Note

Growth Characteristics of Four Fungal Species in Bathrooms

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The growth of four fungal species (four isolates) from bathrooms was examined under various environmental conditions. These fungi were found several times in bathrooms and washing machines, but not in other indoor environments such as house dust or windows. The four species (*Ramichloridium strelitziae*, *Cyphellophora laciniata*, *Phoma fimeti*, and *Exophiala* sp.) were identified using DNA and morphological analyses. These bathroom fungi were able to consume surfactants, soap and shampoo, but were unable to grow well in high-temperature or dry conditions. Soap and shampoo seem to affect fungal flora in the bathroom.

Key words : Bathrooms / Detergent / Fungal contamination.

Several genera of fungi with generally slow growth — *Exophiala*, *Phoma*, *Scolecobasidium*, *Phialophora*, and *Cladophialophora* — are more common in bathrooms than in other indoor areas such as air-conditioners or on windows, which are also humid and warm environments as bathrooms are (Hamada and Abe, 2008). Growth characteristics of common fungi in bathrooms were described in an earlier paper (Hamada and Abe, 2009) (see Table 1). The six predominant species — *Cladophialophora boppii*, *Exophiala spinifera*, *E. salmonis*, *Phialophora europaea*, *Phoma herbarum*, and *Scolecobasidium constrictum* — grew on media of 0.05, and 0.25% nonion surfactant, a component of shampoo, although common airborne indoor fungi such as *Aureobasidium* sp. and *Cladosporium cladosporioides* grew on media containing sodium fatty acids. These characteristic fungi found in bathrooms were able to use detergents as a nutrient. On the other hand, these fungi were unable to grow well in 35°C or on media with 15% NaCl, namely under high-temperature or dry conditions. Therefore, factors controlling fungi found commonly in bathrooms are apparently more specifically associated with nutrients than with environments.

Fungi found more frequently in bathrooms than in other areas were not only those six species. Other fungi that we had never found in other parts of the household were also found on several occasions in bathrooms and washing machines, where detergents, soaps, and shampoo are usually used.

In this study, four species of fungi isolated from bathroom walls or floors were identified as in an earlier study (Hamada and Abe, 2008). Moreover, their respective growth characteristics in culture on various media and under various environmental conditions were examined to confirm the factors determining fungal species growth in bathrooms.

Identification of four species of fungi according to their genetic and morphological characteristics was attempted in this study.

Genomic DNA from each mycelium sample was extracted and purified using the DNeasy Blood and Tissue Kit according to the manufacturer's instructions, as reported previously (Hamada and Abe, 2009). Subsequently, PCR was performed using the ITS1 (or V9D) and ITS4 primers, which amplify the region of the internal transcribed spacers 1 (ITS1) and 2 (ITS2) including the 5.8S ribosomal RNA gene (5.8SrDNA). The PCR products were gel-purified using a QIAquick Gel Extraction Kit and were sequenced in both directions using an ABI 3130 automated sequencer. Sequence chromatograms

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TABLE 1. Effects of three surfactants on the growth of common fungi in dwellings and bathrooms

Fungal species		(Standard)	Temperature*		NaCl*		SO (sodium fatty acid)**		Surfactant AE (non-ion surfactant)**	
		25°C	33°C	35°C	10%	15%	0.05%	0.25%	0.05%	0.25%
<i>Aureobasidium</i> sp. ^a	Count	40	28	13	34	0	34	13	0	0
	Size	+	+	±	—		+	±		
<i>Cladosporium cladosporioides</i> ^a	Count	40	0	0	40	22	46	8	0	0
	Size	+			+	±	+	±		
<i>Cladophialophora boppii</i> ^b	Count	40	0	0	0	0	34	0	25	26
	Size	+					+		+	+
<i>Exophiala spinifera</i> ^b	Count	40	6	0	0	0	39	30	26	21
	Size	+	—				+	±	+	+
<i>Exophiala salmonis</i> ^b	Count	40	0	0	0	0	43	37	46	24
	Size	+					+	±	+	+
<i>Phialophora europaea</i> ^b	Count	40	38	0	0	0	34	39	37	29
	Size	+	±		—		+	±	±	±
<i>Phoma herbarum</i> ^b	Count	40	41	0	21	0	37	26	37	24
	Size	+	±		±		+	+	±	—
<i>Scolecobasidium constrictum</i> ^b	Count	40	0	0	0	0	35	29	32	28
	Size	+					+	+	+	+

The growth of each fungus is indicated using the count and size of colonies.

