* coincides with virulence as revealed by avian and invertebrate infection models, phylogeny, physiological and metabolic flexibility

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Keywords: carbon source assimilation, embryonated hen egg, *Galleria mellonella*, growth kinetics, infection model, Rhizopodaceae, *Rhizopus*, *Syzygites*, *Sporodiniella*, thermotolerance, Virulence, wax moth

Mucormycoses are fungal infections caused by the ancient Mucorales. They are rare, but increasingly reported. Predisposing conditions supporting and favoring mucormycoses in humans and animals include diabetic ketoacidosis, immunosuppression and haematological malignancies. However, comprehensive surveys to elucidate fungal virulence in ancient fungi are limited and so far focused on *Lichtheimia* and *Mucor*. The presented study focused on one of the most important causative agent of mucormycoses, the genus *Rhizopus* (Rhizopodaceae). All known clinically-relevant species are thermotolerant and are monophyletic. They are more virulent compared to non-clinically, mesophilic species. Although adaptation to elevated temperatures correlated with the virulence of the species, mesophilic strains showed also lower virulence in *Galleria mellonella* incubated at permissive temperatures indicating the existence of additional factors involved in the pathogenesis of clinical *Rhizopus* species. However, neither specific adaptation to nutritional requirements nor stress resistance correlated with virulence, supporting the idea that Mucorales are predominantly saprotrophs without a specific adaptation to warm blooded hosts.

Introduction

Zygomycetes belong to one of the oldest fungal groups on earth, with known fossils from the Middle Triassic of Antarctica[1] and a diverging time calculated for their origin of around 600 mya years.[2] Contemporary descendants of these early ancestors can be found all over the world colonizing a wide range of ecological habitats, and are currently classified in several subphyla, namely Mucoromycotina, Kickxellomycotina, Zoopagomycotina, Mortierellomycotina,[3,4] and the phylum Entomophthoromycota.[5] Within the Mucoromycotina, the largest order Mucorales comprises predominantly saprotrophic inhabitants of soil and organic decaying matter. Some species are also able to parasitize on plants, insects and fungi, or they can be found as opportunistic pathogens of man and animals.

The mucoralean family Rhizopodaceae K. Schum. today encompasses 3 genera, namely *Sporodiniella*, *Syzygites* and *Rhizopus*. Although the family comprises only 11 species, saprotrophic, parasitic and pathogenic life-styles are represented within the Rhizopodaceae in a species-specific manner.

While *Sporodiniella umbellata*, sole species of its genus, is a facultative parasite of insect larvae,[6,7] *Syzygites megalocarpus*, also sole species of its genus, is parasitic on members of the Dikarya.[8] In contrast, *Rhizopus* species display a high variability of lifestyles and habitats. Being primarily saprotrophic fungi, several species are important plant-pathogens or spoilage agents of fresh and manufactured food e.g. soft rot caused by *R. stolonifer, R. arrhizus* (syn. *R. oryzae*) or *R. microsporus*.[9-12] Yet, *Rhizopus* plays also an important role in industrial biotransformations or food processing through fermentation, especially in Asia and Africa.[13-15]

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Submitted: 06/04/2014; Revised: 03/04/2015; Accepted: 03/05/2015
http://dx.doi.org/10.1080/21505594.2015.1029219

However, *Rhizopus* species are also the most common cause of life-threatening mucormycoses. [16-19] These infections often develop rapidly, predominately as rhinocerebral and pulmonary manifestations; and are often associated with dissemination and high mortality rates.[16-20] Although mucormycoses are uncommon fungal infections compared to aspergillosis or candidiasis, their incidence is increasing in clinical settings.[17,21] Major risk factors for mucormycoses are diabetic ketoacidosis, immunosuppression and malignancies.[17,18] In addition, infections have been found to be associated with administration of certain antifungals such as voriconazole or iron chelators like deferoxamine.[22,23]

In addition to human predispositions, fungal prerequisites are also required for infection. Such virulence factors include pathways that facilitate adaptation to the host environment, e.g., to elevated temperatures, unfavoured pH, unbalanced osmotic conditions and nutrient limitation.[24] Furthermore, some morphological features are linked to virulence: e.g. fungal spore size is known to be related to fungal pathogenesis in *Mucor circinelloides*.[25,26] Finally, the relative burden of asexual spores in the environment might contribute to the establishment of mucormycoses. In *Rhizopus* the amount of spores produced differs between species and is known to be reduced for *R. schipperae*, a rare causative agent of mucormycosis.[16]

Although mucormycoses are seen as emerging serious fungal infections, with a large number of case reports and studies concentrating on susceptibility to antifungal drugs,[27] comprehensive evaluations of the pathogenic potential at genus- or family level so far only exist for the Lichtheimiaceae.[28] In addition to evaluating fungal traits potentially involved in virulence we investigated the pathogenic potential of the Rhizopodaceae applying the embryonated chicken egg model, a model with proven suitability to assess the virulence potential of fungi, and *Galleria mellonella* as a second alternative infection model.[28-31]

