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Submicronic Fungal Bioaerosols: High-Resolution Microscopic Characterization and Quantification

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Submicronic particles released from fungal cultures have been suggested to be additional sources of personal exposure in mold-contaminated buildings. *In vitro* generation of these particles has been studied with particle counters, eventually supplemented by autofluorescence, that recognize fragments by size and discriminate biotic from abiotic particles. However, the fungal origin of submicronic particles remains unclear. In this study, submicronic fungal particles derived from *Aspergillus fumigatus*, *A. versicolor*, and *Penicillium chrysogenum* cultures grown on agar and gypsum board were aerosolized and enumerated using field emission scanning electron microscopy (FESEM). A novel bioaerosol generator and a fungal spores source strength tester were compared at 12 and 20 liters min^{-1} airflow. The overall median numbers of aerosolized submicronic particles were 2×10^5 cm^{-2}, 2.6×10^3 cm^{-2}, and 0.9×10^3 cm^{-2} for *A. fumigatus*, *A. versicolor*, and *P. chrysogenum*, respectively. *A. fumigatus* released significantly ($P < 0.001$) more particles than *A. versicolor* and *P. chrysogenum*. The ratios of submicronic fragments to larger particles, regardless of media type, were 1:3, 5:1, and 1:2 for *A. fumigatus*, *A. versicolor*, and *P. chrysogenum*, respectively. Spore fragments identified by the presence of rodlets amounted to 13%, 2%, and 0% of the submicronic particles released from *A. fumigatus*, *A. versicolor*, and *P. chrysogenum*, respectively. Submicronic particles with and without rodlets were also aerosolized from cultures grown on cellophane-covered media, indirectly confirming their fungal origin. Both hyphae and conidia could fragment into submicronic particles and aerosolize *in vitro*. These findings further highlight the potential contribution of fungal fragments to personal fungal exposure.

Some saprophytic fungi can colonize building materials and other materials in indoor environments with high humidity. Such invasion is commonly associated with poor indoor air quality and health complaints among exposed people (1, 2). Inhaled fungal spores have been recognized as irritants and allergens and may elicit lung function changes, hypersensitivity pneumonitis, organic dust syndrome, and chronic bronchitis, asthma, and rhinitis (3, 4). However, estimated indoor levels of fungal spores reported in the peer-reviewed literature appear to be too low to explain adverse health effects (4).

Experiments using automatic particle counters have revealed the aerosolization of particles smaller than conidia when agar or building materials colonized by cultures of *Aspergillus versicolor*, *Trichoderma harzianum*, *Ulocladium* spp., *Penicillium melinii*, *Cladosporium cladosporioides*, *Stachybotrys chartarum*, *Botrytis cinerea*, or *Rhizopus* sp. were subjected to air jets under controlled conditions (5–15). These particles have been suggested to be fungal fragments and important sources of allergens (16–18), antigens (7), (1→3)-beta-D-glucans (19–25), and mycotoxins (26, 27). Exposure to fungal submicronic particles may therefore provide an explanation for health effects observed in moldy indoor environments (28).

Aerosolization of fine particles from mold-contaminated building materials by air jets or from cultures grown on agar media has been reported using a variety of automatic particle counters (5, 7, 8, 11, 13, 21–23). Automatic particle counters are based on particle acceleration, electric static charge pulses, or light scattering techniques to count particles as small as 6 nm in diameter. With these instruments, fungal spores could be discriminated from other particles by their limited size range. Furthermore, some instruments apply autofluorescence to separate abiotic particles from biotic particles. However, they cannot morphologically distinguish spores, spore aggregates (SA), hyphal fragments, and fragmented spores.

Alternative approaches to characterizing fungal fragments have used the detection of fungal biomarkers, including (1→3)-beta-D-glucans (10, 19, 21, 23, 25, 29), N-acetyl-glucosaminase (23), fungal antigens (7), and mycotoxins (26, 27), in the submicronic particle fractions (21, 22, 25, 30, 31). Some of these studies were confounded by imperfect size separation of the systems utilized since the presence of larger particles such as spores has been demonstrated in the assumed submicronic fraction (10, 23, 29). Furthermore, (1→3)-beta-D-glucans in environmental samples could derive from bacteria and plants, including algae (32, 33). Although detection of fungal biomarkers may provide more-direct evidence of airborne fungal fragments, it is also possible that these particles originate from the substrate as well as from the fungus (12). Moreover, fungal enzymes, including N-acetyl-glucosaminase, fungal antigens, and mycotoxins, may be released into and lead to the deterioration of the substrate (34) and therefore to the release of fragments. These limitations emphasize the

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need for an alternative approach for identification and quantification of submicronic particles (35, 36).

Large hyphal fragments have been observed in indoor air samples by microscopy following immunostaining (4, 17, 18, 36). Scanning electron microscopy (SEM) has been used to resolve and quantify fungal and actinomycete spores (38–40, 56) as well as large hyphal fragments (41). SEM has also been used to qualitatively assess submicronic particles (7, 13, 26). However, those studies did not enumerate or determine the fungal origin of the submicronic particles. Recognition of the fungal origin of these particles is essential to improve our understanding of the potential adverse health effects associated with exposure to these particles, as experimental studies have shown that fungal hyphae and spores have different toxic properties (4, 42, 43).

Therefore, the present study was initiated to characterize submicronic particles aerosolized from fungal cultures by air jets using field emission SEM (FESEM). Furthermore, submicronic particles were classified among larger fungal particles and enumerated with respect to experimental parameters, including biotic factors (species, growth medium, and culture age) and abiotic factors (generator and airflow).