^{a,b} in fungal species indicated common fungi in dwellings or in bathrooms.

The count of each fungal colony under various culture conditions was compared with that on 1/4PDA at 25°C indicated as 40.

*Colony size was compared with that on 1/4PDA at 25°C, and assessed as follows: +, same; ±, smaller; —, much smaller

**Colony size was compared with that on 0.05% SO, and assessed as follows: +, same; ±, smaller; —, much smaller

This table summarizes the results of an earlier study (Hamada and Abe, 2009).

from each strand were inspected using software (Sequencher ver. 4.1). Homology search using the obtained partial gene sequences was performed with FASTA at DDBJ (<http://www.ddbj.nig.ac.jp/search/fasta-j.html>).

The nucleotide sequence data reported in this paper are available in the International Nucleotide Sequence Database (INSD, GenBank/EMBL/DDBJ) under accession numbers AB488487-AB488490; those voucher cultures were also deposited in the JCM.

The following four species of fungi were collected from bathroom walls or floors (Hamada and Abe, 2008), identified and subjected to examination of growth characteristics: *Ramichloridium strelitziae* (NH102, AB488487, JCM16191), *Cyphellophora laciniata* (NH404, AB488488, JCM16192), *Phoma fimeti* (NH650, AB488489, JCM16193), and

Exophiala sp. (NH1238, AB488490, JCM16194).

After blocks of collected spores were diluted to 1,000 or 10,000 times, suspensions of spores of the four species were inoculated onto media for cultivation. About 25 ml of 1/4 potato-dextrose agar (PDA) was added onto a Petri dish and used as corresponding medium (Hamada and Abe, 2008).

Each fungus, growing slowly, was cultured for 8-14 days in an incubator maintained variously at a temperature of 25 (standard), 30°C, 33°C or 35°C. The temperature deviation was within 1.0°C (Hamada and Abe, 2009). A culture of 1/4 PDA with 5, 10, or 15% NaCl added was used to examine the tolerance of fungi to dry conditions. For comparison of the effect of NaCl, each fungus was incubated in darkness for 8-14 days at 25°C. A culture of 1/4 PDA was also used as the standard of acidic medium to examine the tolerance of fungi to alkali conditions. To adjust

FIG. 1. *Exophiala* sp. (JCM16194) ($\times 400$)
Spores: oval, often falcate.

the pH value of the medium to an alkali level, a modified version of the method of Nagai et al. (1998) was used. The pH of 1/4 PDA was 6.0 (same with 25°C); that of the alkaline 1/4 PDA was 9.7.

Moreover, media containing three main components of commercial detergents were used for cultivation. These components were used as culture media: SO (sodium oleate, sodium fatty acid); AE (polyoxyethylene (9) lauryl ether, nonion surfactant); and LAS (sodium dodecyl benzenesulfonate, anion surfactant), as used in an earlier study (Hamada and

Abe, 2009). These three surfactants were each adjusted to 0.01%, 0.05%, and 0.25% (0.10 g, 0.50 g, 2.5 g/L) and added to 15 g/L of Bacto agar. On various surfactant components, growth characteristics of those four fungi were compared in terms of counts, colony sizes, and colors. The fungi inoculated onto Petri dishes with various added surfactant components were incubated at 25°C for 12-16 days.

To count the colonies found on each medium, media with 10-100 colonies were selected from the cultures that had been inoculated at various levels of dilution. The fungal count of each species shown in the tables and detected on 1/4 PDA at 25°C under the corresponding conditions was around 40 colonies.

The predicted size fragment (approximately 500-700 bp, depending upon the isolate analyzed) was amplified using PCR in black yeast isolates (data not shown). Homology search using the obtained partial sequences revealed that the sequences of isolates NH102 (within 533 bp of overlap), NH 404 (within 597 bp of overlap), NH650 (within 539 bp of overlap), and NH1238 (within 601 bp of overlap) had similarity of 93.2%, 98.8%, 96.1%, and 98.8%, respectively, with *Ramichloridium strelitziae* (EU041803), *Cyphellophora laciniata* (EU035416), *Phoma fimeti* (AY531687), and *Exophiala* sp. (FJ665274).