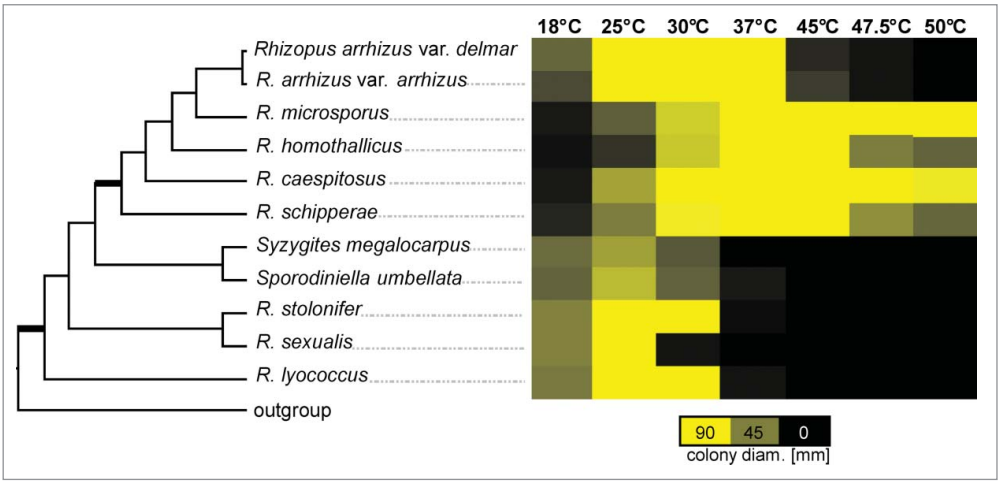

Figure 1. Schematic cladogram of the Rhizopodaceae, modified after Walther et al. 2013.[39] Bold branches show considerable bootstrap support (100 %) in the original phylogram which based on ITS sequences. The second part displays growth at different temperatures after 48 h. Maximum diameter possible for a colony is 90.00 mm, equal to the size of a petridish. The mean diameter was determined by 3 biological replicates, each with 3 technical replicates.

Results

Phylogeny, clinical relevance and infection model

The family Rhizopodaceae comprises the genera *Rhizopus, Syzygites* and *Sporodiniella* with few, closely related species (**Fig. 1**). Only species of the genus *Rhizopus* have clinical relevance, with *R. arrhizus* and *R. microsporus* predominantly described as potential agents of severe mucormycoses.[16,17] The species *R. schipperae, R. caespitosus* and *R. homothallicus* are less frequently observed in human infections.[32-34]

The virulence potential of the different *Rhizopus* species was determined in chicken embryos. The two most-common pathogenic species *R. arrhizus* and *R. microsporus* resulted in high mortality with survival rates of 35 % and 10–22 %, what is comparable to *R. caespitosus* with 17.5 % survival (**Fig. 2**). The less common clinical species *R. homothallicus* and *R. schipperae* produced only small amounts of spores. Therefore infection experiments were performed with a lower infection dose. Both species showed high mortality comparable to or even higher than *R. microsporus* (**Fig. S1**). *Rhizopus schipperae* is the most virulent strain tested in this study with 98 % mortality as early as 3 d after infection (**Fig. S1**). Mesophilic, non-clinical species were found less virulent than the thermotolerant, clinically relevant species with survival rates between 76–90 % at day 7 post infection (**Fig. 2**).

To assess the variability of the virulence potential within a species, 17 additional strains of *R. microsporus* isolated from the environment, food and human patients (**Table 1**) were tested in chicken embryos. No significant difference was found regarding their origin and their potential to cause lethal infections (**Fig. S2**). While some clinical isolates showed higher virulence than food isolates (e.g., CBS124669 [human] vs. CBS228.95 [tempeh] P= 0.5721), there were also isolates from tempeh with higher virulence compared to clinical isolates (e.g., CBS339.62 [tempeh] vs. CBS124669 [human] P = 0 .0428). Overall mortality ranged between 60 to 100 % (average 80 %) for all strains (**Fig. S2**).

Role of temperature adaptation

Growth at elevated temperatures is known to be an important virulence factor in several fungal pathogens. To investigate if thermotolerance of the different species correlated with virulence in the embryonated egg model, growth at different temperatures was determined. A clear shift in the temperature profiles between the virulent and attenuated species was found (**Fig. 1**). While the attenuated

species grew well between 25°C and 30°C, the growth optimum for the virulent clade including *R. microsporus*, *R. arrhizus*, *R. caespitosus*, *R. homothallicus* and *R. schipperae* was 37°C or higher. The mesophilic *R. stolonifer* and *R. lyococcus* were able to germinate at 37°C, but did not grow well (**Fig. 1**).

