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The Occurrence and Persistence of Mixed Biofilms in Automobile Air Conditioning Systems

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Abstract. Twelve automobile air conditioner systems from six manufacturers and three countries, selected mostly because of complaints of unpleasant odors in the passenger compartment, were examined for microbial growth by direct microscopy and enrichment culture. Mixed populations of fungi and bacteria (with occasional protozoa) were observed in biofilms in at least some of the components from all used units. The aluminum heat exchanger fins from ten evaporators demonstrated bacterial biofilms that yielded *Methylobacterium mesophilicum*. *Penicillium viridicatum* colonized components from four units. These bacteria and fungi were recoverable repeatedly from these units during 'dry' storage of up to 27 months. This report associates a bacterial-fungal community with disagreeable air quality in some automobiles.

Microorganisms emanating from automobile air conditioning systems (ACS) have been associated with hypersensitivity pneumonitis and other allergic reactions [11–13]. Recently we demonstrated that foam insulation and glues, in particular, on automobile air conditioner units were colonized by fungi such as *Aureobasidium*, *Cladosporium*, and *Penicillium* [17], fungi often implicated in colonization of similar substrates in buildings categorized with the sick building syndrome [1, 3, 14]. In this report we extend our observations on automobiles identified with odor problems to the occurrence of mixed bacterial-fungal biofilms in automobile air conditioning systems.

Materials and Methods

Eleven used automobile air conditioner units and one unused unit, representing four different foreign and two domestic automobile brands, were obtained through various automobile industry-associated sources. All but one of the used units were associated with unpleasant odors. The various insulation and casing materials and the metal heat exchanger fins were examined for the presence of biofilms or colonized surfaces. Preliminary observations on some of these units and the light microscopic (LM) or scanning electron microscopic (SEM) procedures used in polypropylene adhesive tape-, and whole-mount preparations were described earlier, as were the moisture chambers used for the verification of susceptibility of components to colonization or biofilm development [17]. In these latter procedures, system components (foams, plastics, metal fins) were selected and periodically transferred from a given unit to moisture chambers and thereafter examined by LM and SEM methods. The ACS units and components were stored in plastic autoclave bags in the laboratory between examinations.

Isolation of microorganisms. Sections (1–2 cm^2) and swabs of the various insulation materials and individual aluminum heat exchanger fins that had demonstrated microscopic colonization (LM/SEM) were screened for viable fungi with malt extract agar [33.6g malt extract agar (MEA); Difco Laboratories, Detroit, MI, with 5g agar added per liter], Mycological agar (Difco), Mycological agar with 0.05% chloramphenicol and Czapek agar (Difco) with 20% or 40% sucrose. Nutrient, Tryptic Soy, R_2A agars (Difco) and Actinomyces agar (Difco) were employed for the isolation of bacteria. The sections were drawn across the agar, then placed in the center of the plate. With most materials, another section was placed in an empty petri plate, and cooled liquid medium (about 15 ml at 45°C) was poured directly onto the sample, then allowed to solidify (pour-plate method). The samples were incubated for 7 days at room temperature (approx. 25°C).

Identification of bacteria and fungi. Identification of fungi was made in accordance with standard morphological keys [2]. Identification of the penicillia followed the procedures of Raper and Thom [16] and Pitt [15]. Predominant bacteria were identified with standard methodology and the Microlog 3 System GN database (Biolog, Inc., Hayward, CA). Reference cultures of *M. mesophilicum* and *M. radiotolerans* were obtained from the American Type Culture Collection (ATCC, Rockville, MD).

Electron microscopy. Small (1–2 cm) samples of air conditioning system components were mounted on aluminum stubs, sputter-coated with gold/palladium, and examined in a Leica s420 scanning electron microscope.

Sections of a dried film-like material were removed from the surface of evaporator heat exchanger fins with forceps. The film

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Table 1. Persistent biofilms in air conditioning systems

Code	Status/source	Biofilm/colonization[a]	Site[b]	Time[c]
A	New Unit	None		
B	Used,[d] Ohio	*Penicillium* sp.	F	18
C	Used, Georgia	*Methylobacterium, Flavobacterium*	H	12
		Cladosporium, Penicillium	F	
E	Used, Florida	*Methylobacterium*	H	12
F	Used	*Methylobacterium*	H	
		Cladosporium, Penicillium	F	12
G	Used, Mexico	*Methylobacterium, Flavobacterium*	H, F	11
		Cladosporium, Penicillium	F	
H	Used, California	*Methylobacterium, Flavobacterium*	H	11
		Alternaria, Cladosporium, Penicillium	H, F	
I	Used, Georgia	*Methylobacterium, Flavobacterium*	H	7
		Alternaria, Cladosporium, Penicillium	F	
J	Used, England	*Methylobacterium, Flavobacterium*	H	26
		Alternaria, Cladosporium, Penicillium	F	
K	Used, Florida	*Methylobacterium, Flavobacterium*	H, F	6
		Paecilomyces, Cladosporium	F	
		Acanthamoeba	H, F	
AV	Used	*Methylobacterium*	H	27
		Fungi[e]	F	
BV	Used	*Methylobacterium*	H	27
		Fungi[e]	F	