TABLE 2. Effects of environmental factors on the growth of four fungi

Fungal species		(Standard)	Temperature			NaCl			pH
Factor			25°C	30°C	33°C	35°C	5 %	10%	
<i>Ramichloridium strelitziae</i>									
	Count	40	42	0	0	32	28	0	36
	Size	+	+			+	±		±
	Color	dark	+			+	+		+
<i>Cyphellophora laciniata</i>									
	Count	44	40	0	0	24	8	0	46
	Size	+	+			±	—		±
	Color	dark	+			+	+		+
<i>Phoma fimeti</i>									
	Count	55	55	30	0	34	0	0	53
	Size	+	+	±		±			+
	Color	dark	+	+		+			+
<i>Exophiala</i> sp.									
	Count	41	34	0	0	38	0	0	25
	Size	+	+			±			+
	Color	dark	+			+			+

Fungal count indicates the total number of colonies detected on each of two Petri dishes.

Size of colonies was compared with that on 1/4PDA at 25°C, pH 6.0, and assessed as follows: ++, larger; +, same; ±, smaller; —, much smaller

Color of fungi was compared with that on 1/4PDA at 25°C, pH 6.0, and assessed as follows: ++, darker; +, same; ±, lighter

A direct preparation of dark mold isolated and cultured on a slant appeared dark under microscopic observation. This fungus was identified morphologically using a fungi monograph (de Hoog et al., 2000; Arzanlou et al., 2007; Boerema et al., 2004), *Ramichloridium strelitziae* were colonies with red pigments, with unbranched erect brown conidiophores, and subhyaline aseptate clavate conidia. *Cyphellophora laciniata* were dark colonies, with intercalary phialides, sickle-shaped conidia, and dark 1-3 septate. *Phoma fimeti* were slowly growing colonies with gray aerial mycelia, pycnidia subglobose with 60-160 μm diameter and ellipsoidal conidia. *Exophiala* sp. (see Fig. 1) were dark velvety colonies, with cylindrical conidiogenous cells, and oval to falcate conidia. *Exophiala* sp. was identified as *E. equina* by Dr. de Hoog, CBS (personal communication, soon to be published).

The colony counts of four species cultured at 30 °C were similar to those at 25 °C (Table 2). The sizes and color of all species that were examined was similar at 25 °C and 30 °C. At 33 °C, only colonies of *P. fimeti* were detected, although the counts and sizes of colonies were smaller. Colonies of four species were detected on media with 5% NaCl, but their colony count was smaller than on media without NaCl. Moreover, only colonies of *R. strelitziae* and *C. laciniata* were detected on media with 10% NaCl;

those counts and sizes decreased with the addition of NaCl. In the three fungi – *R. strelitziae*, *C. laciniata* and *P. fimeti* – the colony counts were approximately similar on media of pH 6.0 and pH 9.7. The sizes of *P. fimeti* and *Exophiala* sp. were similar on media of both pH, although the colors of all four species were similar on those media.

The effects of the sodium fatty acid SO on the growth of the four fungi were examined (Table 3). Colonies of all examined species were detected on media of 0.01%, 0.05%, and 0.25% SO within 16 days. The colony count on media of 0.25% SO compared to that on 1/4 PDA was lower in four species, and less than one-third of that on 1/4 PDA for *C. laciniata* and *P. fimeti*. Moreover, the colonies of *C. laciniata* were much smaller than on 0.05% SO. However, the color of colonies of *R. strelitziae* and *P. fimeti* darkened concomitantly with increased SO concentration.

No *C. laciniata* colony was detected on 0.25% AE, but colonies of *R. strelitziae*, *P. fimeti*, and *Exophiala* sp. on 0.25% AE were detected, although the colony count in *P. fimeti* was also lower.

The color of the colonies of *R. strelitziae* was darker on 0.05% AE than on 0.01% AE, and colonies on 0.25% AE were darker than those on 0.05% AE (Table 3), different from *P. fimeti* with a color on 0.25% AE that was lighter than on 0.05% AE (Fig. 2).