While thermotolerance is a prerequisite for a pathogen to cause infections in warm-blooded animals, additional virulence factors have been found to be involved in the infection process of fungal pathogens. To investigate whether the observed reduced virulence of the mesophilic *Rhizopus* species was caused only by their reduced thermotolerance, infection experiments were carried out using wax moth larvae. In contrast to the chicken embryos the larvae could be incubated at 30°C, a temperature at which the growth rate of the mesophilic species was comparable to or even higher than for the thermotolerant species (**Fig. 1**). *Rhizopus arrhizus* and *R. microsporus* as representatives of the thermotolerant species induced high mortality rates in *Galleria* (86–100 %) with *R. arrhizus* being significantly more virulent than *R. microsporus* ($P < 0.0001$; **Fig. 3**). Yet, *R. arrhizus* was faster growing at this temperature than *R. microsporus* (**Fig. 1**), eventually supporting faster spreading within the larvae. The tested isolates of *R. stolonifer* and *R. lyococcus* were significantly less virulent than *R. arrhizus* and *R. microsporus* ($P<0.0001$; **Fig. 3**). Despite the lower incubation temperature, the results from the *Galleria* experiments (**Fig. 3**) resemble those from the chicken model (**Fig. 2**), indicating additional adaptations supporting virulence of the thermotolerant *Rhizopus* species.

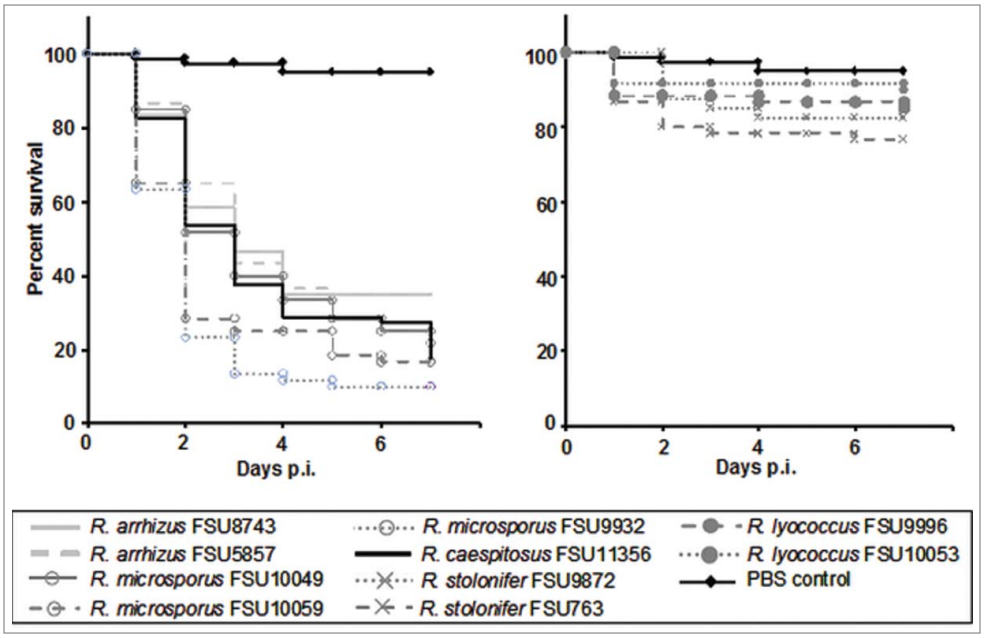

Figure 2. Virulence of different *Rhizopus* species in embryonated chicken eggs. Eggs were infected *via* the chorio-allantoic membrane at developmental day 10 using 10^6 spores (n = 20) from the thermotolerant species (left) and mesophilic species (right). Spore-depleted PBS was used as negative control. Survival was assessed daily over a period of 7 d post infection. Experiments were performed 3 times (except FSU 9872 which was performed twice). Kaplan-Meier-curves represent average survival rates.

Stress resistance and metabolic flexibility

In addition to adaptation to temperature, coping with arising stress conditions in the changing host environment is an important feature affecting virulence in fungal pathogens. Therefore, resistance toward osmotic stress and cell wall stress was tested. Thermotolerant and mesophilic species showed comparable susceptibility to the different stressors and no correlation was found between stress resistance and virulence of the species (**Table S1**).

In order to survive in the host, pathogens have to be able to acquire nutrients from the resources within the host. Thus, metabolic flexibility might influence virulence. Therefore, we analyzed the utilization of different carbon- and nitrogen sources by *Rhizopus* species. As primary soil inhabiting fungi, all species tested were able to utilize carbon sources originating from living or decaying plant material like xylose, xylitol, pectin, cellobiose and common sugars or sugar alcohols like glucose, fructose, galactose, mannose, mannitol and sorbitol. Maltose and starch could not be utilized by *R. stolonifer, R. sexualis* and *Syzygites*. *Sporodiniella* was unable to use soluble starch. None of the tested species could use the complex polysaccharids xylan or cellulose (**Table S2**).