[a] Bacteria and/or fungi detectable by direct microscopy; repeated recovery of viable cells at approximately 3-month intervals.
[b] H = heat exchanger fins; F = foam seals or insulation material and plastics.
[c] Duration, in months, of study of the unit.
[d] Returned after fluid leak, no odor, <6000 miles.
[e] Hyphae only.

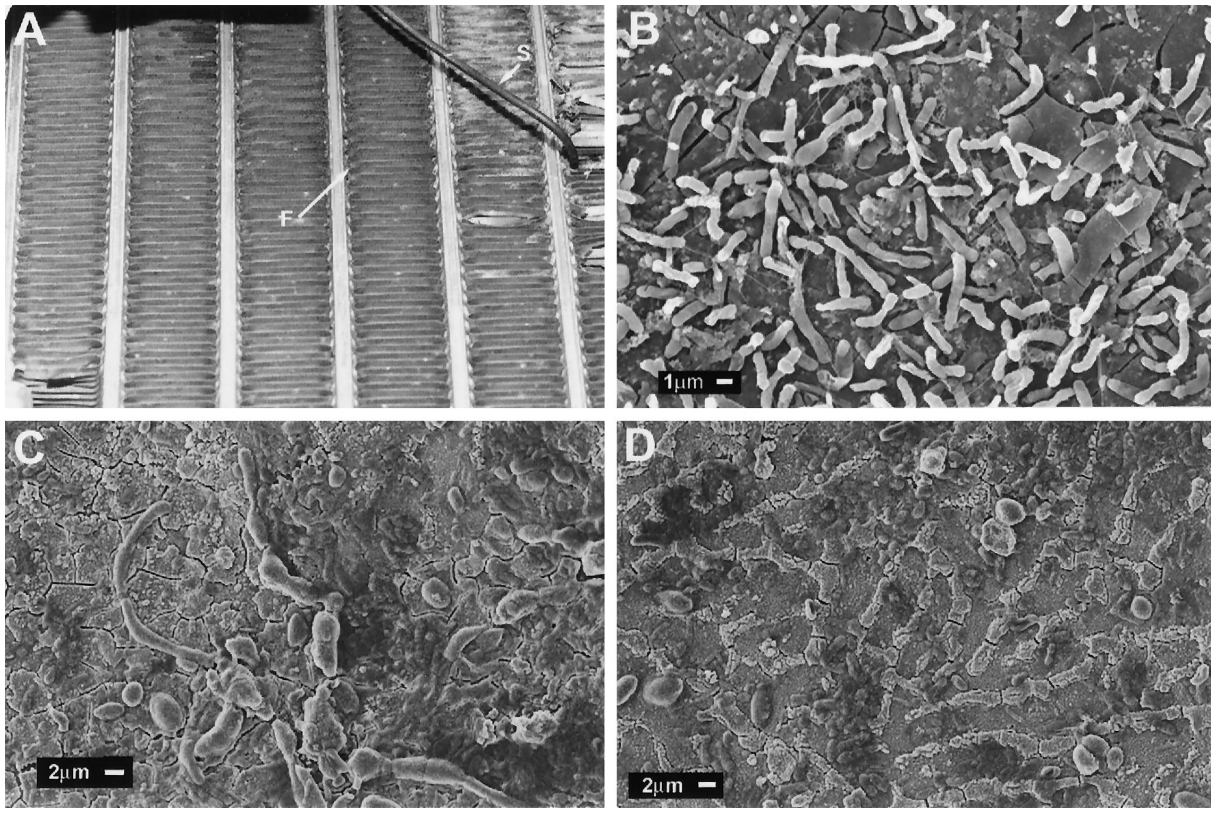

Fig. 1. Unit 'H', aluminum heat exchanger fins. A. A/C system evaporator showing the orientation of the aluminum heat exchanger fins with mixed bacterial-fungal biofilms. S = evaporator temperature probe. F = Fins in the area from which the samples for the SEMs were removed. B. Scanning electron micrograph (SEM) showing bacterial biofilm on an aluminum heat exchanger fin surface; scale bar = 1 µm. C. Germinating fungal conidia within the biofilm matrix; scale bar = 2 µm. D. Fungal hyphae with arthroconidia-like structures embedded in the surface film on an aluminum heat exchanger fin; scale bar = 2 µm.

samples were encased in a thin (1–2 mm) layer of agar (held at 45°C, just above the gel point) on a glass slide. The agar encasement was processed in glutaraldehyde/OsO_4 for transmission electron microscopy after the method of Hayat [6].