TABLE 3. Effects of three surfactants on the growth of four fungi

Fungal species		SO (sodium fatty acid)			AE (nonion surfactant)			LAS (anion surfactant)		
Surfactant		0.01%	0.05%	0.25%	0.01%	0.05%	0.25%	0.01%	0.05%	0.25%
<i>Ramichloridium strelitziae</i>										
	Count	36	42	28	38	26	34	42	0	0
	Size	+	+	±	±	±	±	±		
	Color	±	dark	++	—	±	+	—		
<i>Cyphellophora laciniata</i>										
	Count	36	20	12	0	0	0	30	0	0
	Size	+	+	—				—		
	Color	±	dark	+				+		
<i>Phoma fimeti</i>										
	Count	44	22	8	48	28	9	48	0	0
	Size	±	+	+	+	+	±	—		
	Color	±	dark	++	+	+	±	+		
<i>Exophiala</i> sp.										
	Count	38	43	22	32	34	22	33	0	0
	Size	+	+	±	+	+	+	—		
	Color	±	dark	+	±	±	±	±		

Fungal count shows the total number of colonies detected on each of two Petri dishes, on which the suspension of spores used in Table 2 was inoculated.

Colony size was compared with that on 0.05% SO, and assessed as follows: ++, larger; +, same; ±, smaller; –, much smaller

Color of fungi was compared with that on 0.05% SO and assessed as follows: ++, darker; +, same; ±, lighter

FIG. 2. Growth of *Phoma fimeti* (JCM16193) cultured on AE (nonion surfactant) medium for 14 days. Left, 0.01% AE; center, 0.05% AE; right, 0.25% AE

The colony counts of fungi were 30-48 on 0.01% LAS and were similar to those recorded on the other surfactant media, 0.01% SO and 0.01% AE. The colony, however, was smaller than on the other media, irrespective of the fungal species that was examined.

All four species examined in this study were grown on media of 0.25% SO and with pH 9.7 (Table 2). Therefore, all four fungi that were examined are apparently alkali-tolerant. Those fungi are thought to use soap as a nutrient. Soap apparently has a strong effect on fungal contamination in bathrooms.

The three species, *R. strelitziae*, *P. fimeti*, and *Exophiala* sp., grew on 0.25% AE media (Table 3), as did six species using detergent described in an earlier report (Hamada and Abe, 2008). The fungi that grow on 0.25% AE or 0.25% SO media would seem therefore to make use of the shampoo or soap adhering to or condensed on the walls and floors. Shampoo containing nonion surfactant is therefore thought to have a clear effect on the fungal flora in bathrooms. Some fungi in bathrooms use shampoo, a synthetic surfactant, as a nutrient, which is interesting from an evolutionary viewpoint.

However, the growth rate of *R. strelitziae* and *P. fimeti* on 0.25% AE was lower than on 0.05% SO, different from most bathroom fungi described before (Table 1). Consequently, these fungi were thought to be not as common in bathrooms as the previous six species. It is notable that *C. laciniata* cannot use nonion surfactant, different from the other three fungi. Moreover, *C. laciniata* grows less well than the other three fungi on soap medium: the colonies were much smaller on 0.25% SO. In fact, *C. laciniata* was often

found in both bathrooms and washing machines, although *C. laciniata* was misidentified as *Fusariella* sp. in some earlier studies (Hamada and Abe, 2008; Hamada, 2005). Why *C. laciniata*, which grows slowly, is often found in bathrooms and washing machines remains unclear.

However, fungi were unable to grow on anion medium, including more than 0.01% LAS, which is similar to the 13 most common species collected and examined (Hamada and Abe, 2009).

The four species examined in this study did not grow at 35°C (high temperature) or on media with 15% NaCl (osmotic conditions), as did the six species of bathroom fungi investigated in the previous study (Table 1). These fungi seemed to prefer water at temperatures lower than 30°C, and did not flourish with hot water used in bathtubs or in house dust in dwellings as well as changeable outdoor environments. Consequently, these four fungi, growing slowly, might have difficulty surviving in environments other than most places associated with detergent use such as those of bathrooms, washing machines, and sinks of kitchens.

This study of characteristics of common bathroom fungi suggests a means of controlling fungal contamination in bathrooms. Washing away soap and shampoo, which provide nutrients for fungal contamination, is most important. Drying treatments in bathrooms are also thought to be effective for eliminating contamination.

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