Table 1. List of isolates of *R. microsporus* used for extension of the virulence test in chicken eggs to survey isolate specificity

CBS number	Mating type	Geography	Source
CBS339.62	plus	Indonesia	tempeh
CBS337.62	unknown	Indonesia	tempeh?
CBS130971	plus	Netherlands	wood chips pile
CBS699.68	plus	Ukraine	soil
CBS130967	unknown	Indonesia	tempeh
CBS130968	unknown	Indonesia	tempeh
CBS700.68	minus	Georgia	forest soil
CBS289.71	plus	Italy	starch-containing material
CBS112588	plus	Indonesia	tempeh
CBS112586	plus	Indonesia	tempeh
CBS346.49	unknown		*Eleusine coracana*
CBS631.82	minus	China	bread
CBS537.80	plus	South Africa	*Sorghum* malt
CBS357.93	plus	Java	tempeh
CBS124669	plus	Greece	human
CBS536.80	plus	South Africa	*Sorghum* malt
CBS359.92	minus	Australia	human
CBS228.95	unknown	Java	tempeh
CBS343.29	plus	USSR	air

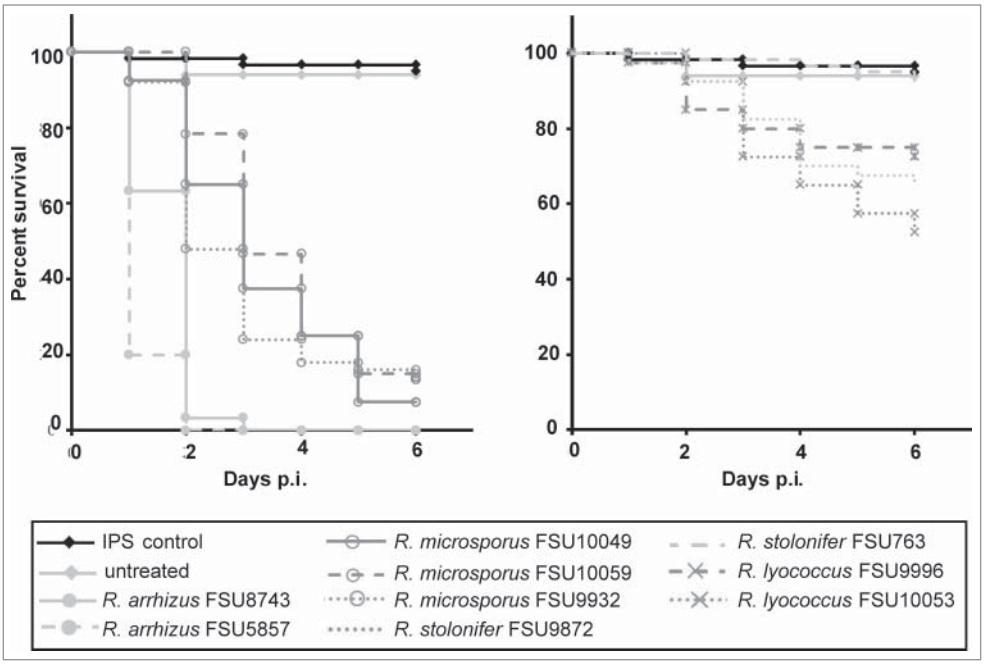

Figure 3. Virulence of different *Rhizopus* species in *Galleria mellonella. Twenty* sixth-instar larvae per group were infected each *via* injection in the hemocoel with 10^6 spores of thermotolerant species (left panel) and mesophilic species (right panel). Spore-depleted IPS was used as negative control. Experiments were performed 3 times; curves represent average survival rates over a period of 6 d post infection.

Rhizopus caespitosus and *Syzygites megalocarpus* are the only fungal species tested capable to utilize citric acid, a common organic acid in mushrooms.[35]

Within animal hosts fermentable sugars like glucose, fructose or galactose have often limited availability. All of them can be assimilated by all *Rhizopus* species. For alternative carbon sources only the amino acids arginine, tyrosine and partially phenylalanine were exclusively metabolized by the virulent species. Most of the other amino acids could not be metabolized by any *Rhizopus* species (**Table S2**).

A similar effect was observed when amino acids were used as sole nitrogen source (**Fig. 4**, **Table S3**). Thermotolerant species were generally able to utilize all 20 tested amino acids while mesophilic *Rhizopus* species lacked the ability to grow on several amino acids, including lysine, cysteine, histidine, isoleucine, threonine and valine. All other nitrogen sources tested revealed no obvious differences.

Infection-related morphological features

Since infections with *Rhizopus* species occur mainly in the respiratory tract, the small size of fungal spores may contribute to the success of fungal infections. In addition, fungal spore size is known to be related to fungal pathogenesis in *Mucor circinelloides* with larger spores being more virulent.[26] For the genus *Rhizopus*, spore size differs largely between species ranging from average volume of 28 μm^3 to 555 μm^3. Spores from thermotolerant species were in general smaller compared to spores from mesophilic species (**Table 2**). However, there was no correlation between spore size and virulence in the thermotolerant species.

In addition to spore size, the burden of fungal spores in the environment can be important for the development of mucormycoses as a high spore burden increases the likelihood that spores are inhaled in sufficient numbers to establish infection. In our artificial setting the relative amount of spores produced in a specific period of time differed considerably between *Rhizopus* species (**Fig. S3**). Within the thermotolerant species *R. schipperae* and the homothallic *R. homothallicus* produced the lowest number of spores. Generally, homothallic species (also *R. sexualis*) produced less asexual spores than heterothallic species.