MATERIALS AND METHODS

Fungal cultures. Fungal isolates evaluated in this study included *A. versicolor* (Vuillenium) Tirobaschi 1908 (strain VI 03554), *A. fumigatus* Fresenius 1863 (strain A1258 FGSC), and *P. chrysogenum* Thom 1910 (strain VI 04528). *A. versicolor* and *P. chrysogenum* were chosen because these species are common in the indoor environment (44). *A. fumigatus* was chosen because this species has been studied extensively as a causal agent of aspergilliosis. *A. versicolor* and *P. chrysogenum* strains were kindly provided by the Section of Mycology at the National Veterinary Institute of Norway. The *A. fumigatus* strain was purchased from Fungal Genetic Stock Center (University of Missouri, Kansas City, KS).

Fungal inocula were prepared by gently scraping 2-week-old cultures grown on 2% malt extract agar (MEA) medium (45) and were submerged in 20 ml sterile phosphate-buffered saline (PBS) containing 0.1% Tween 20. The spore suspensions were collected into 50-ml centrifuge tubes and resuspended by vortex mixing 2 times for 30 s each time followed by sonication for 3 min in an ultrasonic bath (Sonorex RK 510H; Bandalin Electric, Berlin, Germany) at 35 kHz. The suspension was filtered through a 10-μm-pore-size nylon mesh filter (Millipore, Tullagreen Cork, Ireland) and centrifuged (model 4k15; Sigma, Osterode, Germany) (at 1,500 × *g* for 5 min), and the pellet was resuspended in 30 ml sterile Milli-Q water containing 10% glycerol. The spore suspension was kept at 4 ± 1°C until inoculation. The concentration of spores was determined by filtration of 0.1 ml of the suspension through a 25-mm-diameter polycarbonate filter (Millipore, Tullagreen Cork, Ireland) (0.4 μm pore size) and enumeration by FESEM.

For aerosolization experiments, fungal cultures were grown on agar medium or on gypsum board discs (GB). Agar media included 2% MEA, which was chosen because it supports increased biomass production as previously described in several other reports (8, 13, 14). MEAC, i.e., MEA (2%) covered with a cellophane membrane (Visella Oy, Valkeakoski, Finland), was designed as an internal control to prevent potential aerosolization of colonized growth medium and was developed for pure fungal biomass production (46, 47). Gypsum boards (common indoor building material; Coop Bygg, Vinterbro, Norway) were cut into 80-mm-diameter discs, soaked with water for 16 h, autoclaved at 132°C for 1 h, and placed in 90-mm-diameter sterile petri dishes (10). MEA dishes were inoculated with 0.1 ml of the spore suspension containing approximately 1×10^6 spores and gypsum boards with 1 ml of the same inoculum. All inoculated petri dishes were sealed with parafilm and incubated at 25 ± 1°C and 45% ± 5% relative humidity (RH). MEA and MEAC were incubated for 2 and 8 weeks and GB for 8 weeks.

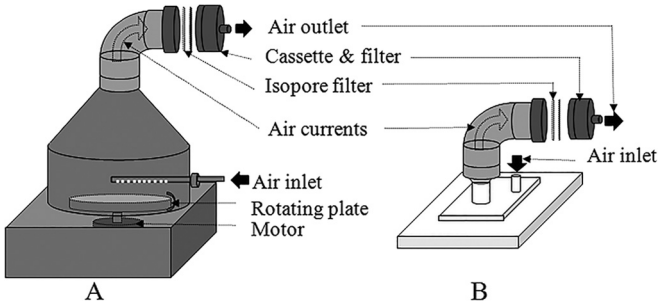

FIG 1 Instruments used for aerosolization and collection of particles from fungal cultures. (A) Stami particle generator (SPG). (B) Fungal spores source strength tester (FSSST).

Instrumentation for generation of fungal particles. Two particle generators were evaluated in this study. The fungal spore source strength tester (FSSST) is a portable bioaerosol generator developed at the University of Cincinnati (48) and has been previously described elsewhere (10, 11, 21, 27). The second generator is a novel generator fabricated in-house termed the Stami particle generator (SPG) that included design features from the generators described by Kildesø et al. (5), Scheermeyer and Agranovski (12), and Lee et al. (13).

The SPG (Fig. 1A) was fabricated at the National Institute of Occupational Health, Norway (NIOH), out of aluminum with smooth joints in order to minimize particle loss from static charging and turbulent flow. The internal cross-sectional area is 156 cm^2. Particles were aerosolized by air jets from a tube outfitted with 10 nozzles of 1.2 mm diameter mounted 16 mm above the culture surface. Air jets were directed perpendicularly to the culture plates. During aerosolization, culture plates were rotated by means of a time-regulated direct current (DC) motor with angular velocity adjustable from 0.05 to 2 rpm. The aerosol was directed through a 90° stainless steel bend onto an in-line 37-mm-diameter polycarbonate filter (Isopore; Millipore, Ireland) with 0.4 μm pore size in an open-face standard aerosol cassette made out of conductive polypropylene (SKC Inc., Eighty Four, PA).

The FSSST (Fig. 1B) is constructed out of polyvinyl chloride with a square internal area of 144 cm^2. Particles were liberated by air jets through 112 orifices of 0.4 mm diameter situated at a distance of 16 mm above and directed perpendicularly to the culture surface. Released particles were aerosolized onto a polycarbonate filter as described above.