Results

The most obvious microorganisms in biofilms in the ACS units were fungal colonizations, i.e., hyphae with mature conidiophores and conidia. These were observed on initial microscopic examinations, usually on plastic foam or insulation components, particularly in association with glue layers. Hyphae often extended over hard plastic or metal surfaces. The fungal colonizations occurred on components of ACS units that visually appeared clean as well as in several systems that contained heavy leaf litter and detritus. Fungi abundant on leaf litter mainly were species different from those consistently found in biofilms in ACS units without organic litter. For example, *Hyalodendron* sp. was cultured from direct swabs of surfaces on ACS ''H,'' and *Aspergillus niger* and *Syncephalastrum racemosus* colonized the organic litter, but these species were not found in persistent biofilms on components of the unit devoid of litter. Mixed fungal/bacterial biofilms were observed on aluminum fins removed from evaporator cores. Fungal conidia were found on and apparently embedded in the biofilm matrix; some conidia appeared to be germinating. Fungal hyphae with arthroconidia-like structures were found also in and on the film matrix (Fig. 1). *Penicillium viridicatum, Aspergillus niger*, and, in the biofilm area, *A. fumigatus* were isolated from ACS unit ''H'' heat exchanger fins for up to 11 months in storage. We have found *Aspergillus fumigatus* on repeated examinations of several automobiles, both from the passenger compartment and the ACS units. We have also isolated *A. fumigatus* and *A. flavus* from potpourri-type air fresheners designed for use in automobiles. *Penicillium viridicatum* occurred in biofilms mainly on foams in 4 of 12 units and was recovered repeatedly from dry material over an 18-month period. Species of *Cladosporium* and *Penicillium* were the most common and persistent fungi among all ACS units, but the most prodigious fungal colonization in an ACS unit was dominated by *Paecilomyces lilacinus* (Fig. 2a). This fungus produced essentially monotypic colonization at certain sites as well as overgrowth of bacterial biofilms. These mixed biofilms were also observed in association with *Acanthamoeba* (Fig. 2b). Trophozoites (in condensate water) and cysts of *Acanthamoeba* were observed in initial observations of the biofilms as well as in repeated examinations that included samples from moisture chambers and culture recovery studies.

The most common and perhaps the most persistent microorganisms in biofilms on the aluminum evaporator fins were bacteria representative of *Methylobacterium*, most probably *M. mesophilicum*, often in association with a *Flavobacterium*-like organism and, occasionally, *Bacillus* sp. (Table 1). These biofilms were observed by SEM on metal heat exchanger fins on both the upstream and downstream sides of evaporators (Fig. 3a). *Methylobacterium mesophilicum* was recovered from 11 of the 12 units, and biofilms were observed in 10 units. The biofilms on fins of several units (C, E, H, I) had been partially covered with an unknown coating material presumably containing substances intended to reduce odors. Transmission electron microscopy of these samples showed the presence of apparently viable bacteria in the film adjacent to the metal surface (Fig. 3b).

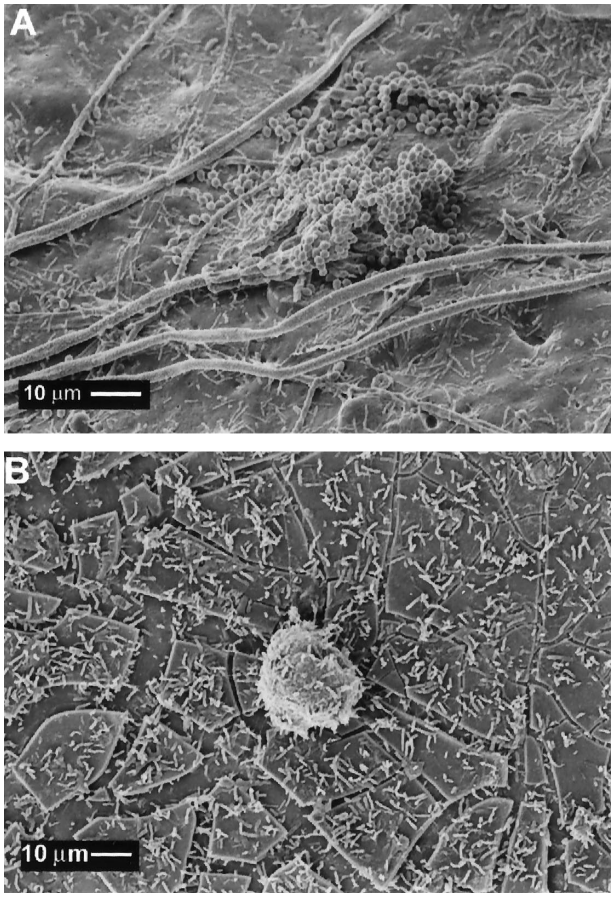

Fig. 2. Unit ''K.'' A. SEM showing hyphae and conidiophore of *Paecilomyces lilacinus* on a plastic foam with underlying bacterial biofilm. B. Cyst of *Acanthamoeba* sp. on aluminum heat exchanger fin. Trophozoites were recovered repeatedly in culture in association with bacteria; scale bars = 10 μm.