Discussion

All recent phylogenetic analyses strongly support separation of mesophilic and thermotolerant species of the Rhizopodaceae (**Fig 1**), although the relationship between the species in each supported group is not finally solved.[36-39] The mesophilic group contains species able to grow around 25°C to 30°C but displaying reduced growth rates at higher temperatures. However, the ability to grow at elevated temperatures of 37°C or above, as seen for the thermotolerant *Rhizopus* species (**Fig. 1**), is a prerequisite for colonization warm blooded hosts.

The genus *Rhizopus* exhibits the largest impact on mankind, being important in agriculture and industry and is furthermore the main causing agent of mucormycoses, followed by *Lichtheimia* and *Mucor*. All three genera being responsible for 70 to 80 % of the reported infections, predominantly as rhinocerebral, pulmonary or disseminated manifestations; associated with high mortality rates.[18-20] From the mesophilic species of the genus *Rhizopus*, only *R. stolonifer* can be found in clinical settings, but is seen rarely; mostly as agents of allergic alveolitis or superficial infections but being predominantly non-invasive.[16,40,41] For the thermotolerant species *R. arrhizus* and *R. microsporus* are reported more frequently in severe infections than any other species from the Rhizopodaceae.[19,42] In our study, *R. schipperae, R. caespitosus* and *R. homothallicus* displays a virulence potential comparable to *R. arrhizus* and *R. microsporus* (**Fig. 2**, **Fig. S1**), yet they were only isolated rarely from human infections.[32-34] This suggests that additional factors might be required for infections in humans. One of those aspects could be the abundance of species and the burden of fungal spores in human environment. Although all species of *Rhizopus* are distributed worldwide, and the natural habitats are similar for nearly all species, like soil, or

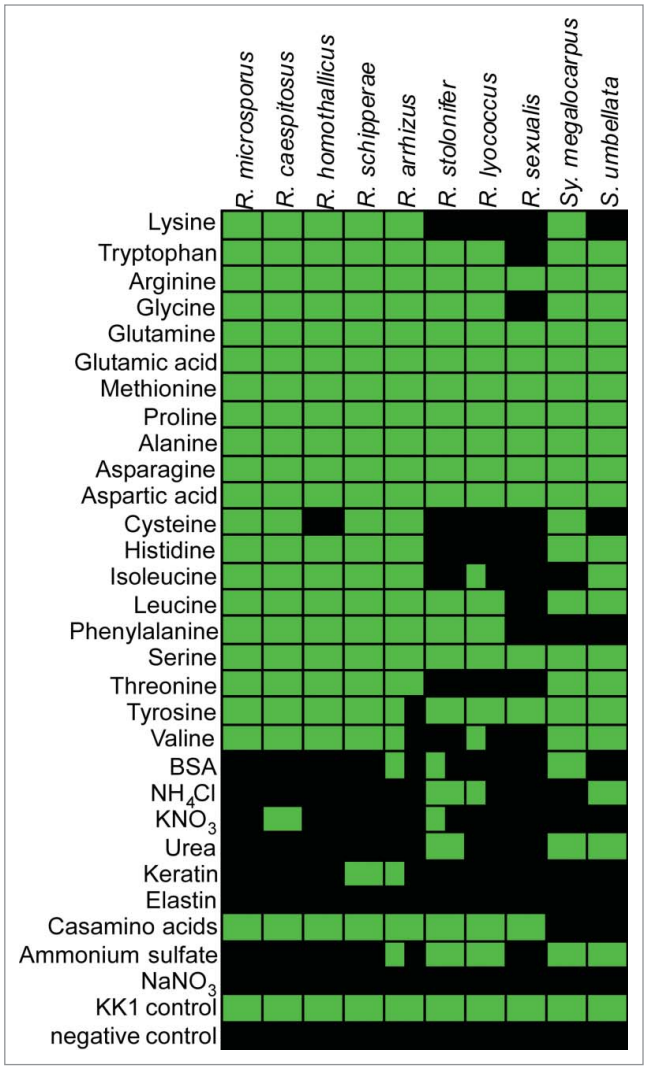

Figure 4. Utilization of different nitrogen sources by different *Rhizopus* species (**Table S3**). Growth is indicated in green and no growth in black.

on decaying organic matter including wood, and especially sugar-rich fruits,[16] they are isolated from environmental samples with different frequencies. *Rhizopus arrhizus* is found most frequently, followed by *R. stolonifer* and less frequently by *R. microsporus*.[16] While *R. arrhizus* and *R. stolonifer* are found to similar extends, the majority of infections is caused by *R. arrhizus* (50%) and *R. microsporus* (15–25%).[16,17,43] This could be explained by the lower virulence potential of *R. stolonifer* observed in this study. In contrast, *R. schipperae, R. caespitosus* and *R. homothallicus* appear to be less abundant in the environment, if judging from the few available specimens from public culture collections, or the fact, that *R. schipperae* is only known from 2 reported cases with no obvious natural substrate presented.[34] Furthermore, *R. schipperae* fails to sporulate on most artificial media,[16] which was confirmed in this study. If sporulation is also low in natural habitats, this could explain the few known isolates. Furthermore, low numbers of spores in the environment would likely result in very limited exposure of humans to *R. schipperae,* thereby explaining the limited number of reported human infections despite the significant virulence potential.