The experimental setup is shown in Fig. 2. Airflow was provided by a rotary vane pump (model G24/27; Gardner Denver Thomas, Bandhagen, Sweden) and controlled by a computer system developed at NIOH. Inlet and outlet air was filtered using HEPA filtration (Hepa Versapor capsules; Pall Corporation, Port Washington, NY), and flow rates were monitored by two mass flow meters (822 Top-Trak; Sierra Instruments, Monterey, CA) mounted before and after the generator. Electrostatic charging was reduced by an in-line alpha ionizer (model P-2021; NRD LLC, Grand Island, NY). Air temperature and RH were monitored in real time using a Picolog 1261 sensor (model AV095/086; Picotech, Tyler, TX). The RH was maintained at 18.8 ± 0.4% using a silica gel in-line dryer (model L144; Air Sentry, Rockwall, TX).

Aerosolization and collection of fungal particles. Experiments were conducted on 2- and 8-week-old cultures grown on agar media (MEA and MEAC). In contrast, only 8-week-old GB cultures were evaluated as fungal growth was not observed on GB after 2 weeks. Flow rates of 12 and 20 liters min^{-1} were applied to each set of culture plates. The tested airflows at the orifices were theoretically equivalent to air velocities of 18 and 29 m s^{-1} in the SPG and 14 and 23 m s^{-1} in the FSSST, respectively. Particles were aerosolized for 120 s at 0.5 rpm from *A. versicolor* and *P. chrysogenum* cultures. To avoid filter overloading in *A. fumigatus* experiments, the

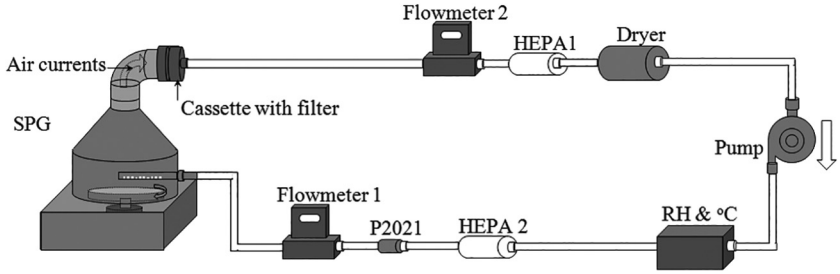

FIG 2 Experimental setup. The generator (SPG) with mounted cassette is connected to the system setup. Flowmeters 1 and 2 measure the airflows at the inlet and outlet of the generator, respectively. HEPA 1 and HEPA 2 filter the airflows from the outlet and to the inlet, respectively. The dryer (in-line tube containing silica gel) maintains a constant relative humidity in the air at the inlet, and the in-line P2021 ionizer reduces electrostatic charges.

aerosolized culture surface was reduced by the use of a cover plate with a central hole of 1 cm diameter, and cultures were aerosolized for 60 s at 1 rpm. The FSSST was operated without rotation of the culture plates. In both generators, culture plates were placed 16 mm below the orifices. All experiments were conducted in a Bio Safe Grade II laminar flow hood (Scanlaf Mars Labogene, Lynge, Denmark). The generator chamber was cleaned with 70% ethanol and purged with HEPA-filtered air prior to each experiment. A total of 16 blank experiments were performed at 12 and 20 liters min^{-1} in an empty chamber during 120 s. The blank experiments were designed to correct for background particles prior to aerosolization experiments.

Sample preparation and analysis by high-resolution FESEM. A quarter-segment (ca. 2.4 cm^2) of the filter specimen was cut from the collection filter and mounted on a 25-mm-diameter aluminum pin stub (Agar Scientific Ltd., Stansted Essex, United Kingdom) using double-sided carbon adhesive discs (Ted Pella Inc., Redding, CA) in a sterile laminar airflow cabinet. Samples were coated with platinum during 20 to 30 s with 40 mA current in a 4×10^{-1} mbar vacuum using a Balzers SDC 050 sputter coater (Balzers, Liechtenstein). The coating time corresponds to a 6-to-10-nm-thick platinum layer according to the calibration curves provided by the manufacturer.

Samples were viewed using a SU 6600 FESEM (Hitachi, Ibaraki-ken, Japan) in the secondary electron imaging (SEI) mode. For the enumeration, the microscope was operated at an acceleration voltage of 15 keV, an extraction voltage of 1.8 kV, and a working distance of 10 mm. Particles were quantified using the counting criteria described by Eduard and Aalen (37). Particles were assumed to be homogenously distributed on the filter, as that has been previously demonstrated for fungal spores collected in electrically conducting filter holders (49).

Particles were recognized by their morphological features as fragments, spores, and spore aggregates, sized by length, and further classified as submicronic particles (0.2 to 1 μm diameter) and larger particles (>1 μm diameter). Four hundred particles or a maximum of 100 fields were counted at ×3,000 to ×10,000 magnification depending on the particle density on the filter and type of particle. The number of particles per culture area (cm^2) was calculated by dividing the total number of particles on the filter by the exposed culture area. The minimal detectable particle numbers were 8×10^3 and 9×10^4 particles per exposed filter at ×3,000 magnification and ×10,000 magnification, respectively.