Discussion

In general, the mixed biofilms observed in this study included the same genera of fungi reported previously

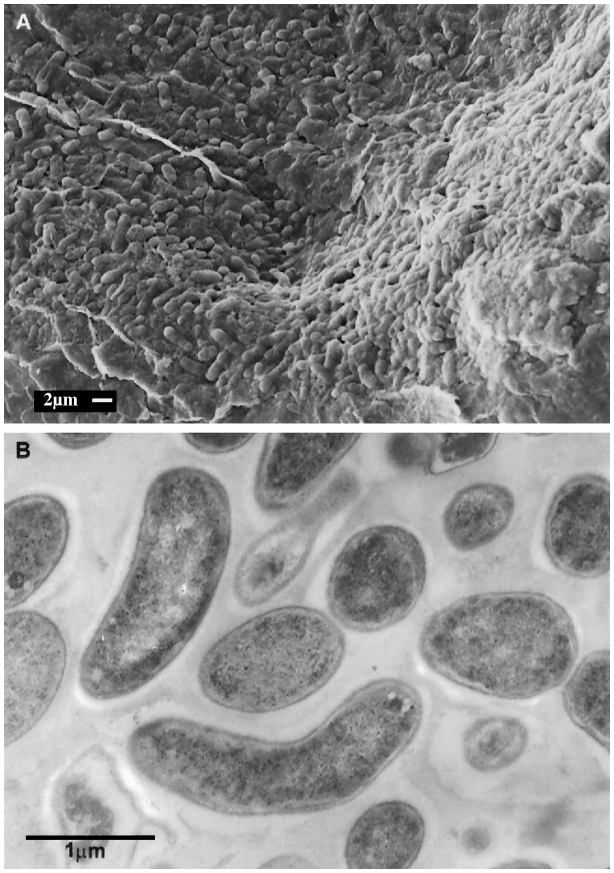

Fig. 3. Unit 'I'. A. SEM of biofilm on an aluminum heat exchanger fin showing a confluent growth of bacteria; scale bar = 2 µm. B. Transmission electron micrograph showing apparently viable bacterial cells in a biofilm removed from the heat exchanger fin; scale bar = 1 µm.

from air conditioning units freshly removed from automobiles [17]. In most instances, the biofilms were not visually distinct, apparent only under microscopy and often hidden within the evaporator core. Bacteria, fungi, and protozoa were recovered from the biofilms repeatedly during ''dry'' storage. Resistance to desiccation and the ability to produce active growth when placed in a moisture chamber were noted, particularly for *M. mesophilicum* and *P. viridicatum*. The ability of fungi to produce resistant chlamydospores is well recognized.

Colonization of the heat exchanger fins of automobile air conditioners by *M. mesophilicum* is previously unreported, though Hugenholtz and Fuerst [9] noted high levels of *Blastobacter* spp., later identified as *Methylobacterium*-like bacteria [10], on the coil fins of a building air conditioner system with no reported odor or health problems. Hugengoltz et al. [10] and Hirashi et al. [7] noted that the genus is resistant to desiccation.

Methylobacterium spp. may be well adapted to the ACS environment. These bacteria, previously identified as species of *Pseudomonas*, *Protomonas*, *Vibrio*, as well as *Blastobacter*, are facultative methylotrophs, members of the α-2 subgroup of the proteobacteria. Their resistance to desiccation may be due to an ability to create an exopolysaccharide (EPS) layer for protection and poly-β-hydroxybutyrate inclusions for energy storage. The possibility exists that this EPS layer may be responsible for resistance to (or protection from) biocides and biostatic agents used to treat odor in ACS units. Species of *Methylobacterium* are common environmental organisms found in fresh water, soil, dust, and air. They are resistant to chlorine treatment, and some species are resistant to penicillin, chloramphenicol, as well as other antibiotics [7]. Rare opportunistic infections by *Methylobacterium* spp. have occurred in immunocompromised patients [4, 5, 8].

This research shows that bacteria and fungi may form mixed, desiccation-resistant biofilms on the metal heat exchanger fins within the core of ACS evaporators as well as on foams, plastics, and other components. These mixed biofilms when moistened may be major contributors to episodic noxious odors in automobile air conditioning systems.

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