No clear correlation between virulence and special nutritional requirements or differences in the ability to cope with stress was observed in our study. Whether the observed small differences in the profiles of C- and N-sources contribute to virulence remains to be determined. A recent study of pathogenic *Lichtheimia* species likewise identified only few differences in nutritional requirements between strains.[28] For *Lichtheimia* and *Rhizopus* the carbon utilization profiles differ for raffinose, lactose, melibiose, inosine, glycine and pyruvate which could be utilized by *Lichtheimia* spp. but not by any *Rhizopus* spp. On the other hand, *Rhizopus* spp. are able to use glycerol and ethanol, whereas *Lichtheimia* spp do not (**Table S2** and ref 28). Yet there are few amino acids which were exclusively used by thermotolerant *Rhizopus* species, a feature which could contribute to the survival within the host, but needs further studies.

Beside adaption to temperature or available nutrients, coping with arising stress conditions in the changing host environment

Table 2. List of species used in this study. Strain numbers, origin, mating type, spore size and sequences generated for identification are given

Species	FSU number	CBS or alternative number	Mating type	Geography	Source	Spore size [μm^3]	28S	18S	ITS
R. arrhizus var. *arrhizus*	FSU5857	CBS112.07	minus	Netherlands	unknown	47.13 ± 21.99	KJ408556	KJ408539	KJ408568
R. arrhizus var *delemar*	FSU8743	RA99–880	plus	Texas	human	50.95 ± 20.62			
R. caespitosus	FSU11356	CBS427.87	minus	India	unknown	49.89 ± 26.58	KJ408564	KJ408548	
R. homothallicus	FSU2530	CBS336.62	homothallic	Guatemala	desert soil	63.48 ± 14.85	KJ408554	KJ408537	KJ408567
R. lyococcus	FSU10053	CBS398.95	unknown	unknown	unknown	72.24 ± 27.58	KJ408562	KJ408545	
R. lyococcus	FSU9996	CBS117.43	unknown	Netherlands	grain	88.39 ± 34.27	KJ408559	KJ408542	
R. microsporus	FSU10059	CBS102277	plus	unknown	human	not determined	KJ408561	KJ408543	KJ408571
R. microsporus	FSU10049	CBS308.87	unknown	Australia	human	27.98 ± 5.36	KJ408560	KJ408544	KJ408570
R. microsporus	FSU9932	CBS294.31	plus	France	cow	55.55 ± 11.89	KJ408558	KJ408541	KJ408569
R. schipperae	FSU10234	CBS138.95	unknown	Texas	human	46.52 ± 24.10	KJ408563	KJ408546	KJ408572
R. sexualis	FSU11355	CBS102880	homothallic	Italy	leave litter	230.87 ± 142.20	KJ408565	KJ408549	KJ408573
R. stolonifer	FSU9872	none	unknown	Germany	human	286.85 ± 146.22	KJ408557	KJ408540	
R. stolonifer	FSU763	DSM63011	unknown	Germany	bread	555.41 ± 246.60	KJ408551	KJ408534	
Sporodiniella umbellata	FSU11407	CBS195.77	unknown	Ecuador	Membracidae	not determined	KJ408566	KJ408547	
Syzygites megalocarpus	FSU728	none	homothallic	Germany	mushroom	2412.29 ± 1712.94	KJ408550	KJ408533	

affects virulence, as demonstrated for e.g., *Candida albicans* and *Aspergillus fumigatus*.[44–46] In our study, mesophilic species showed a trend toward higher tolerance to osmotic stress due to excess of sodium chloride, but without species-specific differences (**Table S1**). A similar concordance was observed between virulent and attenuated species of *Lichtheimia*.[28] No obvious differences could be observed for cell wall stresses, although the applied stress conditions generally led to reduced growth compared to normal conditions in *Rhizopus*. Yet, this is less pronounced than in other mucoralean pathogens (**Table S1** and ref.[28]). Nevertheless, no correlation between tolerance to stress and the observed virulence could be made, in contrast to virulence of evolutionary derived fungi like *Candida*.[44-46] Yet, pathogens of the derived fungi are often adapted to their hosts, whereas mucoralean fungi seem to be not, which could explain why there is not obvious difference in stress tolerance between potential pathogenic and non-pathogenic species.

An additional factor that could affect virulence of fungi is the ability to produce hydrolytic enzymes aiding in the degradation of host tissue, such as glycosidases, lipases and proteases. Previous comprehensive tests for the presence of gelatinase, urease, lipase, amylase, cellulase, laccase and tyrosinase within different isolates of *R. microsporus* sampled from various substrates (environment, food, clinical) revealed no differences in hydrolytic activity. In contrast, significant difference was observed in the production of the iron chelating compounds, the siderophores, by strains of food and clinical origin.[47] As siderophores are important for iron acquisition of some pathogens within the host,[48] this observation suggests a link between siderophore production and clinical relevance of strains. However, no correlation between the presence of siderophores or the origin of the isolate[47] and the observed virulence of *R. microsporus* strains was observed in this study.