The outer cell wall rodlet layer is characteristic of hydrophobins found on the fungal conidial surface (50, 51) and was used to confirm the fungal spore origin of submicronic particles. For classification of particles originating from hyphal biomass, the morphology of freeze-dried mycelial biomass was used for comparison. Morphological classification of 100 randomly selected submicronic particles was conducted at high magnification (×100,000 to ×300,000), an acceleration voltage of 25 keV, and a working distance of 5 to 6 mm.

Experimental design and data analysis. For each fungal species, comparative experiments were run on 2- and 8-week cultures grown on MEA and MEAC and on 8-week cultures grown on GB. The two generators and two airflows were compared in a total of 178 aerosolization experiments. Each combination of these factors was run in triplicate. Particle counts were adjusted for blanks and are described by medians and 25th and 75th percentiles because the data were not normally distributed. Negative values after blank adjustment were assigned a value of zero. Blank counts for submicronic fragments (SF) ranged from 160 to 4,300 particles per cm^2. The effects of experimental factors, including generator, flow, and culture age, on the number of submicronic fragments were compared using the nonparametric Wilcoxon-rank sum test (Mann-Whitney U tests). The effects of species and media were tested by the Kruskal-Wallis test for multiple categories followed by *post hoc* Wilcoxon rank sum tests. The biotic factors (medium and culture age) and abiotic factors (generator and airflow) were individually compared because of the factorial design. A two-sided P value of 0.05 was regarded as statistically significant. For multiple *post hoc* comparisons, the P value of 0.05 was Bonferroni corrected (significance level for 3 comparisons = 0.017). STATA SE 12 (Statacorp LP, College Station, TX) was used for statistical analysis.

RESULTS

Morphology of fungal particles. Figure 3 shows the particle types aerosolized from fungal cultures, including single spores and spore aggregates (Fig. 3A, B, and C), a submicronic particle (Fig. 3A, arrow a), and a larger particle (Fig. 3D, arrow b). Disrupted spores were observed in some of the experiments, indicating that abiotic forces on spores could have been large enough to shear conidia into smaller fragments during aerosolization (Fig. 3D). A spore with typical rodlet structure is shown in Fig. 3E. Submicronic particles with rodlet structures were observed and regarded as being specific for spore fragments (Fig. 4A and B). Fragments without rodlet structures (Fig. 4C, D, and E) may have derived from hyphae, to which they were morphologically similar, or they may have derived from the substrate, as no fungal phenotypic features were observed.

Aerosolized spores and fragments. Collected spores and fragments were identified and classified into four groups: single spores (SS), spore aggregates (SA), larger fragments (>1 μm) (LF), and submicronic fragments (0.2 to 1 μm) (SF). The overall numbers of all particle types with respect to species are summarized in Table 1. The median number of particles per cm^2 ranged between 0.93×10^3 cm^{-2} and 2.0×10^5 cm^{-2} for SF; 0 and 42×10^3 cm^{-2} for LF; 0 and 330×10^3 cm^{-2} for SS; and 0.094×10^3 cm^{-2} and 200×10^3 cm^{-2} for SA. Significant differences between species were observed for all particle types ($P < 0.001$), showing that these particles were aerosolized in much higher numbers (median, 42×10^3 to 200×10^3 cm^{-2}) from *A. fumigatus* cultures than from *A. versicolor* cultures (median, 0 to 2.6×10^3 cm^{-2}) and *P. chrysogenum* cultures (median, 0 to 1.2×10^3 cm^{-2}). Significantly more

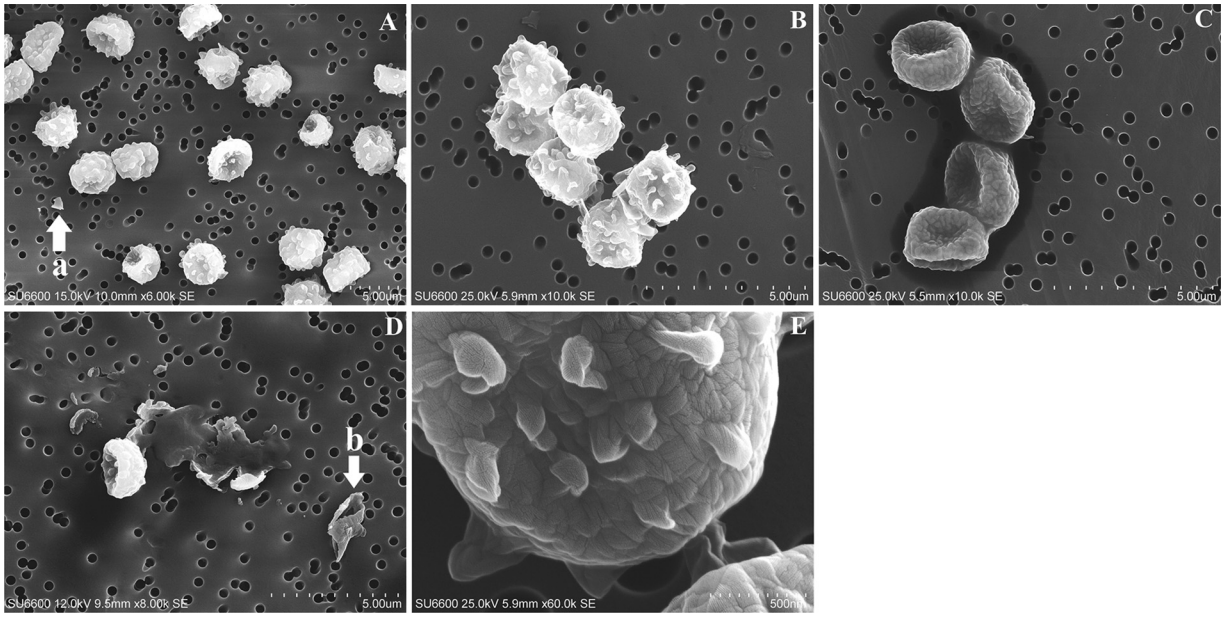

FIG 3 Micrographs of aerosolized particles. (A) Warted to spiny spores from *A. fumigatus* (8-week-old cultures grown on GB and aerosolized by the use of the FSSST at 12 liters min^{-1}). (B) Spiny spores from *A. versicolor* (2-week-old cultures grown on MEAC and aerosolized by the use of the SPG at 12 liters min^{-1}). (C) Rugose spores from *P. chrysogenum* (2-week-old cultures grown on MEAC and aerosolized by the use of the SPG at 12 liters min^{-1}). (D) Shattered spores of *A. fumigatus* (8-week-old cultures grown on GB and aerosolized by the use of the FSSST at 12 liters min^{-1}). (E) Spore with rodlet structure from *A. versicolor* (2-week-old cultures grown on MEAC and aerosolized by the use of the SPG at 12 liters min^{-1}). Arrows shows submicronic (a) and larger (b) particles on the filter membrane. SE, secondary electron.

single spores and spore aggregates were aerosolized from *P. chrysogenum* than from *A. versicolor* ($P < 0.001$). Due to the major differences observed between species, further analyses were performed separately for each species.

Effects of biotic parameters on the emission of submicronic particles. The effects of culture media and age are summarized in Table 2. These experiments revealed that the number of SF collected from cultures grown on MEA and MEAC were not statistically different when cultures of the three isolates were 2 weeks old. However, significant differences were revealed for older (8-week) cultures of *A. fumigatus* and *A. versicolor* (P values, <0.01 and <0.001, respectively). For *A. fumigatus*, the number of SF generated from MEAC (median, 570×10^3 cm^{-2}) was significantly greater than the number generated from MEA (median, 1.2×10^3 cm^{-2}) ($P < 0.01$). The difference between GB results (median, 21×10^3 cm^{-2}) and MEAC results was also significant ($P < 0.01$),

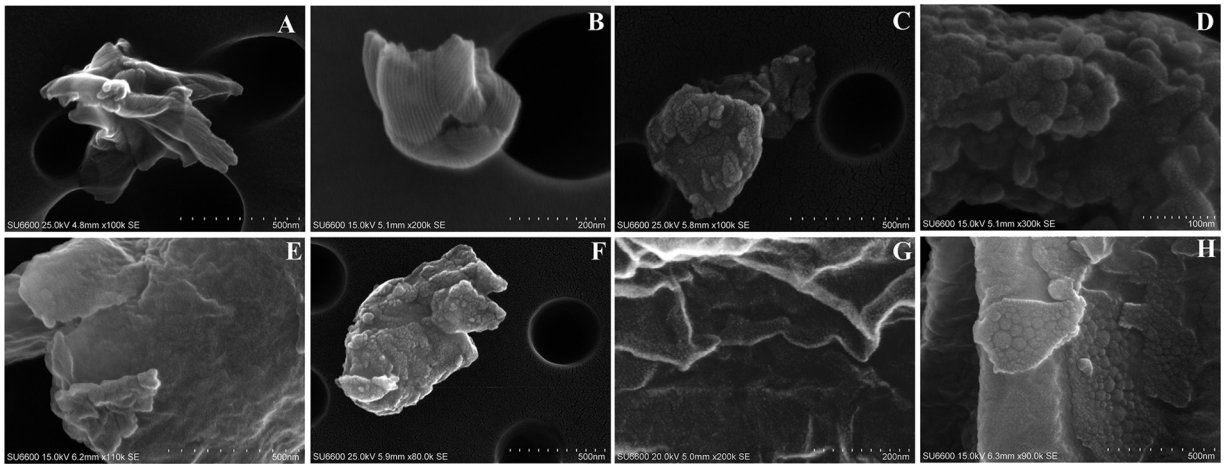

FIG 4 (A to E) Surface structures of submicronic fragments with rodlets from *A. versicolor* (2-week-old cultures grown on MEA and aerosolized by the use of the SPG at 12 liters min^{-1}) (A) and *A. fumigatus* (2-week-old cultures grown on MEA and aerosolized by the use of the SPG at 20 liters min^{-1}) (B) and surface structures of submicronic fragments without rodlets from *A. versicolor* (2-week-old cultures grown on MEAC and aerosolized by the use of the SPG at 12 liters min^{-1}) (C), *A. fumigatus* (2-week-old cultures grown on MEA and aerosolized by the use of the SPG at 12 liters min^{-1}) (D), and *P. chrysogenum* (2-week-old cultures grown on MEAC and aerosolized by the use of the SPG at 12 liters min^{-1}) (E). (F to H) Control micrographs of freeze-dried hyphal fragments without rodlets from *A. versicolor* (F), *A. fumigatus* (G), and *P. chrysogenum* (H).