Another interesting observation in recent studies on the virulence of Mucorales is the relation between differences in spore size and virulence, where larger spores produced by the minus mating type of *Mucor circinelloides* are more virulent than smaller spores produced by the plus mating type.[25,26] A second observation is that hyphal-stage of a fungus is more virulent than yeast-stage.[25] This study by Lee et al.[25] demonstrated for the first time, that morphogenesis is also linked to virulence in zygomycetes. Comparing spore size with the genus *Rhizopus*, no correlation to virulence could be made. Further studies on species level will reveal if spore size in mucoralean fungi is related to virulence as demonstrated for *Mucor circinelloides*. Recent studies revealed also the iron permease FTR1 and the surface protein cotH as known factors contributing to the virulence of these species.[23,53] However, both factors are present in a variety of mucoralean fungi and are not sufficient to explain the clinical importance of certain species. Comparative analyses of closely related virulent and non-virulent species may improve the understanding of the evolution of the pathogenicity mechanisms. Avian infection model-mediated virulence analysis yields objective results that overcome the disadvantages of mammalian infection models being time consuming, laborious and conflicting with ethic aspects. Therefore, it can be expected that the assessment of virulence of *Rhizopus* spp. applied to the embryonated hen egg infection model will play a crucial role in future investigations of host-pathogen interactions by the utilization of knock-out mutant-based identification of virulence factors. Future experiments should also include various, distinctly related zygomycetes to elucidate comparability of virulence factors with the background of long time speciation of microorganisms not specifically adapted to warm blooded hosts.

Material and Methods

Ethics statement

All experiments were performed in compliance with the European and German animal protection law. According to this, no specific approval is needed for work performed in avian embryos before the time of hatching. The experimental protocols were reviewed and approved in regard to ethical and welfare issues by the responsible animal welfare officer. Experiments were terminated latest on developmental day 18, 3 d before hatching, by chilling the eggs on ice for 30–60 min.

Fungal isolates

A total of 34 isolates of the family Rhizopodaceae were included in this study (**Table 1 and 2**). Strains were obtained from the Jena Microbial Resource Collection and from the Centraalbureau voor Schimmelcultures (CBS). Isolates were identified by standard microbiological procedures and by sequencing of 18S rDNA, 28S rDNA, and ITS regions. For DNA isolation strains were grown for 5–10 d on medium KK1, especially composed for Mucorales (1 % glucose, 0.44 % NaCl, 0.3 % KH_2PO_4, 0.125% K_2HPO_4, 0.2 % yeast extract, 0.1 % KNO_3, 0.05 % $MgSO_4$*$7H_2O$, 0.05 % KCl (all Carl Roth)) at room temperature. DNA isolation, PCR and sequencing were conducted as described previously.[38] Primers used for amplification were: NL1 and NL4 (for 28S rDNA),[49] NS1 and NS4 (for 18S rDNA)[50] and ITS1 and ITS4 (for ITS).[50] Sequences generated in this study are given in **Table 2**.

Embryonated chicken egg model

Infections at developmental day 10 was done via the chorioallantoic membrane as described previously[29,30] with 10^5 and 10^6 spores/egg. Twenty eggs were used for each strain. Experiments were performed 3 times, except for *R. homothallicus* FSU 2530 and *R. schipperae* FSU 10234 for which spore concentrations of 10^6 could not be reached and *R. stolonifer* FSU 9872 which was tested only twice. *Rhizopus homothallicus* and *R. schipperae* were tested for 3 times with 10^5 spores/egg in comparison to *R. microsporus*. Spore solution was prepared in PBS, which was used as negative control. Eggs were incubated at 37 °C; survival was assessed daily by candling. Pooled data was analyzed with GraphPad Prism v5.03 and displayed in **Figure 2** and **Fig. S1**. To assess strain dependent differences, 17 additional isolates of *R. microsporus* were checked once (**Fig. S2**). *Syzygites* and *Sporodiniella* were not tested because they do not grow at elevated temperatures and did not produce the necessary amount of spores.

Galleria mellonella infection model

In order to test whether the virulence data observed in the chicken eggs correlate to the elevated temperature used for incubation and the different abilities of the strains to grow at this temperature; a second, widely accepted, infection model was applied. *Rhizopus lyococcus* and *R. stolonifer* were chosen as representatives of the mesophilic group, *R. arrhizus* and *R. microsporus* for the thermotolerant.