TABLE 1 Particles generated from *A. fumigatus*, *A. versicolor*, and *P. chrysogenum* cultures differentiated by particle type[a]

Species	*n*	Submicronic fragments			Large fragments			Single spores			Spore aggregates		
		Median no.	25th and 75th percentiles	K-W test *P* value	Median no.	25th and 75th percentiles	K-W test *P* value	Median no.	25th and 75th percentiles	K-W test *P* value	Median no.	25th and 75th percentiles	K-W test *P* value
A. fumigatus	59	200^{ab}	7.4 and 410	<0.001	42^{cd}	0.00 and 85	<0.001	330^{ef}	21 and 1,200	<0.001	200^{hi}	11 and 640	<0.001
A. versicolor	60	2.6^{a}	0.60 and 5.0		0.40^{c}	0.00 and 1.0		0.00^{eg}	0.00 and 0.3		0.090^{hj}	0.00 and 0.50	
P. chrysogenum	59	0.93^{b}	0.00 and 3.0		0.00^{d}	0.00 and 0.90		0.96^{fg}	0.00 and 5.5		1.2^{ij}	0.10 and 11	

[a] Data represent 10^3 particles cm^{-2}. Results represent combined data from experiments performed under different media, culture age, airflow, and generator conditions. *n* = number of repeated experiments grouped by fungal species; K-W, Kruskal-Wallis. Superscript roman letters a to j indicate the results of *post hoc* Wilcoxon rank sum tests of differences between species: medians with same letter are significantly different ($P \leq 0.001$).

but the difference between GB and MEA results was not. For *A. versicolor*, the highest SF numbers were released from MEA and GB (medians, 5.3×10^3 cm^{-2} and 4.7×10^3 cm^{-2}, respectively) compared to MEAC (0.74×10^3 cm^{-2}). Comparisons between MEAC and MEA and between MEAC and GB revealed significant differences ($P \leq 0.001$).

The effects of culture age were statistically significant for *A. versicolor* grown on MEA (median, 1.3×10^3 cm^{-2} for 2 weeks of growth versus 5.3×10^3 cm^{-2} for 8 weeks of growth; $P < 0.05$) and for *P. chrysogenum* (median, 0.5×10^3 cm^{-2} for 2 weeks of growth versus 2.8×10^3 cm^{-2} for 8 weeks of growth; $P < 0.05$). For *A. fumigatus* grown on MEAC, significantly higher numbers of SF were obtained from 8-week-old cultures (median, 570×10^3 cm^{-2}) than from 2-week-old cultures (130×10^3 cm^{-2}) ($P < 0.01$).

Effects of abiotic factors on the emission of submicronic particles. The effects of generator and airflow are shown in Table 3. No significantly different numbers of SF aerosolized from *Aspergillus* cultures were observed. The *P. chrysogenum* cultures were significantly affected by the generator and airflows used. Higher numbers of SF were released in the FSSST (median, 1.8×10^3 cm^{-2}) than from the SPG (median, 0.1×10^3 cm^{-2}) at 12 liters min^{-1} ($P = 0.01$). At 20 liters min^{-1}, the opposite trend was observed, with higher numbers of SF from SPG (median, 2.1×10^3 cm^{-2}) than from FSSST (median, 0.0 cm^{-2}) ($P < 0.01$). Increased airflow generated more SF in the SPG (median, 0.1×10^3 cm^{-2} at 12 liters min^{-1} versus 2.1×10^3 cm^{-2} at 20 liters min^{-1}) ($P < 0.01$), while fewer SF were observed in the FSSST (median, 1.8×10^3 cm^{-2} at 12 liters min^{-1} versus 0.0×10^3 cm^{-2} at 20 liters min^{-1}) ($P = 0.001$).

Origin of submicronic particles. High-resolution FESEM of SF revealed that most submicronic particles had a surface structure similar to that of the vegetative biomass (Fig. 4F, G, and H). Thirteen percent of the SF released from *A. fumigatus* showed rodlet structure, confirming a conidial origin (Fig. 4B). This proportion was only 2% for *A. versicolor* cultures, while no submicronic fragments with rodlet structure were observed for *P. chrysogenum*.

DISCUSSION

The detection of aerosolized fragments that contained ornamentation of conidium walls provides direct evidence of the presence of fungal fragments among the submicronic particles. The results further suggest that submicronic particles mainly originate from the hyphal biomass and not from the substrate. These data provide new insights into the aerosolization of fungal bioaerosols, as studies using automatic particle counters or detection of biomarkers could not reveal the nature of submicronic particles. Our findings additionally confirm assumptions of researchers in previous studies that submicronic particles released from fungal cultures originate from the fungal biomass and not from the inoculated substrate (5–8, 11, 13–15, 21, 23, 26), but we cannot exclude the possibility that the colonized medium also contributes, as suggested by Scheermeyer and Agranovski (12).

A number of studies have suggested that submicronic particles originate from conidial and hyphal fragmentation. Madsen and coworkers hypothesized that these particles were derived from mycelial autolysis (23), while Kanaani and colleagues associated their origin with spore fragmentation (11). The observation of submicronic particles with rodlet structures indicates that some of

TABLE 2 Effects of culture media and age on submicronic particles generated from *A. fumigatus*, *A. versicolor*, and *P. chrysogenum*[a]

Biotic factors	*A. fumigatus*				*A. versicolor*				*P. chrysogenum*			
	n	Median	25th and 75th percentiles	K-W test *P* value	*n*	Median	25th and 75th percentiles	K-W test *P* value	*n*	Median	25th and 75th percentiles	K-W test *P* value
Age and medium												
2 wks												
MEA	12	270	130 and 450	0.2	12	1.3	0.0 and 4.3	0.6	11	0.50	0.01 and 1.20	0.5
MEAC	11	130	81 and 240		12	2.5	0.8 and 3.4		12	0.097	0.00 and 0.80	
8 wks												
GB	12	21[b]	0.40 and 380	**0.003**	12	4.7[d]	2.5 and 8.7	**0.001**	12	2.8	1.1 and 11	0.3
MEA	12	1.2[a]	0.02 and 320		12	5.3[c]	3.0 and 8.1		11	2.8	0.80 and 4.7	
MEAC	12	570[ab]	260 and 1,500		12	0.74[cd]	0.06 and 2.0		12	0.57	0.00 and 86	
Medium and age												
MEA												
2 wks	12	270	130 and 450	0.06	12	1.3	0.0 and 4.3	**0.03**	12	0.5	0.014 and 1.2	**0.01**
8 wks	12	1.2	0.02 and 320		12	5.3	3.0 and 8.1		11	2.8	0.9 and 4.7	
MEAC												
2 wks	11	130	81 and 240	**0.003**	12	2.5	0.8 and 3.4	0.07	12	0.097	0.0 and 0.8	0.5
8 wks	12	570	260 and 1,500		12	1.7	0.06 and 2.0		12	0.6	0.0 and 86	

[a] Data represent 10^3 particles cm^{-2}. *n* = number of repeated experiments; K-W, Kruskal-Wallis. *P* values representing significant differences are indicated in bold. Superscript roman letters a and b indicate the results of *post hoc* Wilcoxon rank sum tests for differences between media performed with *A. fumigatus*; medians with same letter are significantly different ($P \leq 0.01$). Superscript roman letters c and d indicate the results of *post hoc* Wilcoxon rank sum tests for differences between media performed with *A. fumigatus*; medians with same letter are significantly different ($P \leq 0.001$).

these particles originate from conidia. Rodlet structures have been reported to be specific to the surface of reproductive structures, especially asexual conidia (52, 53). Rodlets were observed on the spore surface of the three tested isolates. However, only a minor proportion of the submicronic particles contained rodlet structures. It is possible that the orientation of particles on the filter can obscure the outer spore wall surface with rodlets and that such a condition could lead to underestimation of submicronic fragment numbers from spores. These findings further our understanding of conidium fragmentation associated with *in vitro* experiments of particle generation from fungal cultures by air jets. The strong air currents in the generator may cause particles to impact on internal surfaces and induce shear forces that may lead to particle fragmentation. Conidium fragmentation, visualized as submicronic fragments with rodlet structures, was observed with *Aspergillus* isolates and not with *P. chrysogenum*, indicating that conidium fragmentation was not common with the tested isolate. The warted and spiny outer-wall morphology of spores derived from the *A. fumigatus* and *A. versicolor* isolates may result in easier shearing into submicronic fragments than the smooth spore characteristics associated with *P. chrysogenum* isolates. It is not clear, however, whether spore fragmentation also occurs under environmental conditions or whether this is an artifact of extreme experimental conditions. The absence of submicronic fragments with a rodlet structure from *P. chrysogenum* seems in contradiction with the results reported by Kanaani and coworkers (11), who reported that most spore fragmentation occurred with *Penicillium* species. Since those authors did not specify the *Penicillium* species,

TABLE 3 Effects of generator and airflow on submicronic particles generated from *A. fumigatus*, *A. versicolor*, and *P. chrysogenum* cultures[a]

Abiotic factors	*A. fumigatus*				*A. versicolor*				*P. chrysogenum*			
	n	Median	25th and 75th percentiles	K-W test *P* value	*n*	Median	25th and 75th percentiles	K-W test *P* value	*n*	Median	25th and 75th percentiles	K-W test *P* value
Airflow and generator												
12 liters/min												
FSSST	15	126	0.0 and 380	0.06	15	2.6	1.6 and 5.7	0.8	15	1.8	0.9 and 8.7	**0.01**
SPG	15	340	130 and 510		15	2.5	1.3 and 5.4		15	0.097	0.0 and 1.8	
20 liters/min												
FSSST	15	81	9.8 and 340	0.8	15	0.3	0.0 and 4.0	0.1	14	0.0	0.0 and 1.1	**0.002**
SPG	14	174	0.9 and 340		15	2.9	0.9 and 6.4		15	2.1	0.9 and 4.4	
Generator and airflow												
FSSST												
12 liters/min	15	126	0.0 and 380	0.1	15	2.6	1.6 and 5.7	0.8	15	1.8	0.9 and 8.7	**0.001**
20 liters/min	15	81	9.8 and 340		15	0.3	0.0 and 4.0		14	0.0	0.0 and 1.1	
SPG												
12 liters/min	15	340	130 and 510	1	15	2.5	1.3 and 5.4	0.1	15	0.097	0.0 and 1.8	**0.004**
20 liters/min	14	174	0.9 and 340		15	2.9	0.9 and 6.4		15	2.1	0.9 and 4.4	

[a] Data represent 10^3 particles cm^{-2}. *n* = number of repeated experiments; K-W, Kruskal-Wallis. *P* values representing significant differences are indicated in bold.

we can only speculate that they used a strain that produces conidia with protrusions, as many species within this genus have spores with surface ornamentation (54). Furthermore, it seems unlikely that those authors were able to discriminate between fragments from spores and those from hyphae using automatic particle counting based on aerodynamic size and autofluorescence measurements.