Sixth-instar larvae of *Galleria mellonella* (Kurt Pechmann, Langenzersdorf, Austria) were stored in the dark at 18°C prior to use. Larvae weighing between 0.3 and 0.4 g were used, each (n = 20) infected with 1×10^6 spores. Inocula were diluted in insect physiological saline (IPS) and a volume of 20 µl was injected into the hemocoel via the hind pro-leg. Untouched larvae and larvae injected with 20 µl of IPS served as control. Larvae were incubated at 30°C, respectively, in the dark and monitored daily up to 6 d Significance of mortality rates was evaluated by using Kaplan-Meier survival curves with the PRISM statistics software (Mantel-Cox log rank test) using pooled data. All experiments were performed 3 times, each time with duplicates. Survival rates are displayed in **Figure 3**. *Syzygites* and *Sporodiniella* were not tested because they did not produce enough spores.

Relation of growth and temperature

Petridishes with medium KK1 were inoculated with 10 µl spore suspension containing 1000 spores. In cases of growth, the initial colony was 6 mm in diameter. *Sporodiniella umbellata* and *Syzygites megalocarpus* were inoculated as agar slants with 6 × 6 mm. Plates were incubated at different temperatures. The diameter was measured 2 times a day across 3 defined lines at the bottom of the petridish[51] for 3 technical replicates. The mean diameters of 3 biological replicates at 48 h are given in **Figure 1**. Maximum diameter possible is 90.00 mm, equal to the size of the petridish.

Relation of growth and stress conditions

Petridishes with medium KK1 were supplemented with 1 M NaCl, 1.5 M NaCl, 30 µg/ml SDS, 7.5 mM caffeine, 100 µg/ml CongoRed (all Carl Roth). Petridishes were inoculated with 1000 spores in 10 µl. Plates were incubated at 30°C (25°C for *Sporodiniella umbellata, Syzygites megalocarpus, Rhizopus sexualis*). The relative growth [%] compared to medium without stress inducers of 3 replicates at 24 h (48 h for *Sporodiniella umbellata, Syzygites megalocarpus*) is given in **Table S1**.

Carbon and nitrogen assimilation profiles

Agar plates with medium MM (0.5 % $(NH_4)_2SO_4$, 0.05 % $MgSO_4$, 0.1 % KH_2PO_4, 2 % agar), supplemented with 0.2 % carbon source were inoculated with 2×10^5 spores in 20 µl and incubated at 30°C (25°C for *Sporodiniella umbellata, Syzygites megalocarpus, Rhizopus sexualis*) for 3–4 d Evaluation of growth was performed visually and categorized in: inhibition (−), growth arrest after germination (0/−), no growth (0, but this includes ´background` growth due to carbon traces contained in the agar), slight or no growth (0/+, difficult to distinguish from the ´background` growth), weak growth (+), normal growth (++), strong growth (+++, similar to the glucose containing media), stronger growth (++++) (**Table S6**). Since *Syzygites* and *Sporodiniella* did not grow in appropriate time on this medium, a different medium (10 mM KH_2PO_4/K_2HPO_4 (pH6.6), 1.25 mM $MgSO_4{*}7H_2O$, 0.3 mM $ZnSO_4{*}7H_2O$, 0.09 mM $FeCl_3{*}6H_2O$, 0.03 mM $CuSO_4{*}5H_2O$ (all Carl Roth), modified after ref 52) was used to analyze a reduced second set of carbon sources. This time, liquid media was used in 96-well plates. Each well was supplemented with a carbon source and 500 spores. Plates were incubated for up to 6 d at optimal temperatures (37 °C, 30 °C, room temperature). Experiment was done up to 3 times, each time with triplicates; except for species where no differences between isolates were observed. In those cases only 2 repetitions were performed. After incubation the plates were analyzed visually for growth (p) or lack of growth (0). Weak growth was considered negative because of the difficulty in differentiation from background growth. Additionally chitin, pectin, citric acid and cellulose were tested as carbon sources. Results are supplemented in **Table S2**.

The liquid medium was also used to analyze the nitrogen utilization profile. Growth was evaluated after 70–88 h at appropriate growth temperatures. Results are shown in **Table S3**.

Size of sporangiospores and amount of spores

The size of the spores for each species was determined according to standard rules after harvest from KK1 medium cultivated for 5 d under optimal growth conditions (**Table 2**).

R. schipperae is known to produce fewer spores on artificial media. To assess the relative amount of spores produced in a specific period of time *R. schipperae* was cultivated on KK1 medium (petridish with 5.5 cm diam.) for 3 days, at appropriate temperatures (room temperature, 30°C or 37°C). Spores were harvested by extensive washing with PBS and counted in a haemocytometer. Mean spore burden out of 3 replicates is given in **Figure S3**.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

Acknowledgment

We thank Birgit Weber (HKI, Jena) for excellent technical assistance in the performance of the virulence tests in the embryonated hen egg model and Caroline Hörtnagl (Medical University Innsbruck) for valuable help with the *Galleria* infection model. We thank Domenica Schnabelrauch (MPI Chemical Ecology, Jena) for technical support in DNA sequencing.

Funding

Research of INy and TP was supported by the grants OTKA PD101613 and OTKA NN106394, respectively.

Supplemental Material

Supplemental data for this article can be accessed on the publisher's website

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