The main proportion of submicronic particles observed in the present study had surface structures similar to those of the vegetative mycelial biomass (hyphae). However, the morphology of hyphal fragments lacked characteristic features suitable for discriminating them from possible fragments from the growth medium. In order to prevent particle aerosolization from the nutrient medium, we studied the aerosolization of submicronic particles from cultures grown on cellophane-covered medium (MEAC). It has been reported that cellophane membranes are permeable only to molecules 90 kDa in diameter or smaller (55), and this cover has been shown to better separate the mycelial biomass from the substrate (46, 47, 57). It is therefore likely that submicronic particles liberated from MEAC are of fungal origin. The observation of similar numbers of submicronic particles aerosolized from 2-week-old MEA and MEAC cultures further suggests that the submicronic particles aerosolized from young cultures grown on MEA were mainly derived from hyphal structures and not from agar (Table 2). This comparison assumes that the characteristics of mycelial growth on MEA and MEAC are similar.

Significantly higher numbers of submicronic fragments were released from 8-week-old cultures of *A. versicolor* grown on MEA and GB than from those grown on MEAC. Similar results were observed for *P. chrysogenum*, although the differences were not significant. This may indicate that some of the submicronic particles were released from the media, as they were morphologically indiscernible. The release of substrate fragments has been suggested by Scheermeyer and Agranovski (12), who ascribed this to desiccation and weakening of the MEA substrate surface during prolonged incubation times. However, this hypothesis is not supported by our results for *A. fumigatus*. Surprisingly, the highest numbers of submicronic particles from *A. fumigatus* were from cellophane-covered MEA rather than from uncovered MEA or GB.

We observed highly significant differences between the three isolates when data from all experiments were combined. The highest numbers were obtained from *A. fumigatus* (median, 200×10^3 cm^{-2}), while *A. versicolor* and *P. chrysogenum* released fewer fragments (median, 2.6×10^3 cm^{-2} and 0.9×10^3 cm^{-2}, respectively). Our results are difficult to compare to those of previous studies due to differences between criteria for fungal fragments and the use of partly different species, different strains, and different aerosolization methods. However, relative differences observed between species within studies are more reliable. We can then compare our results obtained with *A. versicolor* and *P. chrysogenum* to results from the studies by Kildesø et al. (5) and Górny and Ławniczek-Wałczyk (14), who found that *A. versicolor* released more small particles than *P. chrysogenum*, which is qualitatively similar to our findings (7, 11, 14).

Significantly higher numbers of submicronic particles were obtained from older cultures of all isolates. As for the *Aspergillus* species, these differences (obtained on MEAC for *A. fumigatus* and on MEA for *A. versicolor*) have been attributed to desiccation stress (12) and mycelial autolysis (23). This effect was also observed with *P. chrysogenum* cultures grown on MEA and can be explained likewise (58). In addition, the presence of liquid exudate on 2-week-old cultures is a characteristic of this species and may further reduce the emission of fungal fragments (54, 59). In contrast, the abiotic variables, including the type of generator and airflow, showed no significant effect on the aerosolized number of submicronic particles from the *Aspergillus* species. With *P. chrysogenum*, we found significantly more SF with the FSSST at the lowest flow rate, while with the SPG, SF counts were highest at the highest flow rate. Furthermore, at 12 liters min^{-1}, the number of SF was higher with the FSSST than with the SPG, but at 20 liters min^{-1}, the opposite trend was observed. Differences in jet dimensions, laminar or turbulent airflows, and electrostatic properties of the construction material of the generators combined with the structural characteristics of fungal cultures may all play important roles in the aerosolization of SF as suggested by Kanaani et al. (11) and Górny and Ławniczek-Wałczyk (14).

An important aspect for evaluation of indoor fungal exposure is the ratio of submicronic particles to other particles, including spores and larger fragments, which has been reported in several studies. These values were approximately 1:3 for *A. fumigatus*, 5:1 for *A. versicolor*, and 1:2 for *P. chrysogenum* in the present study and are within the range of ratios reported in previous studies (7, 8, 11, 14). High fragment-to-spore ratios are of concern because the exposure to fungal agents is underestimated unless fragments are quantified, which currently is possible only by the detection of biomarkers in the submicronic aerosol fraction. Although these biomarkers may present health risks of their own, they document only indirectly the occurrence of submicronic fragments and not their enumeration.

The fact that submicronic fungal fragments are generated shows that an additional burden of respirable particles can be produced which may contribute to personal exposure to fungi but which is overlooked using current methods of exposure assessment.

This report demonstrates the contribution of spore wall fragments (containing the rodlet layer) to personal fungal exposure, which to our knowledge had not been previously identified. The observation that most fungal fragments originate from the hyphae may also have implications for health risks associated with fungal exposure. Hyphae have been shown to induce allergic inflammation in experimental studies, whereas spores induce mainly nonspecific inflammation (4).

Conclusions. The FESEM enumeration of similar numbers of submicronic fragments from 2-week-old cultures grown on MEA and MEAC indicated that these fragments were of hyphal or conidial origin and were not from the agar. Conidial fragments were observed in experiments performed with *A. fumigatus* and *A. versicolor* but not in those performed with *P. chrysogenum*. It is not clear whether conidial fragmentation is a natural process. Although the fungal origin of submicronic particles generated from fungal cultures was demonstrated under controlled conditions in this study, it still remains unclear whether submicronic fungal fragments occur in sufficient numbers in the indoor environment to represent an additional fungal burden following personal exposure. Future studies should be designed to confirm and enumerate the presence of submicronic fungal fragments in environmental samples.

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